MAMMARY CELL CYCLIC AMP: REGULATION OF BREAKDOWN AND INFLUENCE ON PROTEIN PHOSPHORYLATION

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by

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TO MY PARENTS, FOR THEIR ENDLESS SUPPORT AND ENCOURAGEMENT

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CONTENTS

List of Abbreviations

Abbreviations used are those cited as acceptable in the instructions to authors published by the Biochemical Journal (Biochem. J. [1986] 233, 1-24), with the following exceptions.

ACTH	adrenocorticotrophic hormone
ATPγS	adenosine 5′-O-(3-triphosphate)
BAPTA	1,2-bis (2-aminophenoxy)ethane-NNN′N′-tetra-acetic acid
BCDH	branched chain 2-oxoacid dehydrogenase
BHK	baby hamster kidney
Bt_2 cAMP	N^6,2′-O-dibutyryl adenosine 3′,5′-cyclic monophosphate
cAMP	cyclic AMP
cAMP-PDE	cyclic AMP phosphodiesterase
cAMP-PrK	cyclic AMP-dependent protein kinase
CC-PrK	calcium/calmodulin-dependent protein kinase
CEH	cholesterol ester hydrolase
CKA	casein kinase A
CKG	casein kinase G
CKGI	casein kinase G inhibitor
DFP	diisopropylfluorophosphate
DMBA	9,10-dimethyl-1,2-benzanthracene
DTT	dithiothreitol
EGF	epidermal growth factor
eIF-2	eukaryotic initiation factor 2

F_A	protein phosphatase 1 activating factor
FAS	fatty acid synthase
F_c	catalytic subunit of protein phosphatase 1
Fru 1,6-P_2	fructose 1,6-bisphosphate
Fru 1,6-P_2ase	fructose 1,6-bisphosphatase
Fru 2,6-P_2	fructose 2,6-bisphosphate
Fru 2,6-P_2ase	fructose 2,6-bisphosphatase
Fru 6-P	fructose 6-phosphate
G_i	inhibitory guanine nucleotide binding protein
G_s	stimulatory guanine nucleotide binding protein
GSK	glycogen synthase kinase
GTPγS	guanosine 5'-O-(3-triphosphate)
HMG	3-hydroxy,3-methylglutarate
HMG-CoA reductase	3-hydroxy,3-methylglutaryl co enzyme A reductase
HPLC	high performance liquid chromatography
HSL	hormone sensitive lipase
I-1	protein phosphatase inhibitor 1
I-2	protein phosphatase inhibitor 2
IBMX	3-isobutyl-1-methylxanthine
Mg-ATP	magnesium salt of ATP
NEFA	non-esterified fatty acids
ODC	ornithine decarboxylase
PA	phosphatidic acid
PDH	pyruvate dehydrogenase
PDE	phosphodiesterase
PEP	phospho enol pyruvate
PIA	N^6-[phenyl isopropyl]-adenosine

PFK1	6-phosphofructo 1-kinase
PFK2	6-phosphofructo 2-kinase
PIP	phosphatidyl inositol 4-phosphate (Ptd Ins 4-P or diphosphoinositide)
PIP_2	phosphatidyl inositol 4,5-bisphosphate (Ptd Ins 4,5-P_2 or triphosphoinositide)
PMSF	phenylmethyl sulphonyl fluoride
Poly A-PrK	polyamine-dependent protein kinase
PrK	protein kinase
PrKC	protein kinase C
PrP	protein phosphatase
PS	phosphatidyl serine
Ptd Ins	phosphatidyl inositol
R_I	regulatory subunit of type I cyclic AMP-dependent protein kinase
R_{II}	regulatory subunit of type II cylic AMP-dependent protein kinase
Ro 7-2956	phosphodiesterase inhibitor developed by Roche: 4-(3,4-Dimethoxybenzyl)-2-imidazolidinone
SAM	S-adenosyl methionine
S-H	sulphydryl group
S-S	disulphide bridge
TLC	thin layer chromatography
TLCK	Nα-p-tosyl-L-lysine chloromethyl ketone
TPA	12-O-tetradecanoyl phorbol 13-acetate (PMA or 4β-phorbol, 12β-myristate, 13α-acetate)
TPP	thiamine pyrophosphate
VLDL	very low density lipoprotein

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Summary

This thesis addresses the question of whether certain control mechanisms responsible for metabolic regulation in mammalian tissues such as liver, adipose tissue and muscle, are also functional in mammary tissue.

Cyclic AMP-dependent phosphorylation is considered ubiquitous in animal tissues and has been demonstrated to be capable of regulating numerous major metabolic pathways (see section 1.). In mammary tissue, cellular metabolism is dominated overwhelmingly by lactogenesis: the biosynthesis of lipid is a principal pathway in the collection of activities comprising this function. The cyclic AMP-dependent regulation of this and other metabolic pathways of particular relevance to mammary cell function is discussed at length in section 1., highlighting the apparent necessity for control mechanisms analogous to those found in other cell types. A review of these mechanisms demonstrates the potential importance of cyclic AMP as a regulator of mammary cell metabolism and raises many interesting questions. One of the most intriguing of these is whether or not mammary cell cyclic AMP levels are subject to the same regulatory influences as have been described for tissues such as liver and adipose tissue. An obvious extension of this is whether modulation of intracellular cyclic AMP levels provokes the same metabolic response, in mammary tissue, as has been observed in these other tissues. Both of these questions have been addressed and the results are presented here.

The discovery that the activity of a high affinity form of cyclic AMP-phosphodiesterase found in adipose tissue and liver can be modulated by hormones such as insulin has stimulated a great deal of interest since it provides a possible mechanism for at least some of the metabolic effects of this hormone. Mammary tissue also contains "high affinity" cyclic AMP-phosphodiesterase and its regulatory properties, particularly with respect to insulin-sensitivity, have been investigated. The results show that rat mammary high affinity cyclic AMP phosphodiesterase activity is stimulated by treatment of isolated acinus preparations with insulin but that the effect cannot be reproduced in a broken-cell system. These results are discussed with reference to similar studies in other tissues.

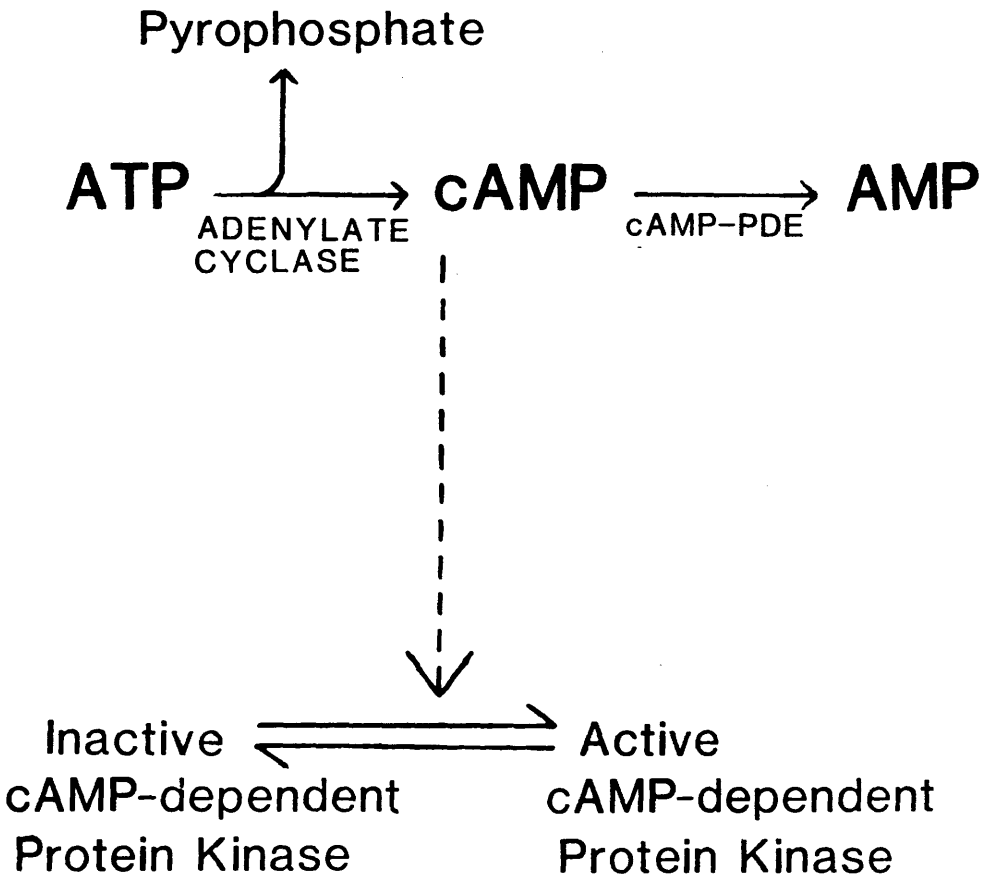
Although intracellular cyclic AMP concentrations can be raised many-fold by treatment of mammary cells with agents such as forskolin and β-adrenergic agonists (in the presence of a phosphodiesterase inhibitor), no discernable effect on the activity of key enzymes such as acetyl-CoA carboxylase (known to be phosphorylated and inactivated by cyclic AMP-dependent protein kinase) has been observed. Consequently, the existence, in mammary tissue, of a competent combination of cyclic AMP-dependent protein kinase and endogenous substrate has been investigated. Complementary to this, a preliminary survey of the existence in mammary tissue, of three other effector-dependent phosphorylation systems (governed by calcium/calmodulin-dependent protein kinase, protein kinase C and polyamine-dependent protein kinase) has been conducted. The major finding was that rat mammary tissue does indeed contain competent

kinase/endogenous substrate combinations for at least two known effectors of metabolic regulation. One of these is Ca^{2+}/calmodulin but of greater significance to the present study, the other is cyclic AMP, for which phosphorylation was shown to display a dose-dependent relationship (in the physiological range) with at least two endogenous substrates. The molecular weights of these and other endogenous substrates for effector-dependent phosphorylation in mammary tissue are compared with substrates for similar phosphorylations already identified (in the literature) in other tissues.

1. INTRODUCTION

The only property of cAMP recognised to be important in the control of animal cell metabolism is its ability to activate cAMP-dependent Protein Kinase (cAMP-PrK) but by this action alone it is able to regulate a number of major metabolic pathways including glycogenolysis, glycolysis/gluconeogenesis and fatty acid synthesis. By virtue of its broad substrate specificity, cAMP-PrK has the potential to regulate the function of numerous cellular proteins of which many are either enzymes themselves or modulators of enzyme activity such as inhibitor 1 (see section 1.3.8.(i)a). In recognition of its role as link between external stimuli and intracellular response, cAMP is termed a second messenger. This nucleotide was the first agent found to possess such a property and is arguably the most important mediator of acute metabolic regulation by external stimuli yet discovered. The control of its accumulation within the cell has, therefore attracted a great deal of interest and as a consequence, the regulation of intracellular cAMP levels is relatively well understood. By the same token, cAMP-dependent phosphorylation has also been extensively characterised in several enzyme systems (see sections 1.3.1.-1.3.7. and 1.3.8.(i)a). The synthesis and degradation of cAMP (represented in Figure 1) is governed by the two enzymes adenylate cyclase and cAMP phosphodiesterase (PDE) respectively, both of which are subject to regulation by various agents.

Figure 1 cAMP Synthesis,Degradation and Action



1.1. Adenylate Cyclase

In recent years in has been established that the activity of adenylate cyclase is subject to dual control mediated by a pair of guanine nucleotide binding proteins (here referred to as G-proteins but also known as N-proteins) termed G_s (stimulatory) and G_i (inhibitory). The catalytic moiety (C) of adenylate cyclase is essentially inactive in the absence of G_s but adenylate cyclase preparations free of G_s can also be activated by the diterpene forskolin [691] suggesting that this compound interacts directly with C. However forskolin induced activation of C is enhanced in the presence of G_s [145] and there is evidence that interaction with intact G-protein is necessary in order for full activation by forskolin to be expressed [145]. Fluoride ions also activate adenylate cyclase in the presence of Al^{3+} and Mg^{2+} but in this instance the presence of G_s is an absolute requirement for activation [712].

Purification of C, a hydrophobic and apparently very labile protein, has recently been achieved using forskolin-Sepharose affinity chromatography [606, 607] but many of the properties of the catalytic component of adenylate cyclase remain poorly characterised. Both G_s and G_i have been purified and studied in some detail by Gilman et al. [253 and references therein]. In most tissues G_s is described as a dimer consisting of a 45kDa α-subunit and a 35kDa β-subunit although variable amounts of a 52kDa subunit have also been identified in G_s from rabbit liver [571, 731]. G_i is also described as a heterodimer and appears to share the same β-subunit [508, 712] but its α-subunit is distinct and has a molecular weight of 41kDa [253]. Both α-subunits contain a high affinity guanine nucleotide

binding site and a site susceptible to NAD-dependent ADP-ribosylation by bacterial toxins. Recently, comparable G-protein purifications conducted by Birnbaumer and co-workers [109] have yielded broadly similar results but this group describe G_s and G_i complexes with identical molecular weights (95kDa), each composed of a 35kDa β-subunit, a 42kDa α-subunit and a 10-15kDa γ-subunit. As previously described, the β-subunits are identical and the α-subunits although now proposed to share the same molecular weight, are distinct; however, a third small (γ) subunit to which no function has yet been assigned, is thought to also be an integral part of both G_s and G_i. The subunit composition of the G- proteins would then be αβγ, analagous to that of the related signal-transducing protein of retinal rod outer segments, transducin, which has been shown to share the same β-subunit as G_s and G_i [362].

Both purified G-proteins have been reported to be activated in an essentially irreversible manner by the non-hydrolysable GTP analogue GTPγS (guanine 5'-(3-O-thio)triphosphate) [253] suggesting that GTP hydrolysis is not necessary for either G_s mediated stimulation or G_i mediated inhibition of adenylate cyclase. Although activation of G_i by non-hydrolysable GTP analogues is a widely observed phenomenon [363, 712], fundamentally contradictory results demonstrating that G_i mediated inhibition of adenylate cyclase can not be induced by such analogues are reported with equal confidence [127, 425]. Activation of G_s is much less controversial and it is generally accepted that non-hydrolysable GTP-analogues are effective substitutes for GTP itself in this respect [127, 712]. The generally accepted mechanism of G_s mediated adenylate cyclase activation and the alternatives proposed for inhibition of adenylate cyclase under

Figure 2

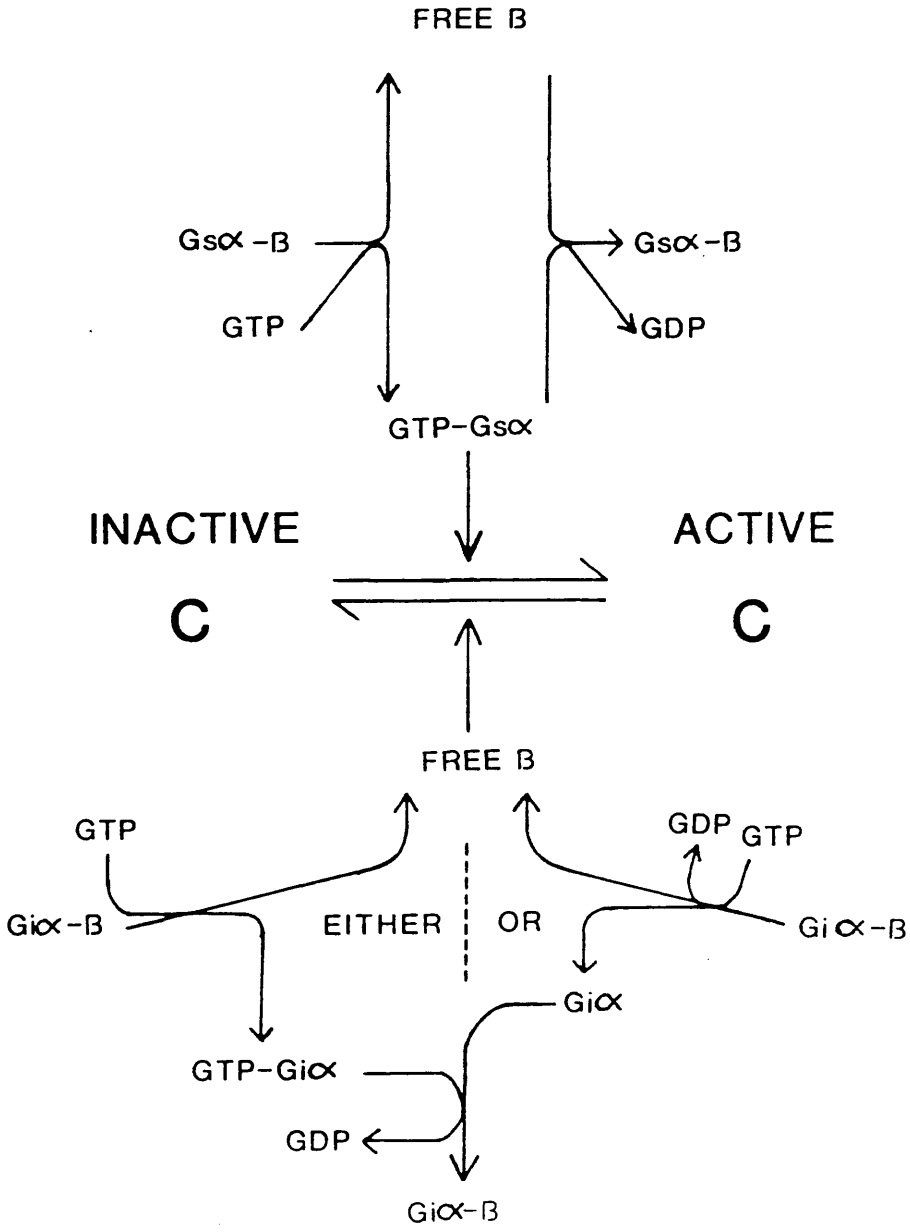
G_s α = α-subunit of stimulatory G-protein (G_s).

G_i α = α-subunit of inhibitory G-protein (G_i).

β = β-subunit common to both G_s and G_i.

c = catalytic subunit of adenylate cyclase.

Figure 2 Schemes Proposed for Modulation of Adenylate Cyclase Activity by G-Proteins



the influence of its counterpart, G_i, are illustrated in Figure 2. G-protein activation results from dissociation of the holo-protein but whereas the adenylate cyclase stimulatory activity of G_s resides in the α-subunit, the inhibitory activity of G_i appears to be accounted for, largely, by its β-subunit though G_i α does possess weak inhibitory activity. However, whether β-subunit inhibition results predominantly from direct interaction with C or is achieved indirectly via binding of free G_s α is uncertain [235]. The relative contributions of the two G-proteins to regulation of adenylate cyclase activity can be probed using cholera toxin and pertussis toxin (also termed Islet-activating protein [IAP]). Cholera toxin-mediated ADP-ribosylation of G_s blocks GTP hydrolysis and thus allows persistent activation of G_s by GTP as observed in equivalent untreated preparations exposed to non-hydrolysable GTP analogues [127, 712]. Treatment with pertussis toxin, on the other hand, promotes ADP ribosylation of G_i and as a result blocks hormonal inhibition of adenylate cyclase in a wide variety of cell types [712] while also often potentiating the effects of stimulatory agonists [300, 390]. This modification presumably blocks G_i dissociation and consequently prevents the expression of its inhibitory activity. The same ultimate effect is implicit in the mechanism for pertussis toxin action proposed by Jakobs et al. [614] involving inhibition of the GDP/GTP exchange required for activation of G_i (see Figure 2). Mg^{2+} is required for activation of both G-proteins but while the concentrations required for G_s activation of adenylate cyclase are in the millimolar range [665], micromolar concentrations of this ion are sufficient to allow G_i mediated inhibition [362, 365].

Many hormones and neurotransmitters are able to modulate adenylate cyclase activity by interaction, via their specific cell-surface receptors, with the G-proteins. Stimulatory receptors include, among others, those for β-adrenergic agonists, ACTH and gonadotrophins while inhibitory effects are initiated by binding of somatostatin, α_2-adrenergic agonists, muscarinic agonists, dopamine and opioids [253, 712, 109]. Binding of agonist to its receptor results in activation of the G-protein associated with that particular receptor type. Whether the response is stimulatory or inhibitory in terms of adenylate cyclase activity therefore depends on the type of receptor involved, while the magnitude of that response may depend on the proportion of G_i to G_s present in the membrane. The latter is particularly important for hormones such as adrenaline which bind to both α and β adrenergic receptors and whose cellular effects will therefore be very much dependent on the predominant G-protein in the target cell membranes. Tissue specific responses to such agents may therefore be achieved either by differences in the proportion of β (stimulatory) and α_2 (inhibitory) receptors or by differences in the membrane complement of G_s and G_i. A further mechanism potentially capable of modulating the response of adenylate cyclase to hormonal regulation has been revealed by recent work with phorbol esters in human platelets [364, 389]. Tumor inducing phorbol esters are direct activators of protein kinase C (PrkC; see section 1.3.8.(i)b) and have been found to impair hormone induced inhibition of adenylate cyclase activity [364]. This effect appears to be mediated by PrKC catalysed phosphorylation of the α-subunit of G_i [389]. Since PrKC is activated by agents that increase phosphatidylinositol turnover such as α_1- adrenergic

agonists, angiotensin II, vasopressin and thrombin (see section 1.3.8.(i)b) this system confers the potential for complex interactions between hormones whose actions are mediated by changes in cAMP levels and those whose primary actions result from an apparently unrelated sequence of events. The response to adrenaline alone is also further complicated since β-adrenergic stimulation of adenylate cyclase may be reinforced by the PrKC induced suppression of G_i resulting from α_1-adrenergic stimulation of phosphatidylinositol turnover. The inhibitory stimulus of α_2 occupancy would then be rendered ineffective. Thus, assuming this system operates in vivo, the degree of adenylate cyclase activation observed following exposure to adrenaline will depend on the abundance of α_1 receptors as well as the factors already discussed above. An additional regulatory feature of the β-adrenergic system, suggested by work with avian erythrocyte receptors [705, 396, 723, 744, 557, 704] and confirmed, recently, in hampster lung [41] is mediated by both cAMP-dependent protein kinase and protein kinase C and involves phosphorylation of the β-adrenergic receptor, resulting in reduced receptor-G_s coupling and, consequently, desensitisation of β-adrenergic response. Amongst other potential physiological functions, this system would represent a means of imposing negative-feedback control on the cellular response to binding of β-adrenergic agonists.

In 1983 Heyworth and Houslay [311] presented evidence that insulin receptors are coupled to a G-protein with properties resembling those of G_i and that via this mechanism, insulin is able to inhibit glucagon stimulated adenylate cyclase in hepatocyte membranes. The same G-protein is proposed to be involved in insulin stimulation of a high affinity membrane bound species of hepatocyte

cAMP-PDE [312, 310] see section 1.2.2.(ii)). The G-protein transducin which mediates between rhodopsin and PDE and has a subunit structure apparently very similar to that of the adenylate cyclase G-proteins (see earlier) also bears functional similarities to these regulatory proteins. The α-subunit (molecular weight 39kDa) of transducin binds GTP and can be ADP-ribosylated by either cholera or pertussis toxins though at distinct, independent sites. Guanine nucleotide analogues cause dissociation of α from $\beta.\gamma$ subunits and the free α subunit activates PDE.

Modulation of the activities of the two enzymes responsible for cAMP synthesis and degradation in cell membranes appears, then, to be achieved by mechanisms involving a common class of regulatory proteins, the guanine nucleotide binding proteins.

1.2. Mammalian cAMP-Phosphodiesterase

Despite the regulatory similarities between the adenylate cyclase system and a particular form of membrane bound PDE, the PDE enzyme system, of which membrane bound activity is often a quantitatively minor though perhaps functionally significant (see section 1.2.2) component, represents a much more diverse group of enzymes both structurally and functionally.

In recent years, a great deal of attention has been focussed on the properties and intracellular distribution of cyclic 3'5'adenosine monophosphate phosphodiesterase (cAMP-PDE), an interest which arises from its role as the only enzyme known to be capable of catalysing degradation of the second messenger, cyclic AMP. As such, cAMP-PDE plays an important part in the regulation of all cellular processes influenced by cAMP and variations in its activity and distribution may,

therefore, have profound effects, particularly in relation to hormonal regulation of cellular activities. As a result of the widespread interest in this enzyme, several reviews of work relating to the characterisation of the various phosphodiesterases and their roles in the modulation of cellular activities have been compiled [including 203, 738, 774, 833], that produced by Thompson and Strada [774] in 1978 being one of the most recent of these. In this short review I have summarised contemporary information gained as a result of recent work by various authors against the background of earlier work.

The bewildering diversity of PDE forms reported in recent years appears to defy categorisation into mutually exclusive groups although attempts have been made at more general classifications [774, 203, 235, 841, 833] on the basis of substrate affinity and specificity. The most widely recognised distinction is that made between high-affinity (low K_m) and low affinity (high K_m) cAMP-PDEs. Although K_m values are often not quoted for the particular PDE being studied, most of the experimental work published to date assumes that activity measured at μM or sub μM cAMP concentrations reflects mainly, if not exclusively, the activity of high affinity cAMP-PDE and that measured at 100μM or above represents the activity of low affinity cAMP-PDE. In some cases, such assumptions may be made with a reasonable degree of safety, particularly if the two types of enzyme are known to have very different K_m values but it must be remembered that unless effective purification techniques have been applied, activity measured at a substrate concentration chosen to highlight high affinity activity is likely to include a certain amount of low affinity activity and PDE activities measured at higher cAMP concentrations (for example, 100μM) will inevitably represent some (depending upon K_m) low affinity

activity and effectively all high affinity activity [833]. A highly activated form of either enzyme might, therefore, easily make a significant contribution to activity measured at a substrate concentration chosen for study of the activity of the other, particularly if the activation involves a large increase in V_{max} of high affinity cAMP-PDE or decrease in K_m of the low affinity enzyme. The potential inaccuracy of these assumptions is highlighted by a review of the range of K_m values reported for cAMP-PDE. High affinity cAMP-PDEs have been described by various authors as having K_m values ranging from 0.2-0.3μM in rat adipose tissue [878, 506] and rat liver [489] to between 5 and 6μM in rat liver [13, 717] and rat heart [13]. Low affinity cAMP-PDEs have been reported with K_m values between approximately 9μM [830] and 300μM [421] or over [315]. So, instead of a population of enzymes falling into two discrete and easily recognised groups possessing characteristically high or low K_m values, the PDE forms found in various mammalian tissues present an apparently near continuous range and it would, therefore, be unrealistic to expect to separate the higher K_m high affinity PDEs from the lower K_m low affinity PDEs on the basis of K_m alone. Despite this, for convenience, the group headings of high and low affinity will be retained although other factors such as substrate specificity and mechanism of activation may be more important in determining the group to which any particular PDE is assigned.

1.2.1. Low Affinity cAMP-PDE

Low affinity cAMP-PDEs generally have K_m values over about 10μM but the range is wide and values vary depending on the tissue under investigation, typical values for rat liver [13, 302, 509, 41] and

adipose tissue [506, 770] being 25-40μM though figures of around 90-100μM and 60μM have been quoted for rat [547, 710] and mouse [14] liver, respectively. Bovine brain PDE appears to display a particularly high K_m with figures between 150 and over 310μM [421, 545, 697, 784] although low affinity PDE of rat brain [14, 146, 674] appears to have similar K_m values to those of rat liver.

Low affinity cAMP-PDE is known to be predominantly a cytosolic enzyme [841] and accounts for the major part of soluble PDE activity [235] but membrane bound low affinity cAMP-PDE may also make a significant contribution to total low affinity activity and has been found in particulate fractions of rat liver [719, 835, 841], heart [235] and brain [766]. In some tissues, for example rat heart [235], particulate low affinity cAMP-PDE may represent 50% of total low affinity activity, although in adipose tissue and kidney the proportion of this activity in particulate fractions is apparently low [674, 774].

The possible roles of low affinity cAMP-PDE as a cytosolic enzyme, predicted by the theoretical models of Fell [212] are discussed later (section 1.2.4.).

Low affinity activity is often reported to coincide with cGMP hydrolysing activity [29, 146, 421, 545, 547, 675, 833]. The low affinity enzyme system is, therefore, sometimes described as consisting of cyclic nucleotide-PDE [29, 235, 547], to indicate its lack of specificity for either substrate, and cyclic GMP-PDE when activity with cGMP exceeds cAMP hydrolysing activity and the proportion of total cellular cGMP hydrolysing activity accounted for by this enzyme is sufficient to justify such a description [13, 91, 235, 378] even though, when assayed at high substrate concentrations (millimolar), activity with cAMP is greater than with cGMP [378]. One of the most

reliable criteria for distinguishing low affinity cAMP-PDE is the susceptibility displayed by certain of this type of enzyme to activation by Ca^{2+}/calmodulin. This property seems to be unique to those enzymes classified as low affinity but it is not a property shared by all such enzymes, since cyclic nucleotide-PDEs have been described with high K_m values for both cAMP and cGMP that may [841] or may not [146, 378] exhibit activation by Ca^{2+}/calmodulin; however, all the members of this latter group are activated by cGMP at micromolar concentrations [146, 378, 841]. Two soluble low affinity PDEs have been isolated from rat cerebral cortex by Davis [146], one of which is Ca^{2+}/calmodulin-dependent and described as a cGMP-PDE, while the other is a Ca^{2+}/calmodulin-independent, cGMP-stimulated cyclic nucleotide-PDE. The Ca^{2+}/calmodulin-independent form appears to represent either a single enzyme with an allosteric site for cGMP binding and two distinct active sites, one for cAMP and one for cGMP, or two separate enzymes sharing the same allosteric site [146]. Enzymes with a greater affinity for cGMP than cAMP have, however, been reported to possess only one catalytic site for both substrates [421, 545]. It seems, therefore, that low affinity cAMP-PDEs may be divided into two groups. One type possesses two distinct active sites [794], one specific to cAMP, the other to cGMP, both with similar, low substrate affinities. This type is the previously described cyclic nucleotide-PDE [13, 29, 547, 841] and is subject to allosteric activation by cGMP. The second type of low affinity cAMP-PDE is probably more accurately described as cGMP-PDE, as discussed earlier, and possesses only one active site [545] capable of the hydrolysis of both cAMP and cGMP. This type of enzyme is activated both by Ca^{2+}/calmodulin and by limited proteolysis [146, 675, 784]. Kakiuchi et al. have found representatives of both

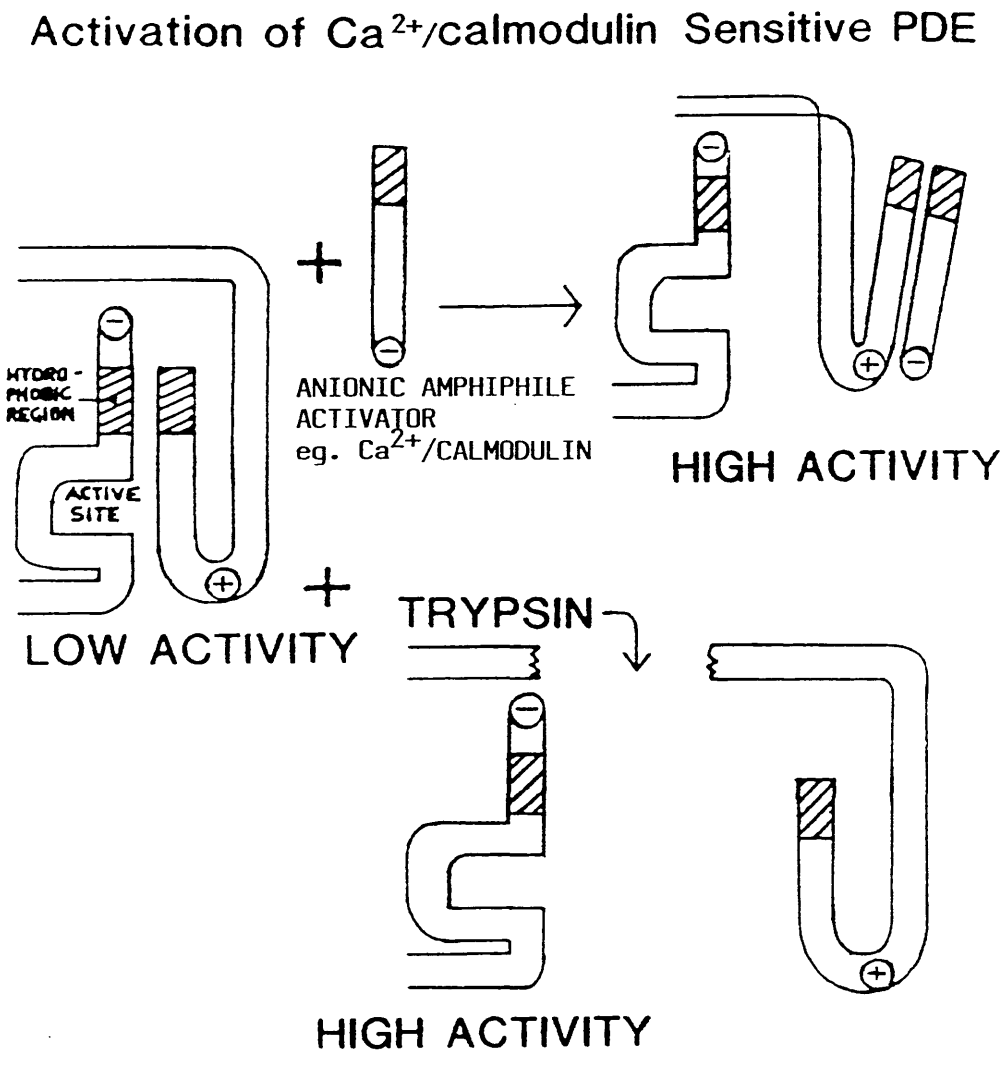
types of low affinity cAMP-PDE in the same tissue preparation (derived from rat cerebral cortex), a situation found by Mullaney and Clegg [549] to be duplicated in rat mammary tissue.

1.2.1.(i) Ca^{2+}/Calmodulin Activation

Calmodulin is an anionic protein of molecular weight approximately 17-18kDa possessing four calcium binding sites. It has been suggested that binding of Ca^{2+} to all four of these sites produces a molecule capable of activating PDE [134, 814] but that binding to only two of the four may result in the formation of an adenylate cyclase activator [814]. Such a distinction is made possible by the existence of two classes of Ca^{2+} binding site in the calmodulin molecule with distinct dissociation constants (10^{-6}M and 10^{-4}M) [423]. The inability of calmodulin alone to active cGMP-PDE is thought to be due to masking of a hydrophobic region required for binding to the enzyme. Binding of Ca^{2+} to the calmodulin molecule is thought to expose this region, allowing binding to and activation of the PDE [146, 458, 761].

A mechanism for the activation of calmodulin-dependent cAMP-PDE and other calmodulin dependent enzymes has been proposed by Gietzen et al. [247] and involves interaction of anionic amphiphiles, such as calmodulin, with a complementary region of the enzyme molecule in such a way as to induce a conformational change allowing greater accessibility to the enzyme active site as shown in Figure 3. Other ionic amphiphiles, such as oleic acid and phosphatidyl serine, have also been shown to activate rat brain PDE and compete for activation with calmodulin, suggesting that the same activation mechanism is common to all three activators [248]. This mechanism also accommodates the activation of a bovine brain calmodulin-dependent PDE by limited

Figure 3


Activation of Ca^{2+}/calmodulin Sensitive PDE
HYDRO-PHOBIC REGION
ACTIVE SITE
ANIONIC AMPHIPHILE ACTIVATOR
eg. Ca^{2+}/CALMODULIN
HIGH ACTIVITY
LOW ACTIVITY
TRYPSIN
HIGH ACTIVITY

proteolysis described by Tucker et al. [784] (see Figure 3). Proteolysis catalysed by trypsin or endogenous protease produces an enzyme with activity equal to the calmodulin-stimulated enzyme but calmodulin will no longer bind to the protease treated enzyme and, therefore, no further activation is observed. The proteolytic activity cleaves a series of peptide fragments from one or both terminal regions of the polypeptide chain constituting the enzyme and calmodulin binding is abolished after removal of approximately 120 residues [784].

Activation of PDE by calmodulin has been reported to involve an increase in the enzyme's V_{max} [625, 684, 832, 842], a decrease in K_m [58, 263] or both [90, 379, 764].

It has been suggested that the function of the calmodulin sensitive enzyme, cGMP-PDE (according to the classification system described earlier), is to remove large excesses of cAMP and cGMP that may collect in the cytosol [718]. The latter, perhaps, is of particular significance since both guanylate cyclase [287] and cGMP-dependent protein kinase [448] are cytosolic enzymes in muscle.

1.2.1.(ii) Molecular Structure and Kinetics

Most of the low affinity cAMP-PDEs purified to apparent homogeneity have been of the Ca^{2+}/calmodulin-dependent type (cGMP-PDE) and have been isolated from bovine brain [697, 421, 545] and heart [329, 457, 558]. There is broad agreement on the molecular weight of the purified enzyme with values, in each instance, being between 121kDa and 135kDa [329, 421, 545, 558, 697] and also that of the catalytically active subunit with figures quoted at 58kDa [697], 59kDa [329, 421, 558] and 63kDa [545]. However, opinion is divided as to the subunit composition of the purified enzyme. A dimeric structure comprising two identical

catalytic subunits, each capable of binding one molecule of calmodulin has been suggested [380, 457, 545, 697] but Klee et al. [421] have isolated three subunits of molecular weights 59kDa, 61kDa and 15kDa of which only the 59kDa unit has catalytic activity and binds calmodulin, the other subunits being described as inhibitory peptides.

The molecular weights of 400kDa and 250kDa determined for cyclic nucleotide-PDE [265, 769] suggest that this enzyme form has a more complex subunit structure than cGMP-PDE but, as yet, there is no information relating to the number and nature of its constituent parts and the possibility therefore exists that such high molecular weights may represent artifactual aggregations.

Anomalous kinetics reported to be displayed by some of the cGMP- and cyclic nucleotide-PDEs are returned to normal Michaelis-Menten kinetics by calmodulin [329, 833] or cGMP [29, 133, 509] respectively. A cGMP-stimulated low affinity cAMP-PDE found in rat liver has a hydrophobic binding site, binding of which to a hydrophobic matrix results in a reduction in the positive cooperativity of the enzyme [133]. Perhaps if bound to a biological membrane, its activity might, therefore, conform to normal Michaelis-Menten kinetics allowing it to be classified as a low affinity intrinsic enzyme of the type described by Marchmont and Houslay [510] in rat liver membranes. Similarly, a cyclic nucleotide-PDE is reportedly released from rat liver membranes following treatment with detergent [841].

Although grouped loosely together, for the purposes of this review, under the heading of "Low affinity cAMP-PDE", the so-called cGMP- and cyclic nucleotide-PDEs have little more in common with each other than they have with "high affinity cAMP-PDEs" and it may therefore be more accurate to recognise them as individual groups in

their own right, as has often been acknowledged in the past [13, 235, 833, 841, 774] and as the proposals for interrelation of enzyme forms presented in section 1.2.3 might suggest.

1.2.2. High Affinity cAMP-PDE

High affinity cAMP-PDE does not represent as diverse a group of enzymes as those constituting the group termed low affinity and may be readily subdivided into two major categories. The two groups share similar physical and kinetic properties and are most easily identified by their response to hormonal stimuli which, in fact, forms the basis of their classification. Enzymes representative of the first group show no response to such stimuli and are therefore termed hormone-insensitive while the second group, hormone-sensitive PDE, has been shown to include enzymes capable of responding to a wide range of hormones (see later; section 1.2.2.(ii)) and has consequently attracted a great deal of interest in the field of metabolic regulation.

1.2.2.(i) General Properties

High affinity (low K_m) cAMP-PDE is the major membrane bound form of PDE [212, 841] but soluble forms of the enzyme have also been reported [274, 742, 772, 830] and may constitute a considerable proportion of the total cellular high affinity PDE in many tissues [235]. Phosphodiesterases classified under the heading of "High affinity" have K_m values below 10μM in the vast majority of mammalian tissues studied, with typical values for rat liver [13, 14, 489, 509, 512, 717] and adipose tissue [506, 878] ranging from 0.2μM to 6.0μM. Neither membrane bound nor soluble high affinity cAMP-PDEs are activated directly by Ca^{2+}/camodulin or cGMP but interactions involving

G-proteins analogous to those of the adenylate cyclase system have, as mentioned earlier (section 1.1), been implicated in the expression of hormonal influences on the plasma membrane bound form of this enzyme.

Many partially purified preparations of high affinity cAMP-PDE display non-linear kinetics [670, 770, 774, 833], suggested by some to imply the presence of multiple enzyme forms. However, doubts have been expressed as to the probability that this is a valid explanation on grounds that the degree of contamination by other kinetically distinct enzyme forms required to achieve the observed effects is unreasonably high [670] and high affinity cAMP-PDE purified to apparent homogeneity has also been demonstrated to display kinetics compatible with negative cooperativity [509]. These observations do not, however, preclude the possibility that the anomolous kinetics displayed by high affinity enzymes in some crude or partially purified preparations are the result of multiple enzyme forms since other high affinity cAMP-PDEs purified to apparent homogeneity have been found to display normal Michaelis-Menten kinetics [193, 194, 538]. A time-dependent loss of negative cooperativity, which is prevented by Mn^{2+} but not Mg^{2+} has been observed in kidney membrane preparations [774]. High affinity cAMP-PDEs have been purified to apparent homogeneity from pig [302] and rat [509] liver, dog kidney [194, 772] and human lung [542]. These enzymes represent a group which, on the whole, appear to share the same, or very similar, characteristics despite the range of tissues and species from which they have been isolated. The dog kidney and human lung enzymes are both described as acidic proteins with molecular weights of 48-60.6kDa [772] and 60kDa [542] respectively and have much greater affinity for cAMP as a substrate than cGMP. Activation has been shown in the presence of Mg^{2+}, Mn^{2+} [302, 542] and Ca^{2+} [302]

but none of the enzymes show any activation by Ca^{2+}/calmodulin. Only purine rings are accepted by all but the pig liver enzyme [302] which shows equal activity with either cAMP or cCMP as substrate despite possessing only one species of active site. Marchmont and Houslay [509] suggest that since the purified rat liver peripheral enzyme is monomeric, the departure from normal Michaelis-Menten kinetics displayed by this enzyme might be explained by the "one substrate, one product mnemonic mechanism" proposed by Ricard et al. [647].

Both soluble [737] and particulate [502, 742, 841] high affinity cAMP-PDEs are susceptible to activation by an endogenous protease. Particulate enzyme solubilised by this and other proteases such as trypsin, chymotrypsin and papain [490, 501] displays a lower molecular weight than that solubilised by detergent [502] and activation is characterised by an increase in V_{max} and a decrease in K_m [774]. However, prolonged exposure to trypsin results in the obliteration of catalytic activity [490]. It has been proposed that the proteolytically solubilised enzyme represents the catalytic subunit or domain of the particulate enzyme [227, 490] and that it is normally associated with an inhibitory domain from which it is released during solubilisation by protease but not by detergent [227, 490]. It would seem reasonable to assume that proteolytic activation of the soluble enzyme is achieved by a similar process but there is, at present, no evidence to support such a proposal. The endogenous protease of rat renal cortex involved in solubilisation and activation of particulate high affinity cAMP-PDE appears to be a lysosomal enzyme similar to the thiol protease, cathepsin L, of rat liver [742]. Stimulation of such thiol protease activity has been proposed to explain the activation of PDE observed in crude adipocyte microsomal fractions incubated in the presence of dithiothreitol [502].

The two major intracellular sites to which particulate high affinity cAMP-PDE activity has been attributed in both adipose tissue [429, 501] and liver [489, 717, 511] are endoplasmic reticulum and plasma membrane although a representative of this group has also been described by Houslay and co-workers in a unique "dense-vesicle" fraction isolated from rat hepatocytes [845]. Redistribution of particulate high affinity cAMP-PDE to the soluble phase appears to occur in mouse embryo fibroblasts (line 3T3 balb C) when a quiescent culture enters log growth phase [774]. Whether this relocation is achieved by a solubilisation mechanism involving the proteolytic effects described earlier or should be attributed to some other process such as comigration with insulin receptors [326] is not known but Smoake and Solomon [718] have observed similar effects in liver cells of diabetic rats.

1.2.2.(ii) Hormone Sensitive Phosphodiesterase

Modulation of high affinity cAMP-PDE activity has been observed in response to a variety of hormones [22, 192, 720, 738, 793] including glucagon [9, 312], catecholamines [64, 597], thyroxine [793] and ACTH [597] but most interest has centered around the ability of insulin to stimulate the activity of certain species of this enzyme [227, 312, 327, 328, 429, 489, 491, 501, 773, 792]. Since insulin is known to be capable of reducing intracellular cAMP concentrations [622, 617], the discovery that the activities of representatives of the enzyme system responsible for cAMP degradation are raised following insulin treatment has lead to speculation that these phenomena are causally linked [491, 506]. Predictions about the ability of variations in the activity of hormone sensitive PDE to modulate cAMP concentrations [212, 646, 313]

support the proposal that the influence of insulin upon this enzyme plays a potentially important role in the regulation of intracellular cAMP concentrations.

In adipose tissue [506, 833] and liver [489, 328], hormone sensitive cAMP-PDE is predominantly, if not exclusively confined to the particulate fraction though a soluble species has also been described in adipose tissue [830]. The soluble enzyme is inhibited by steroids (thought to have cytosolic receptors [774]) and is found in two forms, one of which is stimulated by insulin [670]. Until recently, insulin-stimulated activation of PDE could only be observed in whole cell preparations but in 1980, Marchmont and Houslay [511] demonstrated activation of high affinity cAMP-PDE following incubation of rat liver plasma membranes with insulin, ATP and cAMP. Two plasma membrane enzymes were identified, an integral PDE and a peripheral PDE of which only the latter could be demonstrated to undergo activation in the presence of insulin. The insulin-stimulated activation of peripheral high affinity cAMP-PDE is accompanied by phosphorylation of the enzyme on one site per enzyme molecule [512] and is dependent on cAMP with a Ka for this ligand of 1.6μM. The observation that similar concentrations of cAMP (approximately 1μM) prevail in intact cells [312] explains the ability of such systems to respond to insulin alone [327, 328], though, as would be expected, the magnitude of insulin induced activation of plasma membrane PDE is increased when intracellular cAMP concentrations are raised prior to insulin treatment [312]. This activation is also known to involve guanine nucleotides [310] and has been suggested to be mediated by a specific G-protein with properties similar to those of G_i [310, 311, 312] (see section 1.1.). There is evidence that peripheral cAMP-PDE is closely associated

with and perhaps even bound to plasma membrane insulin receptors [326, 328, 512] and may, therefore be subject to phosphorylation and activation by the same indirect cAMP-dependent mechanism proposed to be involved in the serine/threonine phosphorylation of insulin receptor β-subunit following insulin binding (see section 1.3.8.(ii)a). There is also evidence of close association with the adenylate cyclase system [381, 793], suggesting that the components involved in cAMP metabolism are ideally arranged for effective interaction and for execution of rapid adjustments in net cAMP accumulation. The response of hepatocyte plasma membrane high affinity cAMP-PDE to insulin [327, 328] is blocked by preincubation with glucagon in a dose-dependent manner [312, 813] but insulin sensitivity can be restored by adenosine or its non-hydrolysable analogue, PIA (N^6-[phenylisopropyl]-adenosine) [813]. Various lines of evidence suggest that the adenosine effect is mediated by binding to R-type rather than P-type adenosine receptors [813] and the interactions between glucagon, adenosine and insulin in this system are analogous to those observed on glucose transport in adipocytes [138, 267].

The enzyme responsible for insulin-induced stimulation of PDE activity observed by Loten et al. [489] in a crude hepatocyte particulate fraction differs, in a number of respects, from the peripheral plasma membrane enzyme characterised above [327, 328, 512]. The enzyme identified in crude membrane preparations appears to correspond to an insulin sensitive PDE found to be associated with a unique membrane fraction referred to as a "dense-vesicle" fraction [312] with identical density gradient migration properties to a membrane fraction with which insulin has been reported to be associated following internalisation [402, 403]. Unlike the peripheral plasma

membrane enzyme, the "dense-vesicle" high affinity cAMP-PDE is also activated by glucagon and other agents that increase intracellular cAMP concentrations [312]; indeed, the activation induced by these agents is substantially greater than that achieved by administration of insulin alone or insulin and glucagon simultaneously [312]. Interestingly, however, pretreatment with glucagon but not other agents that raise intracellular cAMP concentrations results in a synergistic effect on the activation of this enzyme following subsequent exposure to insulin, implying a complex interaction between glucagon and insulin which, apparently, is not effected through increased intracellular cAMP concentrations [312]. Despite these effects, "dense-vesicle" PDE is not thought to play a significant role in the insulin-provoked reduction in intracellular cAMP concentrations previously raised by glucagon since this phenomenon is observed even under conditions known to inhibit the activity of this particular PDE [313]. Activation of "dense-vesicle" PDE does not appear directly to involve phosphorylation as observed for insulin dependent stimulation of the peripheral plasma-membrane enzyme and is certainly effected by a distinct mechanism [845]. As might be predicted from its sub-cellular location, activation of the dense-vesicle enzyme is only observed in intact cells [845], implying that the integrity of some as yet undefined messenger system linking insulin binding at the plasma membrane to processes localised in intracellular structures remote from the initial site of insulin action (plasma membrane receptors) is necessary to allow expression of insulin's effect on this form of PDE.

While peripheral plasma membrane high affinity cAMP-PDE appears to be the major insulin sensitive PDE in liver, the major site of insulin-dependent activity in adipose tissue is the endoplasmic

reticulum [429, 501]. Insulin-sensitive PDE has also been attributed to liver endoplasmic reticulum [489] but it has been suggested [312] that this enzyme is in fact "dense-vesicle" PDE and although found in fractions with very similar density gradient migration characteristics to enzymes of the endoplasmic reticulum, it exhibits characteristics distinct from the PDEs associated with rough and smooth endoplasmic reticulum [87, 844]. In common with "dense-vesicle" PDE, the hormone sensitive PDE of adipose tissue is activated by both insulin and agents that raise intracellular cAMP levels; however, the adipose tissue enzyme displays a susceptibility to oxidation/reduction effects during homogenisation not shared by its apparent counterpart [845] in liver [227]. Homogenisation of insulin treated adipocytes has been suggested to provoke air oxidation of S-H groups resulting in stabilisation of the activated enzyme [227]. Treatments that either prevent or reverse such oxidation cause substantial depression of the insulin-stimulated activity but restoration of stimulated activity cannot be effected by subsequent treatment with oxidising agents such as hydrogen peroxide, demonstrating that the activation induced by insulin is not achieved by oxidation of the enzyme [227]. Incubation of a crude adipocyte membrane fraction at high salt concentrations (for example, 0.15M KCl, 5mM $MgCl_2$) raises the basal activity of hormone sensitive PDE almost to the level observed following insulin stimulation (2.5-3 fold increase over unstimulated) while low salt conditions lower insulin stimulated activity to approaching basal level [501]. Although high salt concentrations also raise insulin-stimulated activity and low salt concentrations lower basal activity, these effects are small (< 20%) in comparison [501]. The reducing agent dithiothreitol returns salt stimulated activity to basal levels suggesting that the native enzyme

normally exists in a reduced form in vivo since the intracellular salt composition is probably comparable to that of the high salt condition described above and hormone sensitive PDE would, otherwise, be permanently activated affording little opportunity for further activation in response to hormonal stimuli [501]. Unlike the effect of dithiothreitol on insulin stimulated activity, reversal of its effect on salt stimulation of basal enzyme activity is readily achieved by incubation with hydrogen peroxide [227]. Solubilisation of both liver endoplasmic reticulum and adipose tissue hormone sensitive PDE is accompanied by an increase in activity of the basal enzyme to the level attained following insulin activation but no change in insulin-stimulated activity is associated with such solubilisation procedures [490, 501]. The solubilised forms of basal and insulin-stimulated enzymes show identical physical and catalytic properties [227, 489, 490, 501] indicating that the two forms contain identical catalytic domains [490] and that hormonal regulation of this PDE is therefore probably achieved by modification of the regulatory domain such that its inhibitory effect is abolished [227]. Although direct involvement of phosphorylation in the hormonal activation of PDE associated with membranes other that the plasma membrane (adipose tissue endoplasmic reticulum and liver endoplasmic reticulum or "dense-vesicle") has not been proposed, there is circumstantial evidence to suggest that phosphorylation may participate in some stage of the activation process. Adipocytes deprived of ATP by treatment with uncouplers of oxidative phosphorylation (for example, 2,4-dinitrophenol or dicumarol) or inhibitors of cellular respiration (for example, KCN or sodium azide) fail to show elevated high affinity cAMP-PDE activity following exposure to insulin [430] but recover when

washed and incubated with 2mM glucose, to give a near normal insulin response [430, 803]. The requirement for ATP or metabolic energy has been confirmed by Zinman and Hollenberg [878] and almost complete reversal of insulin stimulated PDE activation has been observed only ten minutes after exposure of insulin treated cells to ATP depleting agents [430]. This information alone gives no indication of the stage(s) at which ATP dependence is imposed on induction of this particular response to insulin and therefore sheds little light on the precise nature of the mechanism(s) involved. Direct action of insulin has sometimes been discounted when discussing activation of PDE associated with intracellular membranes [835] but if, as implied by Heyworth et al. [312], the "dense-vesicle" fraction represents one of the sites to which insulin is directed after internalisation, this view may be misguided. However, assuming the major intracellular localisation of adipose tissue hormone sensitive PDE remains strictly defined as the endoplasmic reticulum which, in contrast, does not appear to be a major target organelle for internalised insulin [402, 403, 503], direct activation would indeed seem unlikely in this tissue. The case for an indirect mechanism is further strengthened by the observations that adipose tissue PDE retains the ability to respond to insulin under conditions known to completely block internalisation [503] and that the chemical mediator released from adipocyte plasma membranes following insulin binding [411, 690] is capable of activating adipose tissue high affinity cAMP-PDE [410]. Various proposals for mechanisms mediating in the response to insulin have been recruited to explain the diverse short and long term effects of this hormone but the systems involved have not yet been characterised and remain the subject of a great deal of controversy. Whether involvement

of one or more of the various kinase activities associated with insulin receptor function (see section 1.3.8.(ii)a) in events leading, ultimately, to PDE activation provides the explanation for the ATP-dependence of insulin induced adipocyte PDE activation or whether the ATP-dependent step(s) is/are yet to be identified is a matter for conjecture but, interestingly, insulin-dependent phosphorylation of a number of proteins whose physiological functions are at present uncertain has been proposed as a possible mechanism for communication between plasma membrane receptors and the intracellular targets of insulin action [18, 39, 221, 464]. In each of four independent studies, phosphorylation of at least one of these proteins was also stimulated by lipolytic agents, for example, a 46kDa molecular weight protein reported by LeCam [464] to be phosphorylated in response to both insulin and glucagon. Observations such as these provide an attractive solution to the problem of how two hormones with well characterised antagonistic effects on cellular metabolism might be envisaged to stimulate the activity of the same key regulatory enzyme, involving, as they do, mechanisms common, at least in part, to the action of both hormones. Evidence for a common mode of activation is provided by the observation that PDE stimulated maximally by insulin cannot be further activated by any of a variety of lipolytic agents [503] but Loten et al. [489] and Pawlson et al. [597] report additivity of the effects of such agents on PDE activity suggesting separate distinct mechanisms. While activation of PDE by agents such as catecholamines, theophylline and ACTH that have little else in common but their ability to raise intracellular cAMP levels [22, 597] strongly suggesting a mechanism involving cAMP-dependent phosphorylation, the factors involved in insulin- dependent activation have yet to be

determined and in both cases, detailed understanding of the events mediating these regulatory phenomena remains elusive.

The apparent paradox of an enzyme whose function is to degrade cAMP being stimulated by agents that raise cAMP levels is perhaps not, in fact, so surprising since it provides an effective negative feedback control of intracellular cAMP levels and would help to ensure that cAMP levels, raised during the response to such agents, are returned to 'resting' levels more rapidly on termination of the stimulus. Conversely, dependence of the insulin response on cAMP [511, 830] ensures that PDE activity is not stimulated when intracellular levels have fallen below a certain threshold, thus protecting against excessive depletion of intracellular cAMP during insulin action.

In addition to the acute regulatory mechanisms described above, insulin also appears to be capable of exerting longer-term control over PDE activity by a process involving protein synthesis [626, 774]. BHK (Baby Hamster Kidney) 21 cells, in which delayed (24-48 hour) insulin activation has been observed, do not, apparently, possess any membrane-bound high affinity cAMP-PDE and high affinity activity is only detected in the cytosol after exposure to foetal calf serum or insulin [626, 774]. Since such a process is obviously dependent on metabolic energy, it might be reasoned that the ATP dependence of insulin activation in adipocytes is a reflection of this requirement and need not involve phosphorylation after all. However, the length of time required for expression of insulin activation by mechanisms involving protein synthesis (24-48 hours) would seem to preclude its having much, if any, bearing on the very much more rapid activation for which the phosphorylation-mediated mechanism was proposed.

1.2.3. Interrelation of Enzyme Forms

There are, undoubtedly, a number of different forms of cAMP-PDE but, as has often been emphasized, many of the enzyme forms reported, particularly after partial purification, may in fact be artifacts of the preparation or isolation procedures and, therefore, not representative of enzymes found *in vivo* [558, 792, 833]. The apparent ease with which various combinations of subunits are seen to associate and dissociate under different conditions to produce PDEs with different properties [274, 541, 558, 609] and the observation that several of these forms cross-react with the same antibody [841] lends some support to the idea that the same catalytic subunit may be common to both low and high affinity cAMP-PDEs and that differences in properties arise from aggregation with other catalytic, inhibitory or activating subunits [542, 772].

Although the catalytic subunit of calmodulin-dependent low affinity cAMP-PDE (cGMP-PDE) is approximately the same molecular weight as the purified high affinity cAMP-PDE, these two enzymes do not share the same substrate specificity or affinities and the cGMP-PDE subunit binds calmodulin whereas the high affinity cAMP-PDE does not. Perhaps, then, there are two basic catalytic subunits, a cAMP-PDE and a cGMP-PDE, both of which have high affinity for their respective substrates but are also capable of the hydrolysis of each other's primary substrate, though with reduced affinity, only the cGMP-PDE being capable of binding Ca^{2+}/calmodulin. The purified high affinity cAMP-PDE would then represent the fundamental cAMP-PDE catalytic subunit and the purified catalytic subunit of calmodulin-dependent, low affinity cAMP-PDE would represent the fundamental cGMP-PDE catalytic subunit. Cyclic nucleotide-PDE might then be envisaged as a

conglomerate composed of both fundamental catalytic subunits associated with inhibitory peptides [424, 826] and allosteric binding sites [491] to produce the high molecular weight attributed to this enzyme form. Cyclic nucleotide-PDE would then have two distinct catalytic sites as suggested by Davis [146]. Their reduced substrate affinities could easily be explained by conformational changes or other interactions resulting from association with the other constituent subunits, one of which might also provide the common cGMP-binding allosteric regulation site. Proteolytic activation of this enzyme might then simply involve partial, if not complete release of the catalytic subunits from constraints imposed upon their activities by virtue of association with the regulatory peptides contained in the parent molecule. Sulphydryl groups and hydrophobic interactions have been suggested to be involved in aggregation of enzyme forms and may be responsible for some of the variation in PDE forms noted under different separation conditions in the same way as pH is known to affect the number of forms isolated by DEAE cellulose chromatography [792]. Other factors known to affect the form, quantity and substrate affinities of PDE include cell-cell contact, cell density and viral transformation [738]. In the light of this information, it is clear that, in an attempt to include as many as possible of the PDE forms so far discovered in a PDE classification, there is a good chance that some of its members will represent forms not found in intact cells. However, besides providing apparently extraneous and potentially misleading data, they must also reveal some clues as to the properties that might be expected to arise from different combinations of subunits. Until truly "physiological" enzymes have been positively identified and their subunit structure determined, predictions about interrelation of PDE forms remain purely

speculative but are attractive in that they seem to impart a certain degree of ordered simplicity to what appears to be an increasingly complicated picture of the PDE enzyme system.

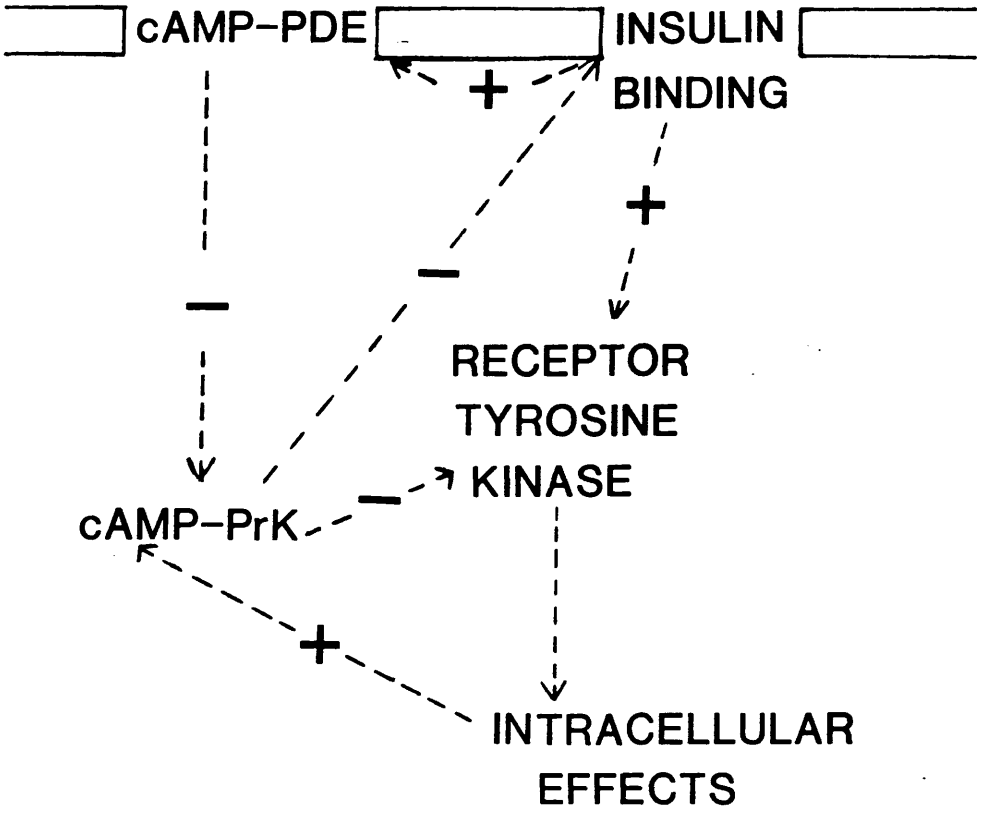
1.2.4. The Role of cAMP-PDE in Regulation of Intracellular cAMP Levels

A theoretical analysis of the functioning of high and low affinity cAMP-PDE in the regulation of intracellular cAMP concentrations has, recently, been conducted by Fell [212]. For the sake of simplicity, Fell's model treats the cell as a sphere with a single pool of cAMP and three populations of enzymes; plasma membrane bound adenylate cyclase and high affinity cAMP-PDE and soluble, low affinity cAMP-PDE. With adenylate cyclase bound to the plasma membrane, soluble low affinity cAMP-PDE activity is sufficient to create a cAMP concentration gradient, lowering the steady-state cAMP concentration in the centre of the cell. In this situation, although ineffective at controlling cAMP concentrations near the plasma membrane, the low affinity form is as effective as high affinity cAMP-PDE further inside the cell, and rate of approach to a new steady state cAMP level after adenylate cyclase modulation is also affected by this form. Because of its proximity to the site of cAMP production, membrane bound high affinity cAMP-PDE will account for most cAMP hydrolysis and at or near the plasma membrane, modulation of adenylate cyclase and/or high affinity PDE alone govern cAMP accumulation. The situation is less straightforward in nervous tissue where prevailing cAMP concentrations are intrinsically high allowing low affinity cAMP-PDE to exercise effective control over intracellular cAMP concentrations. The predicted efficacy of low affinity cAMP-PDE as a regulator of cAMP concentrations in this tissue may explain the apparent preponderance of low affinity cAMP-PDE

observed in rat and bovine brain [91, 146, 278]. Because brain tissue contains much less high affinity cAMP-PDE and much more adenylate cyclase than liver or muscle, the simulation predicts that the cAMP concentration gradient will be exaggerated with cAMP levels falling to almost nothing in the centre of the cell and modulation of high affinity activity will only be a third as effective as in liver or muscle, allowing modulation of low affinity cAMP-PDE activity to execute effective control over cAMP levels throughout the cell, even at the plasma membrane. The result is a more rapid return of cAMP levels to steady state following stimulation of adenylate cyclase activity.

Computer model simulation of the adenylate cyclase/PDE system by Reynolds [646] shows that variation in the activity of cAMP-PDE may be important in the regulation of cellular response to cAMP mediated hormones. Plasma membrane bound high affinity cAMP-PDE may be particularly important. For example, insulin activation of hormone sensitive plasma membrane bound high affinity cAMP-PDE would result in an increase by a factor of 2.6 in the concentration of glucagon required to produce an intracellular cAMP concentration of 1μM. Thus in the light of the discovery that serine/threonine phosphorylation of the insulin receptor by a cAMP dependent mechanism results in modification of receptor function (see section 1.3.8.(ii)a), plasma membrane bound PDE might be expected to be capable of influencing insulin receptor activity. Since such serine/threonine phosphorylation inhibits both insulin binding and receptor tyrosine kinase activity, this system might be imagined, under appropriate conditions, to form part of an indirect feed-forward mechanism antagonising the insulin stimulated loss of receptor function and therefore contributing to maintenance of "normal" receptor function, as shown in Figure 4.

Figure 4 Model for the Regulation of Insulin Receptor Function Mediated by cAMP-dependent Protein Kinase(cAMP-PrK)



Legend: The feedback loop inhibiting insulin receptor function via cAMP-dependent serine/threonine phosphorylation is blocked by stimulation of cAMP-PDE thus potentiating insulin receptor function.

Although low affinity cAMP-PDE activity would have little effect at low cAMP concentrations, at saturating hormone levels, stimulation of low affinity activity by Ca^{2+}/calmodulin could considerably depress the maximal cAMP concentration achieved [646].

The presence of adenylate cyclase on intracellular membranes would reduce the cAMP concentration gradient predicted by Fell's basic model [212] but if adenylate cyclase and high affinity cAMP-PDE were associated with different intracellular membranes, as suggested by Westwood et al. [835], the local cAMP concentrations around these membranes would be quite different and concentration gradients would be accentuated [212]. Local variations in cAMP concentration of this kind might be destroyed by diffusion but the activity of soluble low affinity cAMP-PDE may prevent this by producing gradients away from each cAMP producing site and, therefore, allowing greater independence of one site from the effects of others. Similar effects might result from distribution of the different forms of PDE on different cellular membranes which would then, in effect, be acting on separate pools of cAMP as proposed by Russel et al. [669]. Since uncompartmented enzymes of this type would probably have only marginal effects when independently modulated, such a separation would provide support for the advantage of evolving different PDE forms within the same cell.

Mammary tissue is known to possess an intact, functional adenylate cyclase system responsive to modulation by various agonists (see section 1.1.) and has also been demonstrated to express various classes of PDE activity, including high affinity cAMP-PDE (see section 1.2.). As noted earlier (sections 1.2.2.(ii) and 1.2.4.), the potential importance of hormone dependent modulation of high affinity cAMP-PDE activity in the regulation of cellular metabolism is well recognised,

particularly with respect to mechanisms of insulin action. It is, therefore, of great interest to determine whether the mammary enzyme is sensitive to hormonal regulation and, bearing in mind the reported rapid effects of insulin on mammary lipogenesis [656], an assessement of the ability of insulin to influence mammary high affinity cAMP-PDE activity is of particular significance. Since there is currently insufficient information available concerning the regulatory characteristics of this enzyme to allow comparison with the well characterised hormone dependence of analogous enzymes in adipose tissue and liver (see section 1.2.2.(ii)), the present study was initiated in order to establish whether, in common with high affinity cAMP-PDEs of these other tissues, the mammary enzyme is susceptible to such modulation, particularly in response to insulin.

1.3. Protein Phosphorylation

1.3.1. Glycogen Metabolism

The existence of proteins containing covalently bound phosphorus was established almost a century ago but it was not until the discovery in 1956, by Krebs and Fischer [438] that the enzyme glycogen phosphorylase could be activated by conversion of the dephosphorylated 'b' form to the phosphorylated 'a' form, that interest in protein phosphorylation began to gather impetus. In the following ten years, two more enzymes, phosphorylase kinase [439] and glycogen synthase [229] were found to be regulated by reversible phosphorylation and after the discovery by Walsh *et al*. [817] in 1968 of a protein kinase whose activity is modulated by cAMP, research advanced rapidly, giving rise to the identification of some forty or more enzymes whose activities are regulated by phospho-dephosphorylation *in vitro* as well as inumerable

non-enzymic phosphoproteins. Although the first proteins found to contain covalently bound phosphorus, phosvitin (from egg yolk) and casein (from milk) do not possess enzymic activity, it is those proteins which do possess such activity and exhibit modulation by reversible phosphorylation that have provoked the greatest interest, providing what has come to be accepted as the major mechanism mediating in the intracellular response of mammalian tissue to external physiological stimuli [114]. Early work in this field concentrated on the enzymes of glycogen metabolism and it is the regulatory enzymes of glycogen metabolism in mammalian skeletal muscle that now serve as a model for all other enzyme systems regulated by phospho-dephosphorylation.

The metabolic pathways controlled by the phosphorylation state of phosphorylase and glycogen synthase and the major factors governing their activities in skeletal muscle are summarised in Figures 5 and 6. The key enzyme in the control of glycogen metabolism, by virtue of its ability to control the activities of both phosphorylase and glycogen synthase, is phosphorylase kinase. The enzyme molecule is a tetramer of functional units, each consisting of four subunits and may be represented as $(\alpha\beta\gamma\delta)_4$. The catalytic activity appears to reside in the γ-subunit [711] while phosphorylation of the β-subunit by cAMP dependant protein kinase (cAMP-PrK) results in ~ 15-20 fold stimulation of that activity at saturating Ca^{2+} concentrations and ~ 15 fold reduction in Ka for Ca^{2+} of the Ca^{2+} binding δ-subunit [110, 115]. The α-subunit is also phosphorylated by cAMP-PrK, though less rapidly than the β-subunit [113, 119] and with no apparent effect on the enzyme's activity [113, 119]. Each α and β-subunit is phosphorylated on a single serine residue and activation of phosphorylase kinase correlates with the extent of phosphorylation of the β-subunit [110, 868].

Figure 5 Role of Phospho-dephosphorylation in Muscle cell Glycogen Metabolism and its Modulation by Hormonal and Nervous Stimuli

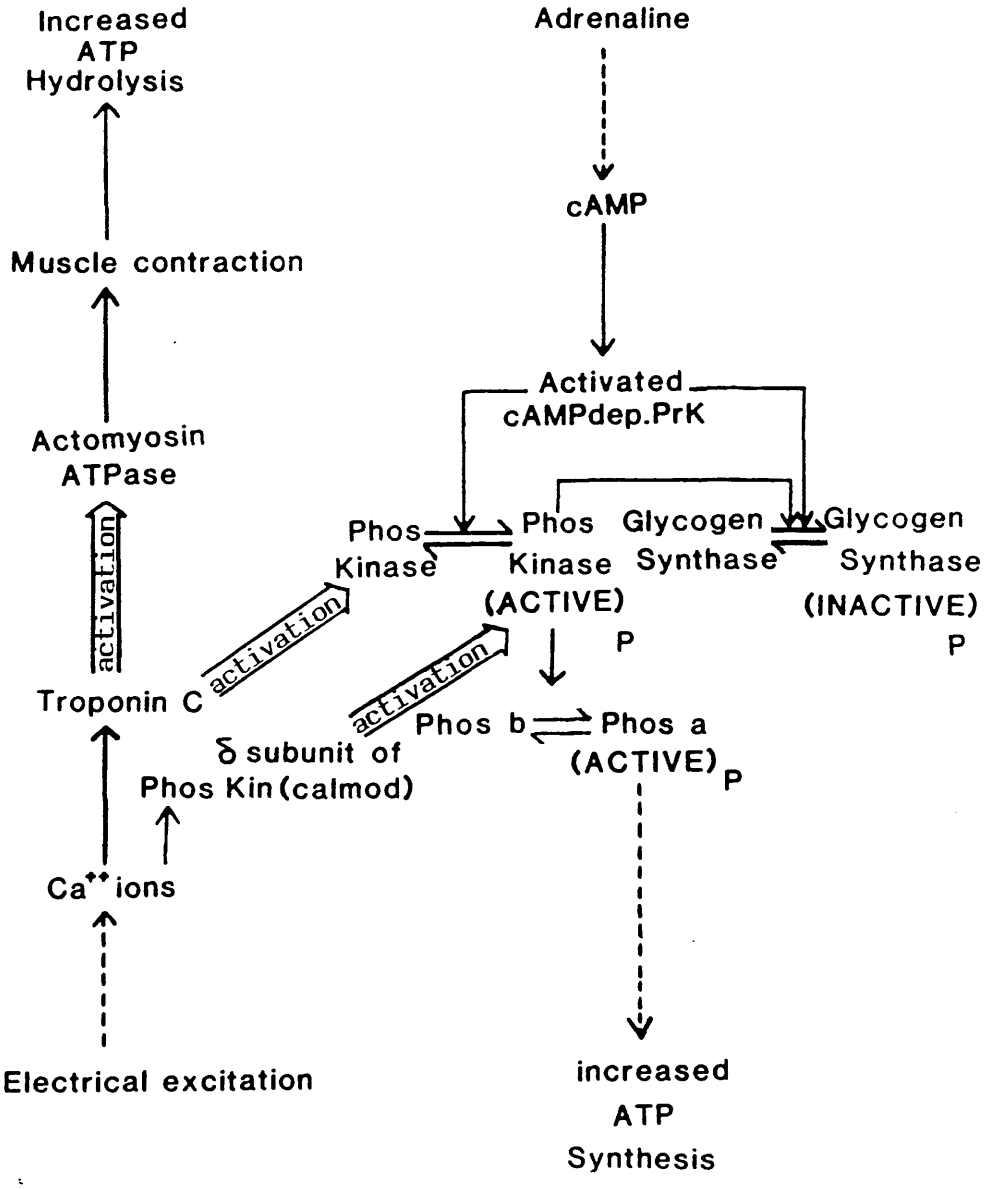
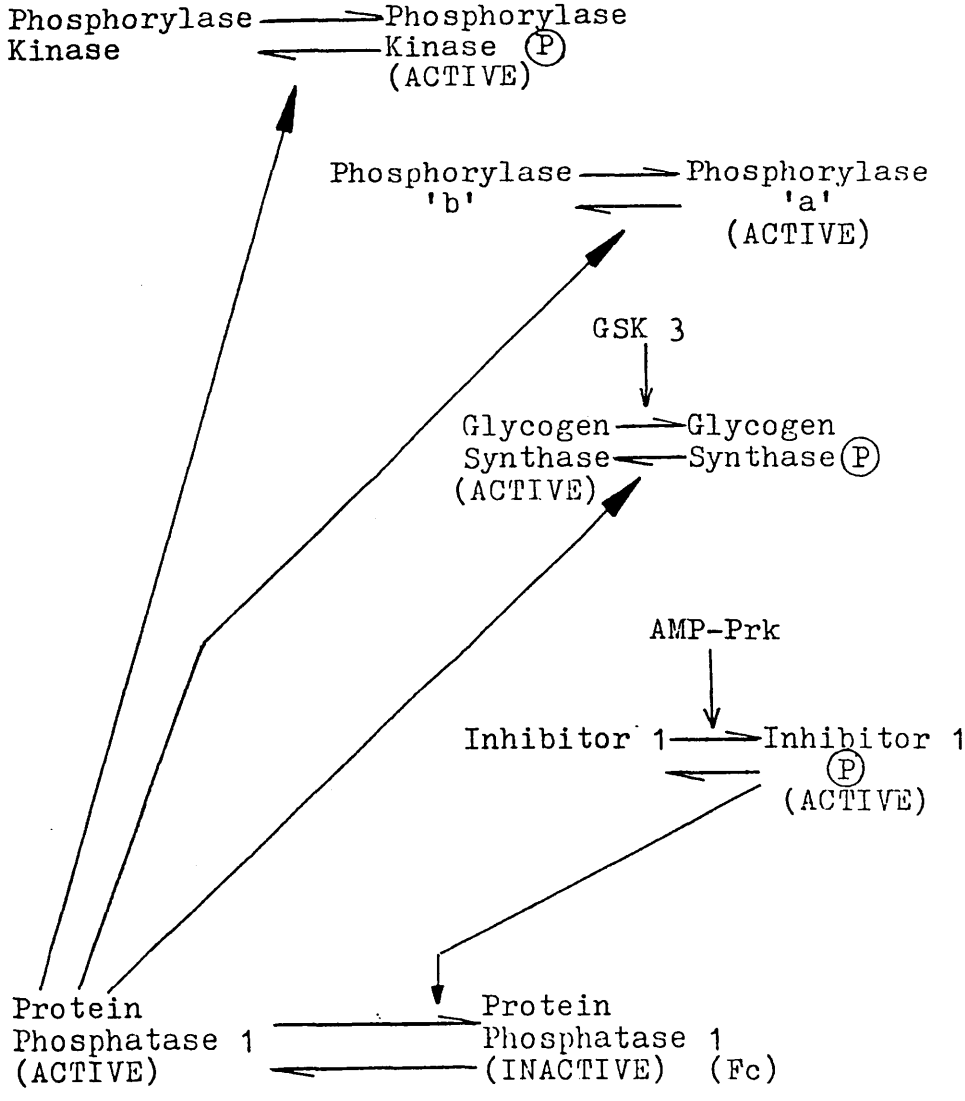


Figure 6. Influence of Protein phosphatase 1 on the activities of regulatory enzymes of Glycogen Metabolism.



In the presence of saturating Ca^{2+}, the dephosphorylated enzyme binds and is strongly activated by calmodulin (originally termed the δ'-subunit since the δ-subunit is identical to calmodulin); however, phosphorylated phosphorylase kinase shows little or no response to calmodulin [112]. There is evidence that troponin C, a Ca^{2+} sensitive protein associated with contractile apparatus function (as illustrated in Figure 5) and closely related in structure and Ca^{2+} binding properties to calmodulin, rather than calmodulin itself, might be the important activator of phosphorylase kinase in vivo [112]. Since the concentration of Ca^{2+} required for half maximal activation of dephosphorylated phosphorylase kinase, either in the presence or absence of calmodulin is approximately 20μM at physiological pH [112] and the physiological range of Ca^{2+} concentrations is approximately 0.1μM to 10μM [275a], the effect on enzyme activity mediated by either calmodulin or the δ-subunit, in response to changes in Ca^{2+} concentration in vivo, should be relatively small. However, troponin C mediated activation has a half maximal activation concentration for Ca^{2+} of only 4μM under conditions when phosphorylase kinase activity is directly proportional to the concentration of active troponin molecules (ie 1/10 saturating concentration of troponin) allowing, at 1μM Ca^{2+}, expression of an activation of around 75% that observed at maximally stimulating Ca^{2+} concentrations (over 100μM) in the absence of troponin. The effect of this is a 75-fold activation of phosphorylase kinase at 1μM Ca^{2+} (15 times greater activation than produced by 1μM Ca^{2+} in the absence of troponin) over the activity at 0.1μM Ca^{2+} and 250-fold activation at 3μM Ca^{2+} (25 times greater activation than in the absence of troponin at 3μM Ca^{2+}).

The Ca^{2+} sensitivity of phosphorylase kinase represents a means of synchronising increased production of ATP with events involving increased ATP hydrolysis. Since both muscle contraction and increased rate of ATP synthesis are initiated by Ca^{2+} acting as a 2nd messenger in the response to nervous stimulation, ATP supply and demand increase simultaneously, helping to maintain ATP levels. A comparison of the influence of troponin and phosphorylation on the response of dephosphorylated phosphorylase kinase to Ca^{2+} concentration is shown in Figure 7, illustrating clearly how the magnitude of increase in glycogen breakdown provoked by nervous stimulation is subject to considerable modification depending on the existing phosphorylation state of phosphorylase kinase. The increased response to changes in Ca^{2+} concentration conferred on phosphorylase kinase by cAMP-dependent phosphorylation may represent an important mechanism whereby the efficiency and performance of muscle might be enhanced as part of the "fight or flight" response to adrenaline (see Figure 8).

The effect of cAMP on glycogen metabolism is further magnified by its effect on inhibitor 1 which is active only after phosphorylation by cAMP-PrK on a specific threonine residue [6, 121, 562]. Active inhibitor 1 converts protein phosphatase 1 (PrP-1), the major enzyme responsible for dephosphorylation of the β-subunit of phosphorylase kinase, glycogen phosphorylase and glycogen synthase in skeletal muscle [12] to its inactive form (Figure 6).

Glycogen synthase is phosphorylated on seven serine residues of which only three (sites 1a, 1b and 2) show cAMP-dependence [116]. After exposure to adrenaline in vivo, phosphorylation of both the cAMP-dependent and three other sites termed 3a, 3b and 3c is increased, the latter three together accounting for half of that increase [591].

Figure 7. Representation of the Effects of Troponin and Phosphorylation State on the Ca^{2+}-Dependence of Phosphorylase Kinase Activity

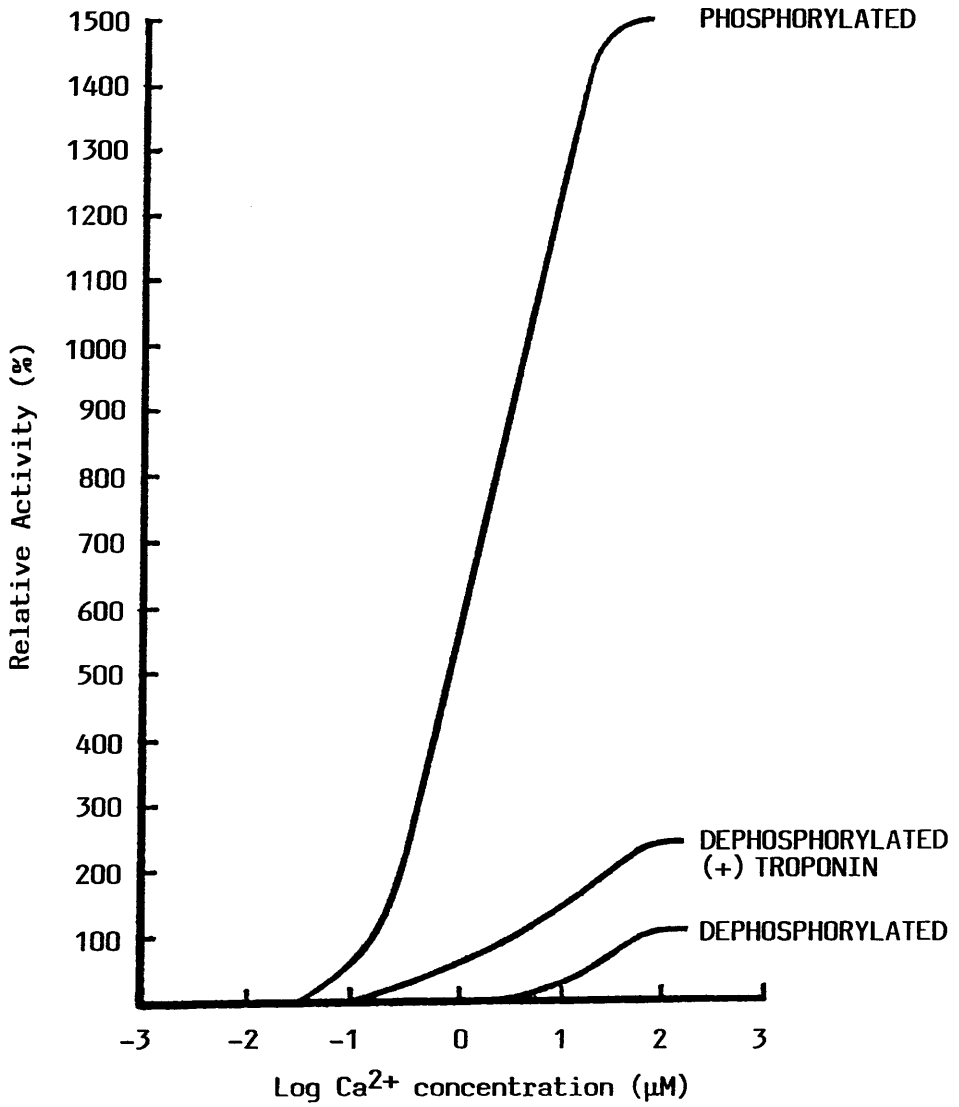
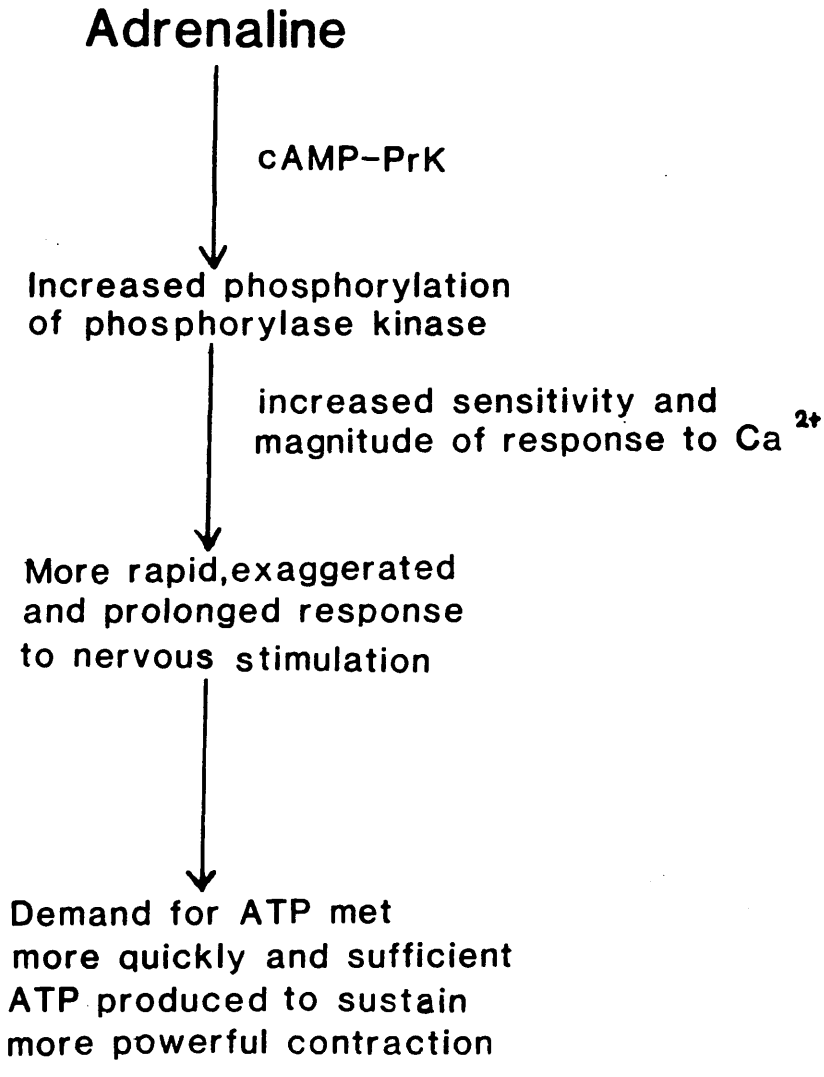


Figure 8 Role of Phosphorylase Kinase Phosphorylation in the "fight or flight" Response to Adrenaline



The phosphorylation state of sites 3 is an important regulator of glycogen synthase activity and has a greater inhibitory effect than phosphorylation at other sites on the enzyme [122, 186]. However, the enzyme responsible for phosphorylation of sites 3a, 3b and 3c, glycogen synthase kinase 3 (GSK 3) is not activated by cAMP [122, 186] and the increase in phosphorylation must therefore be the consequence of a less obvious sequence of events. There are several possible explanations for these findings including a) phosphorylation and activation of GSK 3, although there is as yet no evidence to support this [116], b) conformational change induced by phosphorylation at sites 1a, 1b and 2 allowing more rapid phosphorylation of sites 3 by GSK 3, c) reduction in dephosphorylation of sites 3 due to increased competition with sites 1a, 1b and 2 or other cAMP-dependent phosphoproteins such as phosphorylase and phosphorylase kinase for PrP-1, d) reduction in dephosphorylation of sites 3 by PrP-1 due to conformational change induced in the synthase molecule by phosphorylation of sites 1a, 1b and 2, e) activation of inhibitor 1 by cAMP-PrK resulting in inhibition of PrP-1 and f) activation of GSK 3 resulting from events associated with α-adrenergic stimulation. Consistent with a role for e) above is the observation that activation of inhibitor 1 (as described earlier) is increased markedly in response to adrenaline [404, 491] and occurs at a similar rate to phosphorylation of glycogen synthase and phosphorylase kinase in vitro [121].

In addition to cAMP-PrK, site 2 of glycogen synthase is also phosphorylated by phosphorylase kinase and glycogen synthase kinase 4 (GSK 4). The former was first reported by Roach et al., in 1978 [653a] and later, evidence was presented that phosphorylase kinase and glycogen synthase kinase 2 are in fact the same enzyme [186a]. The

amino acid sequence in the region of site 2 is similar to that around the serine residue phosphorylated by phosphorylase kinase in glycogen phosphorylase [185a] and both of these phosphorylase kinase-catalysed phosphorylations proceed at similar rates in vitro [158a, 185a, 653a]. Glycogen synthase kinase 4 phosphorylates site 2 exclusively [122] and may be largely responsible for the phosphorylation of this site observed [591] under conditions (propranolol treatment) known to inactivate both cAMP-PrK and phosphorylase kinase.

Glycogen synthase kinase 5 phosphorylates the remaining phosphorylation site on glycogen synthase but does not affect the kinetics of the enzyme in vitro [116, 611] and under all conditions so far examined in vivo, site 5 remains fully phosphorylated [590, 591]. However, it has been reported that phosphorylation of site 5 is a prerequisite for phosphorylation of sites 3 by GSK 3 [611].

Although the regulation of phosphorylase, phosphorylase kinase and glycogen synthase activities is of most obvious relevance to liver and muscle cells, there is evidence that glycogen may also have a role to play in the provision of hexose phosphate for lactose, glycolytic and pentose phosphate pathways in mammary epithelial cells [187]. The potential contribution to overall mammary cell function made by enzymes of glycogen metabolism is, however, likely to be small compared with those involved in glucose metabolism and lactogenesis particularly lactose, lipid and cholesterol synthesis. In the non-ruminant, glucose is the major source of acetyl-CoA and NADH for both fatty acid and glycerol synthesis. Pyruvate derived from its passage down the glycolytic pathway enters the mitochondria where it feeds the TCA cycle. The citrate produced may then be exported to the cytosol where it is cleaved by ATP citrate lyase to form acetyl-CoA which feeds both

fatty acid and cholesterol synthesis. During lactation, glucose utilisation for fatty acid synthesis reaches phenomenal proportions and therefore flux through the glycolytic pathway must be increased proportionately, making its acute regulation a necessity in order to maintain the synthetic and energetic economy of the cell. The ability to suppress glucose utilisation by mammary gland in the short term is particularly important to the lactating animal during periods of food deprivation when an inability to control such utilisation would severely compromise its chances of survival.

Examples of regulatory enzymes whose activities are controlled by reversible phosphorylation may be found in, among others, the pathways of glucose oxidation and, of particular significance for mammary tissue, those of lipogenesis and cholesterogenesis. Glycolytic/gluconeogenic activity is influenced by the phosphorylation state of pyruvate kinase and 6 phosphofructo-2-kinase/fructose 2-6 bisphosphatase (PFK 2/F2,6P_2ase), while the rates of fatty acid synthesis and cholesterogenesis are governed by acetyl-CoA carboxylase and 3-hydroxy, 3-methyl glutaryl Co-enzyme A reductase (HMG-CoA reductase) respectively, two more enzymes whose activities are regulated by phospho-dephosphorylation. The characteristics and significance of these and other control mechanisms are summarised below under the relevant headings.

1.3.2. Glycolysis/Gluconeogenesis

The first glycolytic enzyme recognised to display the characteristics of regulation by reversible phosphorylation was pyruvate kinase. There are at least three distinct mammalian pyruvate kinase isoenzymes of which only the L-form, found in hepatocytes, shows significant

allosteric regulation or phosphorylation (for review see [191, 550]). Although allosteric activation of liver pyruvate kinase by free fructose 1,6-bisphosphate (F1,6-P_2) has been described as the most important rapid regulatory mechanism controlling pyruvate kinase activity [219, 790] it is also evident that its deactivation by phosphorylation may be an equally significant factor, particularly in the gluconeogenic response to glucagon. During glucagon stimulated gluconeogenesis, the phosphoenol pyruvate (PEP) to pyruvate concentration ratio is increased [201, 202] and since pyruvate kinase operates at sub-saturating concentrations of PEP in vivo, the increase in its substrate concentration would lead to enhanced pyruvate kinase activity if it were not subjected to control by other means. Deactivation of pyruvate kinase by cAMP-dependent phosphorylation would therefore provide a mechanism capable of preventing significant substrate cycling during glucagon stimulated gluconeogenesis. It has indeed been shown that L-type pyruvate kinase is phosphorylated on a specific serine residue by cAMP-PrK in vitro [484] and that the phosphorylated enzyme has an increased K_m for PEP in the absence of F1,6-P_2, an increased Hill coefficient, increased sensitivity to inhibition by ATP or alanine and a decreased sensitivity to its major activator F1,6-P_2 [179, 483]. In addition, there is evidence that these inhibitory effects might be reinforced by a phosphorylation mediated increase in the sensitivity of pyruvate kinase to proteolytic degradation [191]. Pilkis et al. [624] have recently reported phosphorylation of pyruvate kinase by Ca^{2+}/calmodulin dependent protein kinase at two sites, one of which is the cAMP-PrK site while the other is specific to Ca^{2+}/camodulin-dependent protein kinase. As for cAMP-dependent phosphorylation, inactivation of pyruvate kinase induced

by phosphorylation of the Ca^{2+}/calmodulin specific site is characterised by a decrease in the affinity of the enzyme for PEP. If confirmed, this system will represent another major regulatory mechanism controlling hepatic glycolytic and gluconeogenic pathways, conferring on pyruvate kinase, as it does, sensitivity to Ca^{2+}-linked hormone action. Phosphorylation of pyruvate kinase appears, therefore, to perform an important regulatory function in the liver. However, in non-gluconeogenic tissue such as muscle, where pyruvate carboxylase and PEP carboxykinase activities are negligible, a mechanism for regulation of pyruvate kinase activity, in response to glucagon, such as the one found in hepatocytes would be redundant so it is not surprising that the pyruvate kinase found in non-gluconeogenic tissues displays classical Michaelis-Menten kinetics (review [330]) and is not phosphorylated by cAMP-PrK (review [191]).

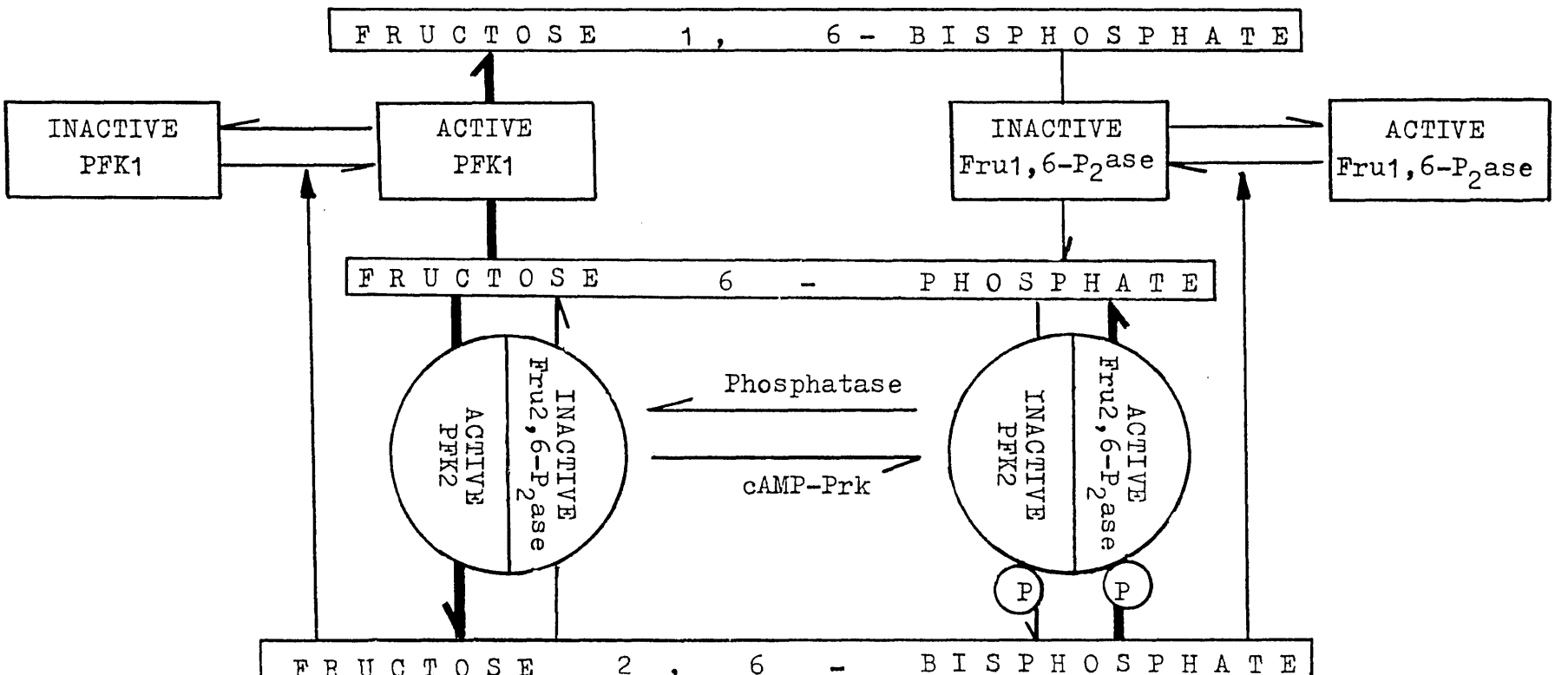
The mechanism of control of the other cAMP-dependent enzymes of the glycolytic and gluconeogenic pathways, 6-phosphofructo 1-kinase (PFK 1) and fructose 1,6-bisphosphatase (F1,6-P_2ase) respectively has, until recently, been uncertain since cAMP-dependent phosphorylation has been shown to have little or no effect on the activities of either enzyme from mammalian sources [97, 376, 622, 652] and yet PFK 1 is inhibited by glucagon treatment of hepatocytes [85, 97, 798]. In 1980, Hers and coworkers reported [798] the presence, in extracts from control livers, of a low molecular weight stimulator of PFK 1 that could restore the activity of PFK 1 derived from hepatocytes incubated with glucagon and went on [801] to identify the stimulator as fructose 2,6-bisphosphate (F2,6-P_2). As well as activating PFK 1 [309, 620, 788] F2,6-P_2 also inhibits F1,6-P_2ase [309, 618, 619, 799] and increases the affinities of both enzymes for AMP [309]. Although AMP

is a potent allosteric activator of PFK 1 and inhibitor of F1,6-P_2ase, its intracellular concentration does not vary with changes in rates of glycolysis/gluconeogenesis except during anoxia when its concentration rises [309, 622]. The increase in AMP levels observed under these conditions probably plays a significant part in the stimulation of glycolysis by anoxia but the role, if any, of F2,6-P_2 in this response is questionable [332].

Fructose 2,6-bisphosphate production and degradation are governed by a pair of enzymes analagous to PFK 1 and F1,6-P_2ase. The relative activities of these two enzymes, termed 6-phosphofructo 2-kinase (PFK 2) and fructose 2,6-bisphosphatase (F2,6-P_2ase) respectively, therefore determine the cellular concentrations of F2,6-P_2 (see Figure 9). Despite sharing the same substrate, fructose 6-phosphate, PFK 1 and PFK 2 represent separate, distinct proteins differing in a number of respects, both structurally and functionally. The same is true of F1,6-P_2ase and F2,6-P_2ase and a summary of their respective differences may be found in the review by Pilkis et al. [622]. Both PFK 2 and F2,6-P_2ase are specific for their respective substrates [181, 622, 798], are activated by inorganic phosphate [180, 798] and display product inhibition [180, 616, 797]. PFK 2 is activated by PEP [798] and inhibited by α glycerophosphate [98], ADP [616] and citrate [798], in contrast to F2,6-P_2ase which is activated by α glycerophosphate [797].

PFK 2 and F2,6-P_2ase activities from both bovine [432] and rat [180] liver copurify through various chromatographic steps [182] to give a single protein of apparent molecular weight 100-110kDa. SDS-disc gel electrophoresis of the purified protein reveals a single peptide band with a molecular weight of 55kDa [432, 622]. It would

Figure 9. Summary of Fructose 2,6-bisphosphate Biosynthesis, Regulation by Reversible Phosphorylation and Effect on Fructose 1,6-bisphosphate Production.



appear, therefore, that the native enzyme represents a dimer possessing both PFK 2 and F2,6-P_2ase activities and should consequently be classified as a bifunctional enzyme [180, 622, 797]. Recent evidence suggests that the PFK 2 and F2,6-P_2ase activities reside in separate, distinct catalytic sites but it is not known whether they are each confined to their own subunit or whether the subunits are identical, possessing catalytic sites for both reactions [184, 621, 623, 676]. The role of this apparently bifunctional enzyme in the biosynthesis of F2,6-P_2 and the effects of its modulation by cAMP-dependent phosphorylation on the activities of PFK 1 and P1,6-P_2ase are summarised in Figure 9.

Phosphorylation of the PFK 2/F2,6-P_2ase complex by cAMP-PrK <u>in vitro</u> occurs on serine residues at one site only per subunit [180, 183] with concomitant inhibition of PFK 2 and activation of F2,6-P_2ase [180, 182, 233, 796]. The same relative effects on enzyme activity are observed after administration of glucagon to hepatocytes leading to a concentration dependent decrease in F2,6-P_2 concentration [180, 333, 797]. It is interesting, however, that these effects occur at glucagon concentrations well below those required to inhibit pyruvate kinase and therefore at very much lower cAMP concentrations, raising the possibility that they may be induced by cAMP-independent mechanisms. However, it appears more likely that phosphorylation of this enzyme system is simply more sensitive to changes in cAMP concentration than that of pyruvate kinase since F2,6-P_2ase is an excellent substrate for cAMP-PrK [622] and is more sensitive to the addition of exogenous cAMP than is pyruvate kinase [617]. Fructose 2,6-P_2ase activation is particularly sensitive to glucagon and shares the same half maximally effective glucagon concentration as the decrease in F2,6-P_2

concentration. In comparison, inhibition of PFK 2 activity is closer in character to that of pyruvate kinase with a half maximally effective glucagon concentration intermediate between the latter and that of F2,6-P_2ase activation [180]. The reciprocal changes in PFK 2 and F2,6-P_2ase activities in response to glucagon [180, 616. 797, 871] and cAMP [182, 233, 796] are both characterised by a reduction in the affinity of PFK 2 for its substrate (Fructose 6-phosphate) and an increase in the substrate (F2,6-P_2) affinity of F2,6-P_2ase, suggesting that the reduction in F2,6-P_2 levels observed in response to glucagon is indeed mediated by cAMP-dependent phosphorylation [182, 622].

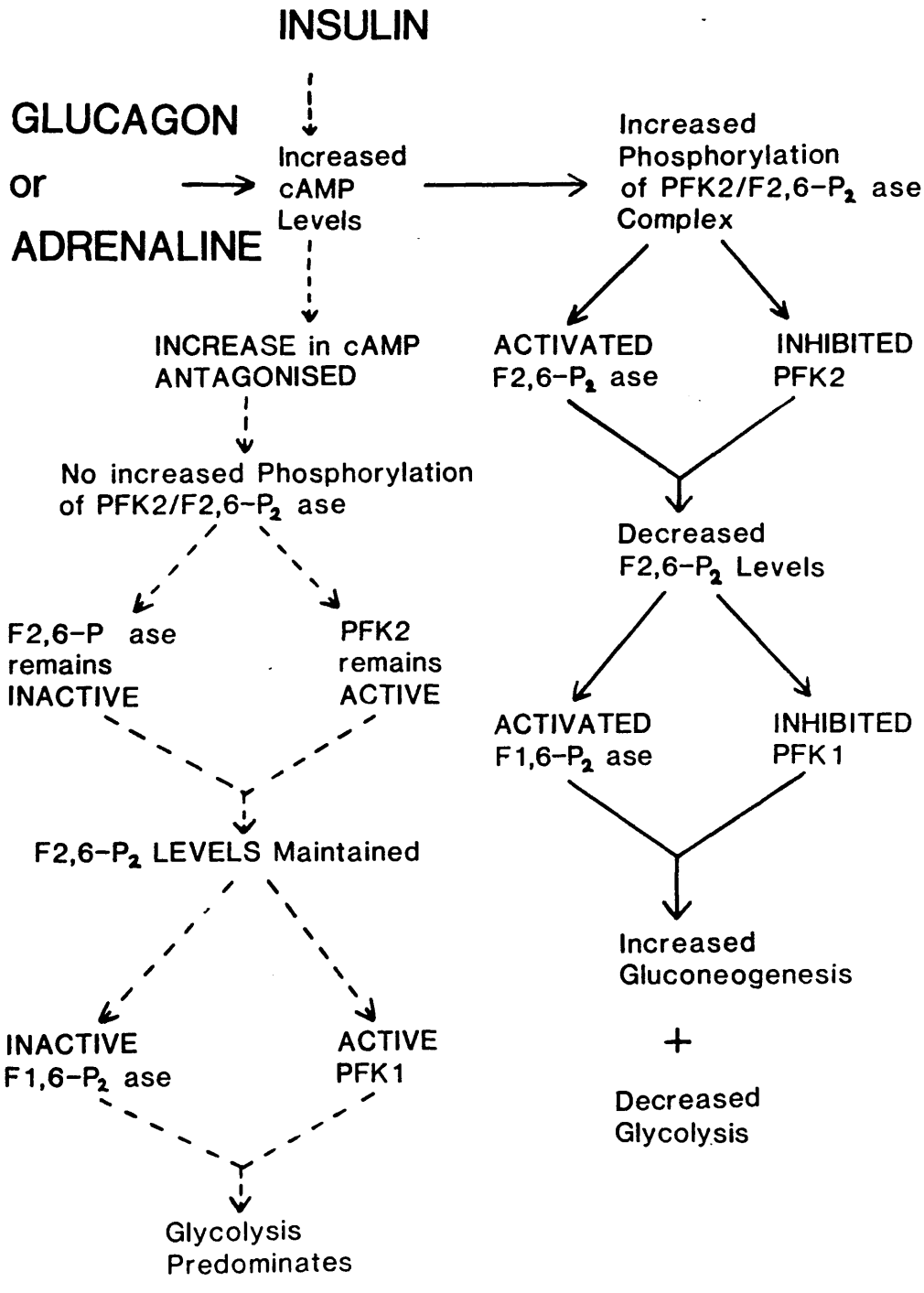
Two other hormones found to influence F2,6-P_2 levels are adrenaline and insulin. Adrenaline lowers F2,6-P_2 levels [617, 648, 649] and although insulin alone has no effect, it is capable of increasing the concentration of either glucagon or cAMP required for half maximal reduction in F2,6-P_2 levels [617]. Although many of the effects of insulin have been attributed to ill-defined cAMP-independent mechanisms, the indications are that its ability to lower intracellular cAMP concentrations is a major factor in its antagonism of glucagon or adrenaline provoked reduction in F2,6-P_2 levels [617, 622] and consequent stimulated of gluconeogenesis [622]. The potential regulatory influences of hormonal modulation of F2,6-P_2 levels are summarised in Figure 10.

1.3.3. 2-Oxoacid Dehydrogenase Complexes

The mitochondrial 2-oxoacid dehydrogenase activity of animal tissues is accounted for by three 2-oxoacid dehydrogenase complexes of which two, pyruvate dehydrogenase (PDH) and branched chain 2-oxoacid dehydrogenase (BCDH), are regulated by phospho-dephosphorylation [207, 477, 478].

Fig 10

Effects of Hormones on $F2,6-P_2$ mediated control of Glycolysis /Gluconeogenesis



The third complex, 2-oxoglutarate dehydrogenase, an integral enzyme of the TCA cycle, is regulated allosterically by ADP, ATP, Ca^{2+} and H^{+} and displays product (NADH and Succinyl CoA) inhibition [236, 523] but is not, apparently, regulated by reversible phosphorylation [637]. PDH and BCDH complexes are both also inhibited by their products (acyl-CoA and NADH) [144, 237, 604, 636] and share a general structure consisting of multiples of three types of enzyme, a 2-oxoacid decarboxylase or dehydrogenase (E_1), an acyl transferase (E_2) and a lipoyl dehydrogenase (E_3) [635, 637]. The E_3 component is a dimer of subunit molecular weight 55kDa, common to both enzyme complexes and although the E_2 components of both also share a common subunit molecular weight of 52 kDa [604, 635], the number of copies of E_2 per complex may differ and the two subunits show distinct migration characteristics on SDS polyacrylamide gel electrophoresis [635]. E_1 components contain multiple subunits of two types, α and β. The α-subunits of PDH are of molecular weight 41kDa [635] and those of BCDH, 46kDa [604, 575], while their β-subunit molecular weights are 36kDa [635] and 31-35kDa [604, 635] respectively.

1.3.3.(i) Pyruvate Dehydrogenase

The PDH complex catalyses the irreversible conversion of pyruvate to acetyl-CoA, generating NADH and CO_2, the overall reaction being represented by:-

$$\text{Pyruvate} + NAD^{+} + \text{CoA} \longrightarrow \text{Acetyl-CoA} + \text{NADH} + CO_2$$

By virtue of its position in relation to the supply of substrate for the TCA cycle in the mitochondrial matrix or, via cytosolic citrate, for fatty acid and sterol synthesis, PDH is an important regulator of net carbohydrate utilisation, particularly in tissues with

insignificant gluconeogenic capacity such as muscle, brain, and mammary gland. The enzyme's activity is subject to control by the availability of oxidisable substrate, an important factor in the preferential oxidation of lipid fuels by muscle and liver during starvation [155]. In addition, insulin has been shown to increase PDH activity, stimulating fatty acid synthesis from glucose in tissues such as liver, adipose tissue (white and brown) and lactating mammary gland [15, 27, 154, 156, 374, 522, 779].

An α-subunit of the PDH complex is phosphorylated on three serine residues by a kinase (PDH kinase) intrinsic to the complex [477, 745, 746, 747]. One particular site designated TA1 in reference to its position in a tryptic peptide of the α-subunit, is phosphorylated much more rapidly than the other two [678, 679, 746, 869]. Greater than 98% of the observed inactivation of PDH accompanying phosphorylation of the α-subunit has been attributed to phosphorylation at this site [678], the proportion of inactive complex being linearly related to its occupancy [399, 677, 679, 869]. The predominance of site TA1 in the control of PDH activity has also been confirmed in vivo [680]; however, it appears that phosphorylation of the other two sites inhibits dephosphorylation and reactivation of the complex [399, 677, 680] by the Mg^{2+}- and Ca^{2+}-dependent PDH phosphatase [157, 478, 679]. The level of phosphorylation of sites 2 and 3, at a minimum in fed, normal rats, is maximal during starvation, leading to a three-fold reduction in the initial rate of reactivation of phosphorylated complex [680]. It has been suggested that this potentially hysteretic mechanism may be capable of inhibiting reactivation of phosphorylated PDH during starvation [635, 680]. The observation that the inhibitory action of site 2 and 3 occupancy on reactivation can not be

demonstrated using PDH phosphatase purified to apparent homogeneity [763] is, as yet, unexplained but may suggest the existence, in less pure phosphatase preparations and mitochondria, of a factor expressing a permissive role in the inhibitory mechanism.

There is evidence that insulin-induced activation of PDH is achieved by an equal reduction in the level of phosphorylation of all three phosphorylation sites on the α-subunit [335] and appears to be a result of increased PDH phosphatase activity rather than inhibition of PDH kinase [156]. Activation of rat heart PDH in response to adrenaline is mediated by increases in intramitochondrial Ca^{2+} concentrations and presumably, therefore involves activation of PDH phosphatase [137, 524]. However, the same mechanism cannot, apparently be invoked to explain the activation of PDH in response to insulin since there is evidence to indicate that mitochondrial Ca^{2+} levels are insensitive to insulin and that the activation of PDH provoked by insulin in the presence of Mg^{2+} persists despite depletion of those levels [515]. The finding that, in the presence of Mg^{2+}, PDH phosphatase activity is stimulated by polyamine [644] may therefore prove important in relation to elucidation of the mechanism(s) involved in this effect of insulin, bearing in mind the insulin-like effects of polyamines (see section 1.3.8.(i)c).

1.3.3.(ii) Branched Chain 2-Oxoacid Dehydrogenase Complex

Branched chain 2-oxoacid dehydrogenase (BCDH) catalyses the conversion of ketoacids derived from the branched chain amino acids (L-isomers of leucine, isoleucine and valine) to their acyl CoA counterparts, generating NADH and CO_2 as summarised below:

$$RCO.COOH + NAD^+ + CoA \longrightarrow Acyl\text{-}CoA + NADH + CO_2$$

The branched chain amino acids are essential amino acids in man and the

rat and this reaction represents the first irreversible step in their degradation. The activity of BCDH therefore determines the dietary requirement for these amino acids and abnormally low activity (as observed in Maple Syrup Urine Disease, an inborn error of human metabolism) can result in increases in plasma concentrations of branched chain amino acids of up to 70-fold, leading to severe brain damage [94, 185].

The existence of interconvertible forms of the enzyme was demonstrated in 1978 by Parker and Randle [594]. Strong evidence for the participation of phospho-dephosphorylation in this process followed soon after [336, 462, 574] and was confirmed by work involving BCDH and its kinase copurified to apparent homogeneity [207, 289, 575]. As with PDH, phosphorylation of the BCDH complex is confined to serine residues on the α-subunit of the E_1 component [207, 463]. Phosphorylation has been observed on three tryptic peptides (designated T_1, T_2 and T_3) of the α-subunit, both in the purified enzyme [124a] and within intact mitochondria [124b]. However, these are derived from only two phosphorylation sites, of which only one (contained in the T_1 peptide) is involved in the inactivation of the enzyme complex [867, 123a]. Inactivation correlates closely with phosphate incorporation into this site [124a] and the phosphorylated form is essentially inactive under all assay conditions [868a]. The BCDH kinase which, like PDH kinase is intrinsic to the enzyme complex [463, 575], apparently loses its ability to phosphorylate the complex once separated from it, since kinase activity, lost during purification, cannot be restored by recombining fractions [575, 637]. In the absence of its own kinase, purified BCDH has, however, been shown to be only minimally phosphorylated by both PDH kinase and cAMP-PrK with no resulting change

in activity [604]. BCDH kinase activity is inhibited by the three physiological substrates of BCDH (4-methyl-2-oxopentanoate, 3-methyl-2-oxopentanoate and 3-methyl-2-oxobutyrate, the transamination products of leucine, isoleucine and valine respectively) but the concentrations required are high in relation to those likely to be encountered *in vivo* [637] implying that such a mechanism might not have any physiological significance. If, however, as proposed by Randle *et al.* [637], ADP can be shown to lower the concentration of BCDH substrate required for half maximal inhibition of BCDH kinase as has been demonstrated for pyruvate inhibition of PDH kinase [632], this system may prove to represent a significant factor in the *in vivo* regulation of BCDH activity. Reactivation of phosphorylated BCDH complex in mitochondria and rat heart is well documented [289, 594, 829] but a phosphatase with properties appropriate to an enzyme responsible for such a function has only recently been identified. One of the broad specificity, cytosolic, type 2 protein phosphatases (see section 1.3.9.(ii)) has been shown to be capable of performing this function *in vitro*, providing direct evidence for reactivation associated with dephosphorylation [289] but due to its cellular location it is unlikely to be the major BCDH phosphatase *in vivo*. Of greater interest, therefore, are the recent reports that certain partially purified preparations of ox-kidney complex contain BCDH phosphatase activity [637] and that mitochondria of the same tissue possess a BCDH complex-specific phosphatase [143, 644]. This enzyme, purified about 8000-fold from mitochondrial extracts [143] has an apparent molecular weight of approximately 460kDa and unlike PDH phosphatase, is active in the absence of divalent cations and shows no response to Ca^{2+} or polyamines but is stimulated by basic polypeptides

and inhibited by nucleoside di and tri phosphates [143, 644]. A potent protein inhibitor of BCDH phosphatase with a molecular weight of 30kDa has also been purified from bovine kidney mitochondria and found to be half maximally effective at nanomolar concentrations [644, 142a].

A higher level of control, not found in the PDH system, is imposed on the BCDH complex by an activating factor identified in high speed supernatant extracts from liver and kidney [208, 637]. The activator does not affect the phosphorylation state of the complex but rapidly activates phosphorylated BCDH to levels comparable with those exhibited by the dephosphorylated form [208, 637]. Activation will only proceed in the presence of NAD^+, CoA, TPP (Thiamine pyrophosphate) and 4-methyl-2-oxopentanoate (transaminated ketoleucine) in addition to the activator and phosphorylated BCDH complex. Neither PDH nor dephosphorylated BCDH complex activities are influenced by incubation with the activator and the other factors required for activation of the phosphorylated BCDH complex [637]. So far the BCDH activating factor, thought to be a protein of molecular weight approximately 100kDa has not been detected in either heart or skeletal muscle mitochondria and its physiological significance has recently been called into question by Yeaman et al. [868a]. These authors have presented strong evidence that the protein activator is in fact free E_1 component, presumably released from the enzyme complex during ultracentrifugation and is therefore unlikely to fulfil any regulatory role in vivo.

1.3.4. Lipid Metabolism

The two pathways responsible for lipid deposition and mobilisation, lipogenesis and lipolysis, are both subject to control by reversible phosphorylation of certain regulatory enzymes. The first and most important step of lipolysis, in terms of rate of lipid mobilisation, is

catalysed by the phospho-dephospho enzyme hormone sensitive lipase (HSL) which catalyses the hydrolysis of 1(3)-ester bonds in tri, di and mono acyl glycerols [228]. The first step in de novo synthesis of fatty acids from acetyl-CoA is catalysed by acetyl-CoA carboxylase and represents a major rate limiting step in the lipogenic pathway [529, 553, 659]. Like hormone sensitive lipase, acetyl-CoA carboxylase activity is also regulated by reversible phosphorylation [78, 79, 282].

1.3.4.(i) Hormone Sensitive Lipase

In recent years it has become apparent that the HSL activity of adipose tissue and cholesterol ester hydrolase (CEH) activity of steroidogenic tissue such as corpus luteum and adrenal cortex may in fact be two characteristics of the same enzyme protein or at least represent isoenzymes [740]. In adipose tissue CEH activity copurifies with HSL [228, 614] and accounts for all the neutral CEH activity present in that tissue [228]. Since CEH and HSL activities in this purified enzyme preparation are also inhibited by diisopropylfluorophosphate to the same extent it seems likely that they are indeed properties of the same enzyme [228]. CEH purified from adrenal cortex cells has a molecular weight of 84kDa [125], identical to that calculated for adipose tissue HSL [228] and corpus luteum CEH [124]. A direct comparison of adipose tissue HSL and adrenal cortex CEH inspired by these compelling similarities demonstrated that the two enzymes are very similar or identical [126].

The primary function of mammary cell lipid metabolism is to supply lipid for lactogenesis, consequently, the rate of lipolysis is necessarily very low to prevent the prodigious waste of metabolic energy that would accompany any significant rate of lipogenic/lipolytic

cycling. However, mammary tissue does store cholesterol as cholesterol ester and has been demonstrated to possess acyl-CoA : cholesterol acyl transferase (ACAT) [664], the enzyme responsible for synthesis of cholesterol ester from cholesterol, so it seems inconceivable that it should not also possess an enzyme with CEH activity to allow mobilization of such stores since 85-90% of bovine and rat milk cholesterol is unesterified [368, 642]. Assuming that CEH and HSL are indeed one and the same enzyme, then the indications are that mammary tissue does, at least, have the potential to express a certain lipolytic capacity but how or if this potential is suppressed are, at present, subjects for conjecture.

The role of this purportedly multifunctional lipase in mammary lipid metabolism is, at best, uncertain but its regulation, in adipose tissue, by hormone dependent phosphorylation represents an important example of the ability of such mechanisms to control the activity of major metabolic pathways. Lipolytic hormones were shown to increase glycogen phosphorylase activity in adipose tissue over twenty years ago [802] and when it was demonstrated, later, that adrenaline stimulated both lipolysis and intracellular accumulation of cAMP, the former also being achieved when dibutyryl cAMP was substituted for adrenaline [74], by analogy with liver phosphorylase it was inferred that HSL was subject to control by reversible phosphorylation. The discovery of cAMP-PrK in adipose tissue [128] and the demonstration that HSL in adipose homogenates is activated by cAMP and Mg-ATP [340, 341, 781] but blocked by cAMP-PrK inhibitor [130, 408] while activation of partially purified particulate HSL has been shown to be almost completely dependent of addition of exogenous cAMP-PrK [342, 343] are all consistent with the proposed model for activation of HSL by cAMP-

dependent phosphorylation. Confirmation that this system operates in intact cells came from work showing activation of HSL in cell-extracts by cAMP and Mg-ATP before but not after preincubation of isolated fat pads [341, 342, 781] or adipocytes [405, 408, 614] with adrenaline. Subsequent treatment of adipocytes with insulin restores the ability of the isolated enzyme to be activated by cAMP and Mg-ATP. The involvement of protein phosphorylation in this mechanism was further demonstrated by studies on deactivation of the enzyme. Reversal of cAMP and Mg-ATP dependent activation was observed in adipose tissue preparations after dialysis to remove these factors [694], a process that was stimulated by Mg^{2+}, inhibited by inorganic phosphate, could be mimicked by exogenous phosphatase from various sources [694] and was completely blocked by preparations of phosphatase inhibitor from either adipose tissue or skeletal muscle [695].

Because of the difficulties associated with obtaining sufficient quantities of purified HSL to enable studies to be made of the properties of the isolated enzyme [228, 740], absolute confirmation of the importance of HSL phosphorylation in its activation by cAMP-PrK has only been presented very recently [228, 739]. HSL is phosphorylated on a single serine residue per 84kDa subunit by cAMP-PrK and phosphorylation of this site is closely paralleled by the corresponding increase in HSL activity [739], the rate of its phosphorylation being comparable with that observed in intact fat cells after exposure to lipolytic hormones [37, 561]. Glycogen synthase kinase 4, partially purified from skeletal muscle is reported to phosphorylate a second site at a rate similar to that obtained with cAMP-PrK but occupancy of this site is not accompanied by an increase in HSL activity [740]. Cyclic GMP-dependent protein kinase appears to be capable of

phosphorylating both sites and invoking a similar parallel increase in HSL activity to that observed for activation by cAMP-PrK [740].

Deactivation of HSL has been shown to be most effectively executed by protein phosphatase 2C (PrP2C) of skeletal muscle but phosphatases 1 (PrP1) and 2A (PrP2A) also show significant activity and it has been speculated that in adipose tissue the activities of all three towards HSL might not differ by more than a factor of three [740]. However, there is insufficient evidence to predict the major phosphatase(s) responsible for HSL deactivation in vivo with any degree of confidence.

Due to the hydrophobic nature of its substrate, characterisation of the kinetic parameters affected by phosphorylation of HSL is difficult and many different factors can influence the results obtained, as evidenced by the conflicting reports of the enzyme's behaviour in systems with various emulsified substrate compositions and ill-defined interface structure [406, 407, 693, 740].

Through work with isolated adipocytes equilibrated with $^{32}P_i$, it has been established that the lipolytic hormones noradrenaline [37, 38, 740], ACTH [740] and glucagon [740] induce an increase in phosphorylation of HSL at the site phosphorylated by cAMP-PrK accompanied by a simultaneous increase in its activity, as evidenced by release of non-esterified fatty acid into the incubation medium. Both the activity and the degree of phosphorylation of HSL are rapidly returned to control levels by addition of the β-adrenergic antagonist propranolol to noradrenaline stimulated adipocytes [740] and the increases in both induced by exposure to varying concentrations of noradrenaline follow the same response curve with a half-maximal noradrenaline concentration of 25nM [37]. Insulin treatment of cells

previously exposed to noradrenaline restores the ability of the HSL activity in cellular extracts to be activated by cAMP-PrK [409] implying that insulin is able to reduce the level of HSL phosphorylation observed in response to noradrenaline. Further manipulations of the $^{32}P_i$-equilibrated adipocytes revealed that insulin does indeed reduce noradrenaline stimulated HSL phosphorylation with a concomitant fall in the enzyme's activity [37]. In the absence of lipolytic hormone, a "basal" phosphorylation is observed apparently at the site previously shown to be phosphorylated by GSK 4 but occupation of this site has no effect on HSL activity and is completely independent of insulin [37, 740].

The mechanism of action of insulin in this system is controversial but it has been asserted recently that the antilipolytic action of insulin may be entirely explained by its ability to reduce intracellular cAMP levels [852, 853]. Although this is an attractive explanation, bearing in mind the well established ability of insulin to activate high affinity cAMP-PDE in several tissues including adipose tissue (see section 1.2.2.(ii)) and the reported inhibition of adenylate cyclase in response to insulin (see section 1.1.) in adipose tissue [373], adipocyte homogenates [344], fat cell "ghosts" [308] and other preparations [452], the possibility that insulin action is mediated, at least in part, by a cAMP-independent mechanism, perhaps involving activation of specific phosphatases (see section 1.3.9.), cannot be discounted. An effect mediated by decreased cAMP levels would, however, be reinforced by the increase in PrP1 activity resulting from reduced inhibition by the cAMP-dependent inhibitor 1 (see sections 1.3.8.(i)a and 1.3.9.(i)) and may, therefore, be subject to sufficient amplification to obviate the need for a parallel, cAMP-independent mechanism.

1.3.4.(ii) Acetyl-CoA Carboxylase

The demand for fatty acids for incorporation into milk fat by the lactating mammary gland cannot be met entirely from dietary lipid and in order to maintain the high rates of lactogenesis prevailing during lactation, the rate of de novo fatty acid synthesis must be capable of responding to bridge that deficit. The daily glucose uptake of lactating rat mammary gland is comparable with the whole body glucose turnover of a male rat and as much as 70% of that uptake is used for fatty acid synthesis. However, de novo synthesis makes large demands on metabolic energy and if the pathway were not strictly controlled, substantial energetic inefficiency would result. Both acetyl-CoA carboxylase and fatty acid synthase (FAS) are subject to control by changes in the rates of their synthesis and degradation, leading to changes in the absolute quantity of individual enzyme protein molecules, thus effecting long term regulation of fatty acid synthesis (for reviews see [51, 659]). In addition to these chronic modifications of the total lipid biosynthetic capacity of mammary tissue, characteristically most marked at the onset and cessation of lactation, acute control of flow through this pathway is also afforded by covalent modification of acetyl-CoA carboxylase [280, 415].

The active form of acetyl-CoA carboxylase, purified to apparent homogeneity from rat liver [354, 556a], bovine adipose tissue [548], rat mammary gland [2] and rabbit mammary gland [281, 507] shows the same linear polymeric structure with a molecular weight of about 10^7, irrespective of its source. The polymer isolated from rat liver and mammary gland and rabbit mammary gland appears to be an aggregation of identical subunits of molecular weight 230-250kDa [2, 68, 281, 497],

each one possessing two active sites [280]. Separate, distinct catalytic sites are, therefore, assumed to account for the two partial reactions i) biotin carboxylation and ii) carboxyl transfer that have been demonstrated to constitute acetyl-CoA carboxylase activity [518]. Under specific conditions (eg high ionic strength at alkaline pH), the active polymer dissociates to give inactive "protomeric" components of molecular weight 410-560kDa which probably represent dimers of the 230-250kDa subunits [284]. An early study of the biotin binding characteristics and substrate and allosteric binding site complement of each 230-250kDa subunit suggested that the minimal binding unit of acetyl-CoA carboxylase would consist of two such subunits and, therefore, that the dimeric "protomer" represented the basic unit of the enzyme [269]. However, more recent work in three separate laboratories has indicated that each subunit binds one molecule of biotin [2, 354, 760], suggesting that the basic unit of acetyl-CoA carboxylase may not in fact be the dimeric "protomer" but rather the 230-250kDa subunit itself.

Whether the reversible transition between active polymeric and inactive protomeric forms operates in vivo is uncertain. Dissociation of the polymer may be induced in vitro by incubation of acetyl-CoA carboxylase with its substrates in the absence of its allosteric activator, citrate [270] suggesting that low cytosolic citrate concentrations may be capable of shifting the polymer/protomer equilibrium towards the inactive protomeric form. Indirect evidence that such an equilibrium might exist in intact cells has been generated by work with chicken liver cell monolayer cultures treated with digitonin to perforate the plasma membrane without disrupting the cell [533]. In such preparations leakage of acetyl-CoA carboxylase is much

slower than that of other cytosolic enzymes such as LDH. Moreover, pretreatment of these cells with dibutyryl cAMP which should promote dissociation to the protomeric form by precipitating a reduction in cytoplasmic citrate concentrations [827], causes rapid leakage of acetyl-CoA carboxylase following digitonin treatment implying that a greater proportion of enzyme was indeed present in its protomeric form [533]. The mechanism by which citrate influences the protomer/polymer transition *in vivo* may be more complex than anticipated since phosphorylation both *in vivo* [251] and of partially purified acetyl-CoA carboxylase [470] leads to an increase in the proportion of the enzyme found in its inactive protomeric form and this phosphorylation is inhibited by high citrate concentrations [470]. At lower concentrations of citrate, phosphorylation is enhanced by cAMP [470] so the increase in protomeric/polymeric ratio observed after treatment of chicken liver cells with dibutyryl cAMP [533] may be the result of a combination of direct allosteric effects and both direct and indirect (via attenuation of inhibition) effects on extent of cAMP-dependent acetyl-CoA carboxylase phosphorylation.

Animal acetyl-CoA carboxylases are completely inactive in the absence of citrate or isocitrate. Activation by citrate is characterised by an increase in the V_{max} rather than a decrease in K_m [268, 827] and involves both partial reactions [518]. Since citrate, an intermediate of the TCA cycle, transported from mitochondrial matrix to cytoplasm, forms the substrate for ATP citrate lyase, giving rise to the major source of cytoplasmic acetyl CoA, it may also be regarded as the basic precursor of all *de novo* fatty acid synthesis. The concentration of citrate required for half maximal activation of acetyl-CoA carboxylase *in vitro* is of the same order as the range of

concentrations (0.2-2mM) estimated to prevail in the cytoplasm [706]. It has therefore been proposed that citrate activation of acetyl-CoA carboxylase represents a mechanism for feed forward control of fatty acid synthesis governed by the concentrations of TCA intermediates but there is no direct evidence to either confirm or discredit this suggestion [280]. A similar situation exists concerning the inhibition of acetyl-CoA carboxylase by palmityl-CoA and other fatty acyl-CoAs observed in vitro [52, 261, 573, 576] but the indications are that this mechanism is, at least potentially, a physiological one providing negative feedback control of acetyl-CoA carboxylase activity by what may be regarded as end products of fatty acid synthesis [261, 576].

The first direct evidence that acetyl-CoA carboxylase may be phosphorylated and that this event is accompanied by reduced enzyme activity was provided by Carlson and Kim [78] who demonstrated that time dependent activation of a partially purified acetyl-CoA carboxylase preparation could be blocked by the phosphatase inhibitor NaF and that inactivation required Mg-ATP and the presence of a supernatant protein fraction [78, 79, 465]. Several factors have since been proposed to account for the Mg-ATP dependent inactivation observed by these workers including a well documented inactivation initiated by slow carboxylation in the presence of Mg-ATP and traces of dissolved CO_2 [270, 276, 453] but this [470] and other proposals have either proved invalid or cannot be demonstrated to explain all of the observed effects. Using the same acetyl-CoA carboxylase preparation, Carlson and Kim have characterised more fully the change in kinetics associated with its phosphorylation. Under such conditions, dephosphorylated acetyl-CoA carboxylase expresses five-fold greater activity, even at saturating citrate concentrations, and displays a half-maximal

concentration for activation by citrate an order of magnitude lower than that exhibited by the phosphorylated form [80]. Despite the early scepticism, regulation of acetyl-CoA carboxylase activity by reversible phosphorylation has been confirmed in isolated cells [69, 323, 849], tissue homogenates [528, 873] and the purified enzyme [282, 283, 551, 777]. Because of the relative abundance of acetyl-CoA carboxylase in lactating mammary tissue [280] and the benefits associated with its apparently low intrinsic level of proteolytic activity [281], lactating rat and rabbit mammary gland have often been chosen as a source of acetyl-CoA carboxylase for studies involving the purified enzyme. The mammary gland enzyme can be phosphorylated by at least five different protein kinases; cAMP-PrK [103, 282, 283], acetyl-CoA carboxylase kinase 2 [551], Ca^{2+}/calmodulin-dependent protein kinase [856] and casein kinases I and II [551]. Of these, at least two (cAMP-PrK and acetyl-CoA carboxylase kinase 2) have been shown to be capable of inactivating acetyl-CoA carboxylase [283, 551]. Both phosphorylate sites located on identical peptides and their dephosphorylation leads to complete reactivation of the enzyme [283, 286, 551]. Both this and the equivalent site in rat liver acetyl-CoA carboxylase are dephosphorylated by PrP1, PrP2A and PrP2C (see section 1.3.9.) [283, 346, 347]. Phosphorylation of purified rat mammary acetyl-CoA carboxylase by cAMP-PrK decreases the V_{max} of the enzyme two-fold and increases the concentration of citrate required for half-maximal activation two-fold [283], qualitatively similar, though less pronounced effects to those observed by Carlson and Kim for phosphorylation of partially purified rat liver enzyme [80]. In hepatocytes exposed to glucagon, increased phosphorylation of acetyl-CoA carboxylase at the cAMP-dependent site accompanies the

decrease observed in activity of acetyl-CoA carboxylase purified rapidly by avidin-Sepharose chromatography and entirely accounts for the effect of the hormone on fatty acid synthesis in such preparations [323]. This and comparable correlations between phosphorylation of acetyl-CoA carboxylase and decrease in activity of the purified enzyme, observed in adipocytes exposed to glucagon or adrenaline [322], provide strong evidence that phosphorylation of acetyl-CoA carboxylase by cAMP-PrK represents an important physiological mechanism for the control of fatty acid synthesis. Incubation of adipocytes with adrenaline has been shown to cause a decrease in V_{max} and an increase in K_a for citrate of acetyl-CoA carboxylase in crude extracts [69] and purified enzyme [322] prepared from such cells. These changes are also very similar to those associated with phosphorylation of purified rat mammary acetyl-CoA carboxylase by cAMP-PrK [283]. Although an analogous glucagon induced decrease in acetyl-CoA carboxylase activity is also observed in hepatocytes, in this instance the major component of inactivation is a decrease in the enzyme's susceptibility to activation by citrate [323], the decrease in V_{max} being less pronounced than that reported for similarly treated adipocytes [322]. Differences in basal phosphorylation site occupancies have been recruited to offer an explanation for this discrepancy [322]. Hepatocyte acetyl-CoA carboxylase displays a higher level of phosphate incorporation per subunit under basal conditions than its counterpart in adipose tissue [322, 323] and phosphorylation of acetyl-CoA carboxylase on a particular peptide (peptide 1) phosphorylated during exposure to glucagon in both hepatocytes and adipocytes is known to affect mainly the concentration of citrate required for activation of this enzyme [551]. However, this site is only one of a number of sites found to be

phosphorylated in response to adrenaline and glucagon in adipocytes [322] and the proposal is that it is these extra sites that are responsible for the large decrease in V_{max} characterising the adipocyte acetyl-CoA carboxylase response to these hormones [322]. The explanation for the lack of such an effect in hepatocytes being that the extra sites are phosphorylated even under basal conditions in liver cells thus accounting for the higher phosphate content per subunit, lower V_{max} and comparatively small change in V_{max} following hormone treatment. Whether phosphorylation of these other sites is also cAMP-dependent is not known but it has been emphasised that such modulation need not occur directly and might equally well be achieved via activation of protein phosphatase inhibitor proteins [322] (see section 1.3.9.(i)). Though plausible, this elegant explanation for the discrepancies between hormone induced acetyl-CoA carboxylase deactivation in adipocytes and hepatocytes does not appear consistent with the recent findings of Swenson and Porter [751] who report inactivation of hepatocyte acetyl-CoA carboxylase by glucagon involving a marked decrease in V_{max} (of the order observed in adrenaline treated adipocytes) accompanied by a percentage change in $A_{0.5}$ for citrate of a similar magnitude reported for acetyl-CoA carboxylase in adrenaline treated adipocytes (though-absolute values were depressed in comparison). These authors point to the inclusion of protease inhibitors in their own incubations as the key to explaining the disparity between theirs and previous reports of glucagon dependent effects in hepatocytes [323, 849] and have shown how the observed phosphate incorporation and acetyl-CoA carboxylase activity can both be dramatically reduced by proteolytic activity, obscuring any changes resulting from exposure to glucagon. The role, if any, of

phosphorylation at sites other than peptide 1 in response to glucagon or adrenaline remains, therefore, uncertain but the available evidence seems to suggest that under conditions where proteolytic activity is minimised, liver and fat cell acetyl-CoA carboxylase might respond to hormonal elevation of intracellular cAMP in a very similar manner. Such studies have proved impossible in lactating mammary gland since both acetyl-CoA carboxylase activity and lipogenesis are apparently insensitive to glucagon in this tissue [656]; however, effects of insulin have been reported in all three cell types (liver [848], adipose tissue [65, 66, 874] and mammary gland [656]). Activation of the enzyme in liver and adipose tissue is accompanied by a slight increase in phosphorylation [66, 848, 850]. However, although exposure of rat adipose tissue to insulin results in a 2-3 fold increase in the acetyl-CoA carboxylase activity of crude tissue extracts [276, 466, 726] and activation is accompanied by an increase in the phosphorylation of a site distinct from those affected by exposure to adrenaline [66, 850], Witters et al. [850] have observed no effects of the increased phosphorylation on the activity of purified acetyl-CoA carboxylase. The effect of insulin on the degree of phosphorylation of mammary acetyl-CoA carboxylase freshly prepared from cellular extracts was, until recently, uncertain [286]. In common with work by Witters et al. in adipose tissue, Clegg et al. [102] have reported no effect on the acetyl-CoA carboxylase activity of purified enzyme from insulin treated mammary acini despite previous work demonstrating a dose dependent stimulation of lipogenesis by insulin in the same type of preparation [656]. While it is still accepted that there must be at least one other rate-limiting step in the pathway for fatty acid synthesis (as suggested by these findings), Munday and Hardie [552]

have now demonstrated that manipulation of insulin levels *in vivo* has marked effects on the phosphorylation state and activity of this enzyme. In this study, refeeding rats starved previously for 24h resulted in a decrease in phosphate content of purified acetyl-CoA carboxylase and a concommitant activation (increase in V_{max} and decrease in concentration of citrate required for half-maximal activation) of the enzyme to control (fed) levels. These effects were blocked completely by treatment of the starved rats with streptozotocin prior to refeeding, suggesting that the increase in plasma insulin that occurs on refeeding was responsible for the activation of the enzyme. Furthermore, this activation could also be achieved by treating acetyl-CoA carboxylase purified from the starved rats with protein phosphatase-2A *in vitro*, providing support for the involvement of enzyme phosphorylation in these regulatory processes.

In conclusion, it seems certain that phosphorylation/dephosphorylation of acetyl-CoA carboxylase represents a major mechanism for acute regulation of fatty acid synthesis in both liver and adipose tissue though the significance of this regulatory mechanism for the control of fatty acid synthesis in mammary gland remains uncertain. The relevance of insulin effects on acetyl-CoA carboxylase phosphorylation in terms of regulation of its activity is controversial. The demonstration of a loss of activation during purification of the enzyme while the increased phosphorylation is preserved [850] tends to suggest that phospho/dephosphorylation is not an important factor in the control of adipocyte-acetyl-CoA carboxylase activity by insulin. However, the apparently insulin-dependent effects observed *in vivo* by Munday and Hardie [552] on both phosphorylation and

activity of acetyl-CoA carboxylase, provide strong evidence that, at least in mammary tissue, the phosphorylation state of this enzyme plays an important role in the regulation of its activity in response to hormonal stimuli.

1.3.5. HMG-CoA Reductase

Though not a quantitatively major component of milk, cholesterol is essential for the maintenance of fat droplet membrane structure [163] and considerable quantities (16mg/day) are secreted in rat milk [95]. The cholesterol requirements of mammary tissue for milk production may be met from two sources, i) circulating serum cholesterol, ii) cholesterol synthesis. The relative contributions of these two sources is controversial but sterol synthesis by mammary gland has been demonstrated both *in vivo* [95] and *in vitro* [135, 627] and, more recently it has been suggested that a major proportion of rabbit milk sterol is supplied by mammary cholesterol synthesis [536] though Gibbons *et al*. [244] have calculated that cholesterol synthesised by the mammary gland provides a maximum of only 40% of the daily total secreted in milk. Whatever the actual proportion of milk cholesterol derived from its synthesis in mammary tissue, in order to meet the varying demands for cholesterol imposed on the tissue by diurnal variation in milk production, cholesterol synthesis must be amenable to short-term regulation. The third step in mammalian cholesterol synthesis from acetyl-CoA, conversion of HMG-CoA and NADPH to mevalonate, CoA and NADP, is catalysed by HMG (3-hydroxy, 3-methylglutaryl)-coenzyme A reductase (HMG-CoA reductase). Generally believed to be the rate limiting enzyme in *de novo* biosynthesis of cholesterol [60, 162, 209] it can be safely assumed that under most

circumstances the activity of HMG-CoA reductase will be an important determinant of the rate of cholesterol synthesis in the liver though exceptions include fasting [657] and dexamethasone treatment [634]. High HMG-CoA reductase activities have been observed in both rat [224] and rabbit [536] lactating mammary gland but the major site of sterol synthesis is the liver [786] and consequently it is the HMG-CoA reductase of the latter tissue which has been most extensively studied.

In mammalian cells, HMG-CoA reductase consists of a single 90-100kDa glycosylated polypeptide [61, 92, 175, 279, 479, 786] though whether it exists as a monomer or an oligomer in vivo is not yet known. The enzyme is integrally associated with endoplasmic reticulum [61, 479] but its active site is exposed to the cytosol [608] and limited proteolysis releases a catalytically active soluble peptide of molecular weight 50-56kDa [398].

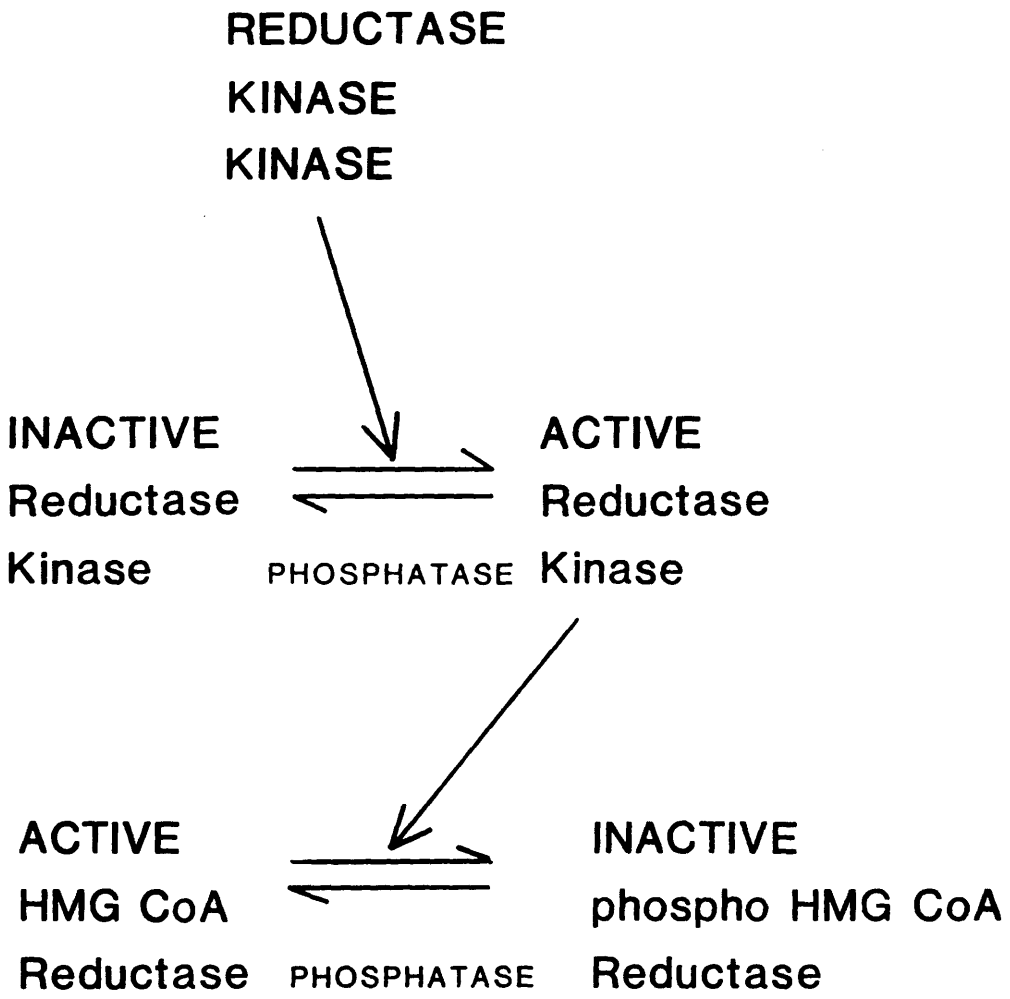
Early work on modulation of HMG-CoA reductase activity concentrated on mechanisms associated with long-term regulation involving changes in the amount of enzyme protein. Regulation of this kind results from manipulation of synthesis at both transcriptional [96, 480] and post-transcriptional [366, 419, 428] levels and variation in the rate of enzyme degradation [89, 209, 383, 708]. Mechanisms implicated in short term control of HMG-CoA reductase include the influences of membrane composition and fluidity [214, 540, 650, 709], thiols [165, 658], microtubules [807, 808] and cytosolic lipid inhibitors and their binding proteins [272, 467, 532] on the enzyme's catalytic activity but perhaps the most significant acute control mechanism, particularly with respect to hormonal effects, is provided by modulation of its phosphorylation state. The first indication that HMG-CoA reductase activity is susceptible to modulation by reversible

phosphorylation came from work by Beg et al. [30] who reported that HMG-CoA reductase activity of washed rat liver microsomes was reduced dramatically by prior treatment with Mg-ATP and a fraction from rat liver cytosol. The degree of inactivation was time-dependent and the lost activity was restored by treatment with a second cytosolic fraction. The Mg-ATP dependent decrease in HMG-CoA reductase activity in the presence of cytosolic or endogenous microsomal factors has been confirmed in several laboratories [53, 62, 93, 348, 569, 579, 696] as has its reversal following treatment with a cytosolic factor [195, 337, 348, 569, 579, 696]. Inactivation of the microsomally bound enzyme by incubation with [γ-^{32}P]ATP results in the formation of ^{32}P-phosphoserine labelled HMG-CoA reductase [32, 249, 394, 395] and parallel inactivation and phosphorylation of purified HMG-CoA reductase has also been demonstrated using purified or partially purified HMG-CoA reductase kinases from rat liver microsomes [34, 213] and cytosol [292]. The latter kinase preparation exhibited a requirement for ADP, consistent with previous reports that inactivation of HMG CoA reductase by Mg-ATP was dependent on ADP [62, 659]. The inactivation produced is characterised by a decrease in the enzyme's V_{max} with no change in the K_m values for either HMG-CoA or NADPH and appears to be provoked by phosphorylation of a single site [63, 213] though at least one other structurally distinct site has also been identified [220, 394]. No function has as yet been assigned to phosphorylation of this second or other sites but it has been suggested that such phosphorylations may control the susceptibility of the "primary" site to the action of kinases or phosphatases or may influence the rate at which HMG-CoA reductase is proteolytically degraded [398]. Activation of ^{32}P labelled HMG-CoA reductase accompanied by concomitant release of ^{32}Pi

from the enzyme has been observed during incubation with various purified phosphatase preparations [32, 63, 213, 250, 736]. However it has been estimated that PrP2C would represent the major HMG-CoA reductase phosphatase in liver, though by virtue of its cellular distribution (a significant proportion is associated with the microsomal fraction), PrP1 may also be an important HMG-CoA reductase phosphatase [345].

The enzyme responsible for phosphorylation of HMG-CoA reductase, HMG-CoA reductase kinase is cytosolic and is itself, apparently, the substrate for another cytosolic protein kinase, termed reductase kinase kinase. Following the discovery by Ingebritsen et al. that the rate of inactivation of reductase kinase was reduced in incubations containing phosphate and completely blocked by 50mM NaF (both inhibitors of phosphatases, the latter being the more effective) [245, 348, 351] but that kinase activity could not be restored by addition of these ions after inactivation [345, 348], it was proposed that regulation of HMG-CoA reductase is achieved via a bicyclic phosphorylation cascade as shown in Figure 11. Active reductase kinase is inactivated in the presence of a broad specificity phosphatase and can be reactivated by incubation with Mg-ATP and reductase kinase kinase [348, 351]. In addition, incubation of homogeneous reductase kinase with its kinase in the presence of [γ-^{32}P] ATP, Mg^{2+} results in incorporation of ^{32}P into reductase kinase, while subsequent incubation with a purified protein phosphatase precipitates a 70% loss of reductase kinase associated ^{32}P [33] further strengthening the proposal that HMG-CoA reductase kinase activity is regulated by reversible phosphorylation. Beg et al. [33] have purified HMG-CoA reductase kinase to apparent homogeneity and reported a molecular weight of

Figure 11 Bicyclic Phosphorylation Cascade Involved in Inactivation of HMG CoA Reductase



380kDa with a subunit weight of 58kDa suggesting that the native enzyme is a hexamer.

Despite reports that treatment of isolated rat hepatocytes with glucagon or cAMP markedly reduces HMG-CoA reductase activity [176, 246, 307, 349, 592], it has been demonstrated that both reductase kinase and reductase kinase kinase are cAMP-independent protein kinases [31, 36, 351] suggesting that the effects observed in intact cells may be due to phosphatase inhibition rather than activation of the kinases comprising the bicyclic phosphorylation system (Figure 11). Microsomal phosphatase 1 activity might then prove to be an important factor in regulation of HMG-CoA reductase since the PrP1 inhibitor, inhibitor 1, is activated by cAMP ([212, 247, 274], see also sections 1.3.8.(i)a and 1.3.9.(i)) though, as for HMG-CoA reductase itself, the major reductase kinase phosphatase is thought to be PrP2C [345]. Little is known about the factors governing the activities of reductase kinase and reductase kinase kinase but it has recently been reported that the latter is modulated in vivo by mevalonolactone [35]. It has also been discovered that reductase kinase is not the only protein kinase capable of phosphorylating and inactivating HMG-CoA reductase. Beg et al. [35] have described inactivation of both the native reductase (molecular weight 100kDa) and its 53kDa soluble catalytic fraction by protein kinase C (see section 1.3.8.(i)b) and have established that this inactivation is accompanied by the incorporation of approximately 1 mole phosphate per mole of native 100kDa enzyme subunit.

Regulation of HMG-CoA reductase in intact animals is complicated and the activity expressed in vivo depends upon both the total amount of enzyme protein present and the state of phosphorylation of that enzyme population. Both parameters are under hormonal control (see

reviews by Ingebritsen [345], Dugan and Porter [168] and Rodwell et al. [657]) and have been shown to vary in a diurnal cycle [173, 657]. Consequently, phosphorylation of HMG-CoA reductase is now accepted as a major regulator of cholesterol synthesis in vivo.

1.3.6. ATP Citrate Lyase

ATP citrate lyase catalyses the conversion of cytosolic citrate to acetyl-CoA and therefore governs the supply of substrate for de novo synthesis of both fatty acids and sterols but is not rate-limiting for either pathway. Although ATP citrate lyase is phosphorylated in intact cells and is one of a small number of identified proteins for which insulin increases the degree of phosphorylation [284], there is, as yet, no evidence that these events have any effect on its catalytic activity.

1.3.7. Non-Enzymic Targets for Protein Phosphorylation

1.3.7.(i) The Histones

The range of non-enzymic proteins known to be substrates for protein kinase activity is vast and expanding all the time but in general the possible significances of such phosphorylations are unclear and consequently, most have attracted only limited interest. Among the non-enzymic targets for protein phosphorylation, which include proteins of cytosolic, membrane, nuclear, ribosomal, contractile and cytoskeletal origin, one of the most extensively studied groups, the histones, particularly H1 and H3, show interesting correlations between degree of phosphorylation and stage of cell cycle. Histone H1 which appears to have an important influence on chromatin packing, particularly during mitosis [302, 378, 866] shows increased

phosphorylation at up to six sites on serine and threonine residues during interphase and metaphase [380, 451, 459]. There is evidence that the phosphorylation of one of these sites in the C-terminal region of the molecule accompanies DNA synthesis and it has been suggested that this may have a role in distinguishing replicated DNA from unreplicated DNA and ensuring that each region of the chromosome is replicated only once per cell cycle [421]. Phosphorylation of H1 proceeds under the influence of at least three different protein kinases or groups of protein kinases, one of which was discovered to be cAMP-PrK [454, 505] and to predominantly phosphorylate only one of the N-terminal region serine residues (serine-37) [455]. Phosphorylation of the remaining sites is independent of cAMP and accounted for by another kinase specific to one particular, though different serine residue (serine-106 in the globular region) [455] and an enzyme or group of enzymes known as growth associated histone kinase (kinase-G), capable of phosphorylating multiple sites on H1 [285, 321] including the DNA synthesis dependent site [285]. Although the links between protein phosphorylation and control of cell division are, at best, tentative, it is such correlations (including that observed between ribosomal protein S6 phosphorylation and stimulation of protein synthesis [see below]) and the known significance of phosphorylations involving enzyme modulator proteins such as inhibitors 1 and 2 (see section 1.3.9.(i)) that provide the most promising indications of a role for phosphorylation of non-enzymic proteins in the control of cellular activities.

1.3.7.(ii) Ribosomal Protein S6

Stimulation of ribosomal protein S6 phosphorylation on multiple sites has been observed following treatment of cells with a variety of hormones and growth factors including insulin [293, 461, 560, 599, 713, 768, 838], epidermal growth factor (EGF) [560, 600, 768], prostaglandin $F_2\alpha$ [768], platelet-derived growth factor [88, 565, 836] and insulin-like growth factor [293]. The rapid activation of S6 phosphorylation observed in response to EGF in quiescent swiss mouse 3T3 cells has been shown to be accompanied by stimulation of protein synthesis, followed, 12 hours later, by a surge in the rate of initiation of DNA synthesis, leading, ultimately, to cell division [765, 768]. The dose responses of S6 phosphorylation, protein synthesis and DNA synthesis for activation by EGF are closely related [768] and, together with the findings of other studies both *in vivo* and *in vitro* (see [572] and references therein), these observations provide evidence in support of the hypothesis that multiple phosphorylation of S6 is a prerequisite for the activation of protein synthesis accompanying initiation of cell division.

Phosphorylation of S6 has been reported to be catalysed by several protein kinases, including cAMP-dependent protein kinase [170, 837, 839], a protease activated protein kinase [234], protein kinase C [605] and an S6 kinase whose activity is regulated by reversible phosphorylation [572]. The first three kinases have been shown, *in vitro*, to phosphorylate sites related to those phosphorylated in cells exposed to insulin [838, 839] or platelet derived growth factor [836] and treatment with phorbol ester (a potent activator of protein kinase C; see section 1.3.8.(i)b) is known to markedly stimulate S6 phosphorylation in hepatoma cells [780] suggesting that these kinases

may also be involved in S6 phosphorylation in vivo. The remaining S6 kinase is interesting in that its activity appears to be dependent on tyrosine phosphorylation [572], raising the possibility that receptor associated tyrosine kinase might directly activate this S6 kinase; however, further work, presented in the same report, suggests a more indirect sequence of events leading to S6 phosphorylation. Besides phosphorylation of S6, phosphorylation-activated S6 kinase activity appears to be involved in down-regulation of EGF receptors [572] and may, therefore, represent an important link in the regulatory mechanisms governing both intracellular propagation of hormone or growth factor initiated stimuli and feedback control of cell-surface receptor activity.

1.3.8. Protein Kinases

Protein kinases catalyse the transfer of the γ phosphoryl-group of ATP to serine, threonine or tyrosine residues of their polypeptide substrates. Phosphorylation of histidine and lysine residues of histones IV and I respectively has also been reported in nuclei of a carcinoma cell line [714] but the functional significance of these modifications is unclear and the phenomenon has received little attention.

Since many of the known protein kinases have broad and sometimes overlapping substrate specificities, it is often more convenient to classify these enzymes according to their specific regulatory effectors though some continue to be named with reference to the protein with which their activity was originally associated. This system enables protein kinases sharing the same mode of regulation or those with unknown regulatory properties to be distinguished from other members of

the same general group. Examples of such enzymes include phosphorylase kinase (Ca^{2+}/calmodulin-dependent), myosin light chain kinase (Ca^{2+}/calmodulin-dependent), casein kinases 1 (unknown regulatory properties) and 2 (polyamine-dependent) and glycogen synthase kinases 3, 4 and 5 (unknown regulatory properties). However, as the functions of enzymes named according to this system are more fully characterised, it becomes apparent that a number might equally well be described as kinases of proteins other than those suggested by their original nomenclature as evidenced by phosphorylase kinase (glycogen synthase kinase 2), casein kinase 2 (glycogen synthase kinase 5) and GSK 3 (factor FA).

1.3.8.(i) Serine/Threonine Protein Kinases

The vast majority of phosphorylations so far identified are specific to serine or threonine and the enzymes which catalyse these reactions form the most extensively characterised group of protein kinases.

1.3.8.(i)a Cyclic Nucleotide-Dependent Protein Kinases

cAMP-Dependent Protein Kinase

The most intensively studied of all protein kinases is cAMP-dependent protein kinase (cAMP-PrK) which appears to be ubiquitous among animal species and tissues [447, 456]. Modulation of cAMP-PrK activity provides the only known link between changes in intracellular cAMP concentrations and the metabolic consequences of such changes and therefore fulfils a key role in the cellular response to a number of hormones including glucagon, catecholamine and ACTH (see section 1.1.). Although cAMP-PrK is largely cytosolic in most tissues including muscle and liver [318], the brain contains approximately equal amounts of

soluble and particulate enzyme [318, 499, 668, 787a] and endogenous enzyme has been detected in nuclear fractions of various tissues [556]. Soluble and particulate forms share virtually identical biochemical and immunological properties [486, 667, 668] suggesting that the same enzyme can exist in both environments but it remains possible that partitioning is determined by some minor, as yet undetected difference [556]. All mammalian cAMP-PrKs have catalytic (C) subunits with very similar physical and functional properties [563, 687, 879]. The tetrameric holoenzyme consists of two catalytic and two regulatory subunits (R_2C_2) arranged as a regulatory subunit dimer associated with two catalytic subunits [437, 588] and exists in two forms designated type I and type II on the basis of their order of elution, with increasing ionic strength, from DEAE cellulose columns [437, 729]. The two classes differ functionally in a number of respects but share the same substrate specificities [437, 663, 821]. The differences between type I and type II cAMP-PrKs appear to be wholly attributable to differences in the R-subunits of each [317, 879] which have molecular weights of 49kDa and 52-55kDa respectively [556]. There is immunological evidence to suggest that the R-subunits of muscle and brain type II cAMP-PrK also differ structurally [197, 215, 668]. Furthermore, type II cAMP-PrKs in these two tissues give rise to specific, distinct tryptic peptide maps [291, 727] and appear to interact differently with the C-subunits [290]. No such tissue differences have been reported for type I cAMP-PrK [556]. Subunits of type II cAMP-PrK (R_{II}-subunits) are autophosphorylated on one serine residue per subunit when incubated with Mg-ATP resulting in an increased responsiveness of the kinase to activation by cAMP [82, 196, 317, 639]. The autophosphorylation site of bovine heart R_{II} is serine

95 in the primary sequence and although further phosphorylation has been observed on serines 74 and 76 by casein kinase 2 (also sometimes termed GSK-5) [82, 303] and on serine residues at positions 44 and 47 by glycogen synthase kinase 3 [303], no significant function has been assigned to occupation of the sites phosphorylated by either of the latter two kinases [82]. Both autophosphorylation and casein kinase 2 sites are phosphorylated in vivo [82, 638, 688] and there is evidence that the level of in vivo autophosphorylation is sensitive to cAMP levels, implying that phosphorylation/dephosphorylation may be an important physiological regulator of PrK activity [688]. Unlike R_{II}, the R-subunit of type I cAMP-PrK (R_I) does not undergo autophosphorylation in vitro [317, 824] but does bind Mg-ATP with high affinity [275, 317] and can be phosphorylated on two sites per subunit by cGMP-dependent protein kinase, one of which is homologous with that autophosphorylated in R_{II} [242, 243, 295]. Phosphorylation of R_I does also occur in vivo though not, apparently, at the site showing homology with the R_{II} autophosphorylation site [243, 728, 730]. Changes in the phosphorylation state of R_{II} in response to steroid hormones have been proposed to have a possible mechanistic role in some of the actions of these hormones [481, 482] thus extending the regulatory potential of cAMP-PrK and allowing modulation of the action of agents that influence cellular cAMP levels by steroid hormones. None of the R_I phosphorylations have been reported to be associated with changes in either cAMP binding or catalytic activity of type I cAMP-PrK.

Both types of R-subunit consist of two proteolytically resistant domains connected by a proteolytically sensitive "hinge" region [131, 628, 831]. The major domain, accounting for about 2/3 of the subunit

structure contains the cAMP binding sites [628] and the second proteolytically stable region, comprising a further 1/4 of the R-subunit appears to be involved in R-R interaction in the regulatory subunit dimer [630]. The remaining "hinge" region contains the autophosphorylation site of type II cAMP-PrK and its homologous (cGMP-PrK dependent) phosphorylation site in type I cAMP-PrK [629]. Several lines of evidence suggest that this region plays a critical role in interaction with the C-subunit [131, 241, 266, 630, 759]. Each R-subunit possesses two cAMP binding sites [70] distinguishable by differences in dissociation rates of bound cAMP and in relative affinities for analogues of cAMP [400, 640, 641]. Although both cAMP binding sites of purified R-subunit apparently have equal affinities for cAMP [640] it is bound preferentially to one of the two in the holoenzyme [437, 71, 367] but the precise stoichiometry of cAMP binding required to achieve full activation is uncertain. Despite reports that occupation of only two of the four cAMP binding sites present in the R-subunit dimer is sufficient to elicit full activation of the protein kinase [129, 437, 641], there is evidence to suggest that the remaining two binding sites also contribute to the activation process [129, 577, 655].

The cAMP-PrKs are inhibited by a soluble heat stable protein found in many tissues but most extensively studies in rabbit skeletal muscle [816]. Widely known as the Walsh inhibitor after the team responsible for its discovery, cAMP-PrK inhibitor binds to the C-subunit of cAMP-PrK and is competitive with respect to protein substrates for the kinase [149]. In common with all other cAMP-PrK inhibitor proteins, the Walsh inhibitor is acidic, a property which has been suggested to be significant in relation to binding to the highly

basic C-subunit [729]. The physiological function of this inhibitor is uncertain since even in skeletal muscle where it is relatively abundant, the concentration of inhibitor protein is such that only 1/5 of the cellular cAMP-PrK could be blocked [815]. At such levels the inhibitor might nullify the effects of low concentrations of cAMP [28] but could not be a significant factor at high cAMP concentrations [437]. Whether the reported variations in inhibitor concentration under different physiological conditions [132, 445, 815] would be capable of making an effective contribution to the regulation of cAMP mediated events is therefore debatable but the purified protein is nevertheless a valuable tool for distinguishing the actions of cAMP-PrK from those of other protein kinases.

cGMP-Dependent Protein Kinase

Unlike cAMP-PrK which is present in similar concentrations in most mammalian tissues [318, 444, 822, 823], cGMP-PrK is unevenly distributed with relatively high concentrations being found in lung, heart, smooth muscle and intestine [83, 147, 476, 819, 820]. cGMP-PrK appears to be predominantly cytosolic in most tissues [446] and the purified enzyme of bovine lung [148, 252, 255, 474] and heart [217] consists of a dimer of identical 74-81kDa subunits containing two cGMP binding sites within a region referred to as the cGMP-binding domain [319, 320, 498] and a catalytic domain [556]. Binding of cGMP activates the enzyme by inducing a conformational change which exposes the catalytic domain [251, 475]. Although cAMP is also capable of activating the enzyme, much higher concentrations are necessary to achieve activation than are required for cGMP induced activation [319]. Autophosphorylation of purified cGMP-PrK is stimulated by either cGMP

or cAMP [148, 222, 251, 319, 320, 475]. The latter is reported to be more effective in this respect [222, 251, 319] though cGMP stimulates autophosphorylation at lower concentrations than those required for stimulation by cAMP [319] and cAMP stimulated autophosphorylation is inhibited by cGMP [251, 319, 475]. Originally no change in the activity of cGMP-PrK was observed following its autophosphorylation [222, 475] but recently autophosphorylation has been shown to be accompanied by an increase in the phosphotransfer V_{max} of the enzyme [319, 320] and over 10-fold decrease in the concentration of cAMP required for its activation [319]. The concentrations of cAMP required to achieve full (4mol phosphate per mol subunit) autophosphorylation in vitro are above the normal physiological range but the enzyme is known to be phosphorylated to a certain extent in vivo [319] so it remains at least possible that this modification is a significant modulator of cGMP-PrK activity in vivo [319].

Relatively few physiological substrates have been identified for this kinase [556] but in vitro, it shows a similar substrate specificity, with respect to histones and synthetic peptides, to that displayed by cAMP-PrK [218, 255, 256, 257]. Although little is therefore known about the regulatory significance of cGMP-PrK in vivo the wealth of evidence supporting an important role for cGMP in neuronal function (see [556] and references therein) suggests that cGMP-PrK activity may, at least in nervous tissue, represent an important factor in the control of cellular metabolism.

1.3.8.(i)b Calcium-Dependent Protein Kinases

In contrast to the cyclic nucleotide-dependent protein kinases which have been characterised primarily in muscle and liver, much of the work

on calcium dependent protein kinases has been carried out on enzymes from brain [556].

Calcium/Calmodulin-Dependent Protein Kinase

Unlike the cAMP-PrKs the Ca^{2+}/calmodulin-dependent protein kinases (CC-PrKs) have distinctly different catalytic subunits with different substrate specificities but are all activated by a calmodulin-mediated Ca^{2+}-dependent mechanism and therefore may be considered to share identical regulatory subunits [436]. Ca^{2+}/calmodulin-dependent activation, originally described for phosphorylase kinase (see section 1.3.1) and myosin light chain kinase of muscle [140, 624a, 810, 860] has also been observed for liver glycogen synthase kinase [436] and two brain synaptosomal membrane kinases termed CC-PrKs I and II [685, 686]. Both phosphorylase kinase [582, 753] and myosin light chain kinase [139a, 296] have also been identified in brain. Myosin kinase (molecular weight 130kDa) has been partially purified from this tissue and found to be a highly specific enzyme which preferentially phosphorylates smooth muscle myosin light chain [296, 556]. Myosin light chain kinase activity has been shown to be Ca^{2+}/calmodulin-dependent in several other tissues besides smooth muscle and brain [295a, 818, 870] and phosphorylation of the smooth muscle enzyme by cAMP-PrK has been reported to be accompanied by a decrease in its activity [1]. Although not as yet purified from brain, the phosphorylase kinase identified in this tissue appears to be similar to the skeletal muscle enzyme and is activated both by Ca^{2+} and by phosphorylation by cAMP-PrK [753] but only partially cross-reacts with antibody raised against skeletal muscle phosphorylase kinase indicating

that the two forms are distinct iso-enzymes [753]. CC-PrKI appears to have a limited substrate specificity with highest activity expressed towards the neuronal proteins synapsin I and protein III [556]. Of these, synapsin I is reported to be the better substrate. CC-PrKI and cAMP-PrK share similar K_m values for phosphorylation of both substrates and the two kinases have been shown to phosphorylate the same site(s) on protein III [556]. Although there is little evidence that CC-PrKI is an effective protein kinase in tissues other than brain, the enzyme does display some activity against smooth muscle light chain and there are indications that physiological substrates in addition to synapsin I and protein III may exist in both neuronal and non-neuronal mammalian tissues [556]. CC-PrKI activity is predominantly cytosolic and has been observed in several tissues including spleen, heart, adrenal gland and skeletal muscle though specific activities in these tissues range from less than 1/20 (skeletal muscle) to around 1/4 (spleen) of the value obtained for brain [397, 556]. Molecular weights of 37kDa, 39kDa and 42kDa calculated for polypeptides separated by SDS PAGE of CC-PrKI purified to apparent homogeneity [367, 556]. In contrast, a single symmetrical peak of CC-PrKI activity with a molecular weight of 49kDa was obtained by gel filtration suggesting that the enzyme protein is a monomer. It is not known whether the three lower molecular weight forms which also each display kinase activity are proteolytic products derived from a higher molecular weight native enzyme or represent iso-enzymes of CC-PrKI [367]. In the presence of Ca^{2+}, CC-PrKI binds calmodulin and is autophosphorylated on threonine residues though at a lower rate than that displayed by the enzyme for phosphorylation of synapsin I [556]. In common with CC-PrK I from bovine brain, CC-PrK II of rat brain demonstrates greatest activity with synapsin I as

substrate [40, 526, 527] though phosphorylation occurs preferentially at a site distinct from that phosphorylated by CC-PrKI [556]. CC-PrKII is reported to have a relatively broad substrate specificity unlike the other Ca^{2+}/calmodulin-dependent protein kinases, phosphorylase kinase, myosin light chain kinase and CC-PrKI. Good substrates include synapsin I, MAP 2, glycogen synthase, smooth muscle light chain [40, 230, 526, 527, 684, 862], tau protein [684, 861], tyrosine hydroxylase, tryptophan hydroxylase [809, 855, 863], myelin basic protein [230, 258], ribosomal protein S6 [262] and Ca^{2+}/calmodulin-sensitive cyclic nucleotide PDE [231] of which all but glycogen synthase, myosin light chain and ribosomal protein S6 are predominantly neuronal. CC-PrKI purified to apparent homogeneity from rat forebrain has a native molecular weight of 55-56kDa composed of 50kDa and 58/60kDa subunits in the ratio 3:1 [40, 526] all of which are autophosphorylated in a Ca^{2+}/calmodulin-dependent manner, bind calmodulin in the presence of Ca^{2+} and are labelled by the photoaffinity label 8-N_3-ATP [40, 526, 556] suggesting that protein kinase activity is associated with all three subunits. As with all other Ca^{2+}/calmodulin-dependent protein kinases, CC-PrKII is activated by calmodulin in the presence of Ca^{2+}, probably in a similar manner to that described for Ca^{2+}/calmodulin activation of Ca^{2+}/calmodulin-sensitive PDE (see section 1.2.1.(i)). Although autophosphorylation of the enzyme inhibits its activity [556] and has been reported to increase its affinity for calmodulin [703], CC-PrKII does not appear to be phosphorylated by other protein kinases [556]. CC-PrKII is present in very high concentrations in both soluble and particulate fractions of brain [40, 526] and it appears that the subcellular distribution is subject to modification by exposure of certain neurons to seratonin [672]. It has also been suggested that

autophosphorylation may regulate subcellular distribution [40]. The CC-PrKII of rat forebrain and cerebellum share similar purification characteristics, physical properties and substrate specificities [525, 526, 537] and are composed of identical (by peptide mapping) subunits though the ratio of 50kDa to 58/60kDa subunits comprising the native enzymes differ markedly [525, 526, 537]. Rabbit skeletal muscle glycogen synthase kinase which is composed of a polypeptide doublet of molecular weight 58/60kDa also displays a number of properties in common with the CC-PrKII of brain including substrate specificity, peptide maps, physical properties and immunological properties [527, 556]. Other tissues found to contain kinases with similar substrate specificities, native molecular weights and autophosphorylation characteristics to CC-PrKII are rabbit liver [3, 598], rat pancreas [262], bovine heart [584], turkey erythrocytes [586], Aplysia nervous system [160] and Torpedo electric organ [587]. In view of the apparently widespread tissue and species distribution of CC-PrKII isoenzymes, it seems likely that further substrates will be found and this kinase may prove to be an important mediator in a number of cellular responses to changing Ca^{2+} concentrations. There is some evidence that CC-PrKII phosphorylates synapsin I in intact nerve cells [559, 783] but strong correlations between kinase-dependent phosphorylation and associated in vivo events of the kind described for cAMP-PrK have not yet been reported for either CC-PrKI or Cc-PrKII and the physiological significance of their activities must, therefore, remain uncertain.

Calcium/Phospholipid-Dependent Protein Kinase (Protein Kinase C)

First purified from cerebellum as a cyclic nucleotide-independent protein kinase that could be activated by Ca^{2+}-dependent protease [353, 754], the holoenzyme was subsequently found to be activated by Ca^{2+}, diacylglycerol and various membrane phospholipids [377, 420, 755, 756]. The enzyme from brain is a monomer of molecular weight 80-87kDa and is thought to consist of a hydrophilic catalytically active domain and a hydrophobic domain which is involved in the activation of the enzyme [413]. Protein kinase C (PrKC) is widespread throughout the phyla of the animal kingdom from anelids to higher chordates including mammals [444] and shows very similar physical, kinetic and catalytic properties in a variety of tissues [444, 539]. In most rat tissues including liver, adipose tissue, heart and skeletal muscle, specific activities of cAMP-PrK and PrKC measured under similar conditions are approximately equal but in several tissues (platelets, brain, lymphocyte and granulocytes) PrKC activity far exceeds that of cAMP-PrK [444, 539]. PrKC activity is highest in spleen with comparable activities in cerebral cortex and cerebellum [444], while activities in other non-neuronal tissues such as liver, skeletal muscle and adipose tissue vary between 1/10 and 1/200 of these values [444, 539]. The proportion of total cellular enzyme activity found to be membrane associated in non-nervous tissue ranges from approximately 2% in muscle and platelets to between 22% and 39% in liver, kidney, heart and spleen [391, 413, 444]. However, particulate PrKC activity in brain consistently accounts for a higher proportion of total activity (45-64%) than in any of the non-nervous tissues studies [391, 413, 444]. The two forms are biochemically indistinguishable [413] and the significance of the tissue specific distributions is not yet known.

Besides a number of uncharacterised protein substrates [393, 846, 857, 858, 859], the broad substrate specificity of PrKC encompasses proteins of both predominantly neuronal and non-neuronal origin including EGF receptor [106], somatomedin C receptor [361], insulin receptor [139, 361, 757], β adrenergic receptor [396, 705], nicotinic ACH receptor [334], eukaryotic initiation factor 2 (eIF-2) [683], smooth muscle myosin light chain [188, 564], troponin T [392], glycogen synthase [4, 654], ribosomal protein S6 [471], vinculin [834], tyrosine hydroxylase [8], GABA-modulin [847], myelin basic protein [787, 846], MAP-2 [811, 812], the B-50 protein [10] and histones 1, 2 and 3 [755].

Activation of PrKC in vivo [433, 434, 529, 710] or by micromolar concentrations of Ca^{2+} in vitro [413, 595] is accompanied by redistribution of the enzyme from cytosol to membrane fractions and is dependent on the presence of certain membrane phospholipids [115, 420, 539]. Experiments with purified phospholipid have shown that both the concentration of Ca^{2+} required for activation of PrKC and the degree of activation achieved at a given Ca^{2+} concentration depend upon the nature of the phospholipid present, phosphatidyl serine and phosphatidyl inositol being the two most effective [377, 420, 755]. In the presence of diacylglycerol, the Ka for Ca^{2+} is reduced by an order of magnitude (from between 1 and 10 x 10^{-5}M to between 1 and 8 x 10^{-6}M) [420, 544, 756] and the affinity of the enzyme for phospholipid is also increased [420, 544] so that in the presence of both diacylglycerol and phosphatidyl serine, PrKC is fully active at Ca^{2+} concentrations in the 10^{-7}M range [377, 413]. Diacylglycerols possessing at least one unsaturated fatty acid are the most effective PrKC activators and those with unsaturated fatty acyl moieties esterified at position 1 or 2 are

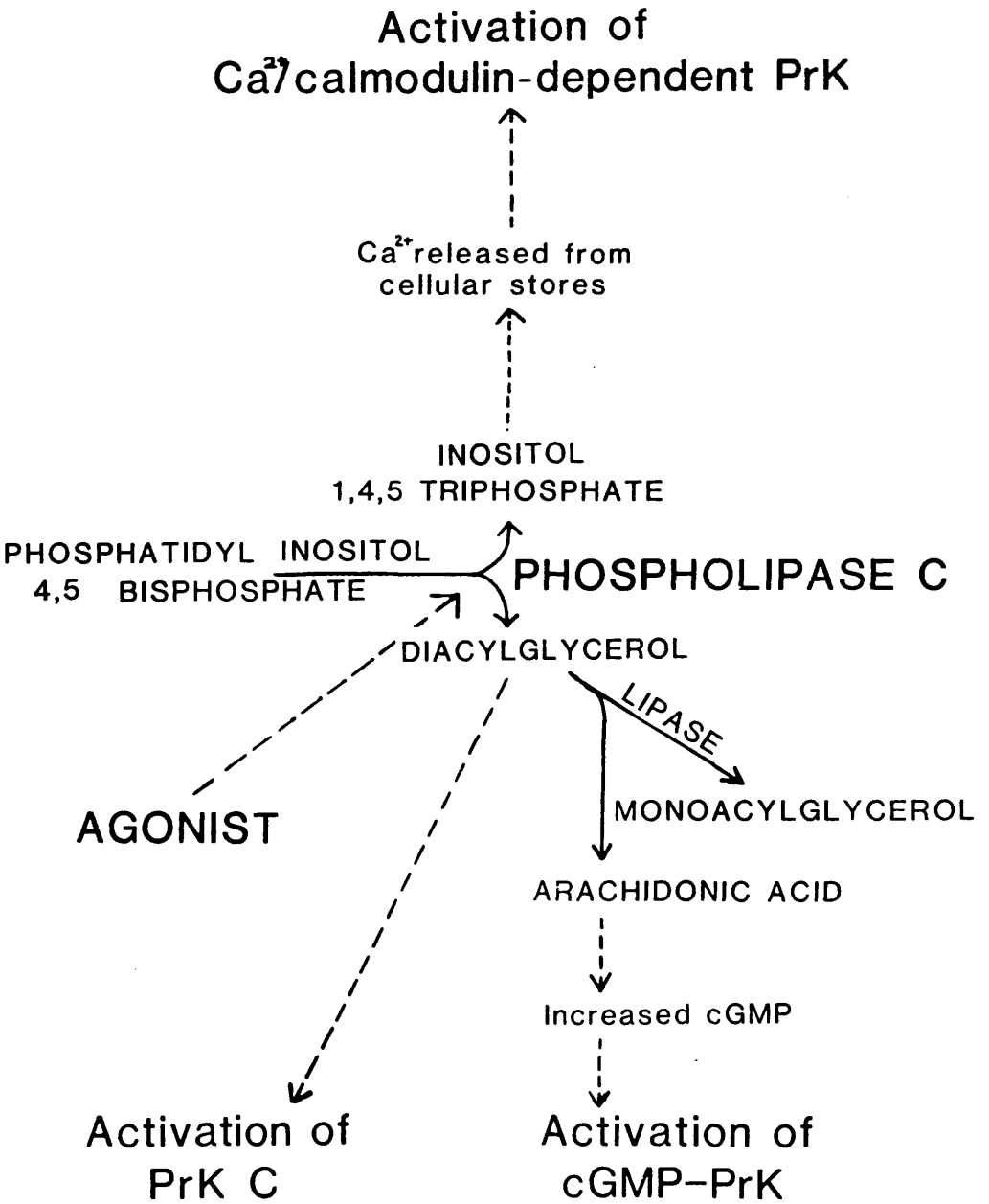
equally active [544]; however, monoacylglycerols and triacylglycerols are both ineffective [420, 544, 756]. Activation of PrKC, studied using purified enzyme and reconstituted vesicles containing phosphatidyl serine and diacylglycerol, appears to be a complicated process involving the simultaneous intraction of Ca^{2+}, a hydrophobic domain of the enzyme molecule and the phospholipid vesicle surface [413, 556]. This conclusion is based on the observation that association with membranes does not in itself guarantee activation of PrKC [413, 543] and that Ca^{2+} binds to phosphatidyl serine vesicles [178, 589] but not, apparently, directly to the enzyme itself [556]. There is indirect evidence to suggest that interaction of Ca^{2+} with certain membrane phospholipids is necessary to create a molecular configuration capable of interacting in a specific manner with the hydrophobic domain of PrKC and as a result, activating the enzyme.

Tumor-promoting phorbol esters such as TPA (12-0-tetradecanoyl phorbol 13-acetate or 4β-phorbol 12-myristate 13-acetate) act as a substitute for diacylglycerol and activate PrKC directly both in vitro and in vivo [84] though related non-tumor promoting phorbol esters are unable to activate the kinase [84]. There is evidence that this relationship is not coincidental since some, if not all, of the tumor promoting actions of these agents are known to be mediated by PrKC [414, 568]. The number of phorbol ester receptors in the brain roughly matches the amount of PrKC present in this tissue and the two entities are now considered to be one and the same [414]. A kinetic study of phorbol ester-stimulated activation suggests that activation of one PrKC molecule results from interaction with one molecule of phorbol ester [414]. This does not necessarily imply that the effect of phorbol ester is a direct consequence of ligand-ligand interaction but

may instead involve modification of the phospholipid microenvironment to create the configuration necessary for formation of a fully active enzyme/Ca^{2+}/phospholipid complex [414, 568]. Because phorbol ester substitutes for diacylglycerol in this activation process the possibility is raised that "normal physiological" activation of PrKC by diacyl glycerol may involve a similar stoichiometry [568]. A number of drugs inhibit the activity of PrKC but none are specific and several, including trifluoperazine, fluphenazine and W-7 also inhibit Ca^{2+}/calmodulin dependent protein kinase [568]. These inhibitors interact with phospholipid and inhibit PrKC activation in a competitive manner [568].

Identification of the role of diacylglycerol in PrKC activation and work relating to polyphosphoinositide metabolism in thrombin-stimulated platelets have led to the construction of a model for regulation of cellular responses to hormonal stimulation involving activation of PrKC [46, 115, 568] (Figure 12). It is envisaged that stimulation of polyphopshoinositide turnover by hormones such as thrombin, vasopressin, angiotensin II and α_1 adrenergic agonists [45, 47, 136, 416, 417, 418, 534, 535, 767, 778] leads to the generation of increased cellular concentrations of both inositol 1,4,5 triphosphate and diacylglycerol (mostly 1-stearyl, 2-arachidonyl diacylglycerol [324]) by the action of phospholipase C on phosphatidyl inositol 4,5 diphosphate. Since inositol 1,4,5 triphosphate stimulates the release of Ca^{2+} from intracellular stores [25, 46, 72, 198, 199, 200, 372, 741], two potential second messengers are generated in response to a single hormonal stimulus. The increases in Ca^{2+} concentration accompanying such hormonal stimulation are probably physiologically irrelevant in relation to PrKC activation since diacylglycerol reduces

Figure 12 Model for the Generation of Factors Capable of Modulating PrK Activity as a Result of Changes in Phosphatidyl Inositol Turnover



the Ca^{2+} concentration required for full activation to a level low enough to allow activation of the kinase even at basal intracellular Ca^{2+} concentrations [568]. However, the increased Ca^{2+} would lead to activation of the Ca^{2+}/calmodulin-dependent protein kinases while increases in diacylglycerol would also result in an increase in prostaglandin synthesis via arachidonic acid which would, in turn, activate cGMP-PrK (Figure 12) [46, 568].

The possibility is emerging that PrKC activation provoked by increased phosphoinositide turnover may be involved in the mechanism(s) of action of insulin. Evidence in support of this role is afforded by the observation that phospholipase C, the enzyme responsible for production of diacylglycerol (see Figure 12), is activated following insulin-treatment of fat cells [427], a finding which is consistent with previous reports that insulin stimulates phosphoinositide turnover [205]. Furthermore, both phorbol ester and phospholipase C have been shown to elicit various metabolic effects similar to those produced by insulin [325, 427]. Together these observations provide quite compelling though circumstantial evidence for an important role for PrKC in insulin action and it now appears that this kinase may also be capable of modulating adenylate cyclase activity [364, 389]. The latter effect of PrKC is reported to involve suppression of G_i function resulting from PrKC catalysed phosphorylation of its α-subunit [389] (see section 1.1.). As discussed earlier, this system provides a mechanism for modulation of the action of adenylate cyclase-linked hormones by hormones whose primary action involves manipulation of phosphoinositide metabolism and thus has the potential to allow considerable hormonal interaction leading to greater flexibility of response to various combinations of hormonal stimuli.

1.3.8.(i)c Polyamine-Dependent Protein Kinase

The two major classes of cell surface receptor found in most tissues are receptors related to cellular cAMP levels and those which induce rapid polyphosphoinositide turnover and Ca^{2+} mobilisation [568]. However, not all of the diverse effects of hormones such as insulin, glucocorticoids, progesterone and prolactin can be adequately explained by these mechanisms alone. The discovery of polyamine-stimulated protein kinase has therefore provoked a great deal of interest since cellular levels of the polyamines are known to be influenced by a large number of hormones [20, 301, 441, 546, 578, 752, 843], are increased dramatically during the early phase of cell growth [578, 752, 843] and are high (up to millimolar) in non-proliferating tissues actively engaged in protein synthesis, such as lactating mammary gland, pancreas and prostate gland [578, 752, 843] (see also section 1.3.9.(i)).

Polyamines are aliphatic polycations found in all living cells. Through ionic interactions and H-bonding they can complex with nucleic acids, proteins and phospholipids while their aliphatic character suggests a capacity to interact with hydrophobic environments such as those of membranes [441]. The three major naturally occurring polyamines are putrescine, spermine and spermidine (for structures see Figure 13) and all three are synthesised by nucleated eukaryotic cells but prokaryotes are apparently unable to synthesise spermine [752, 843]. Prokaryotes generally contain higher concentrations of putrescine than spermidine but eukaryotes contain little putrescine and relatively higher concentrations of spermidine and spermine [441]. The major biosynthetic pathway leading to polyamine formation in mammalian

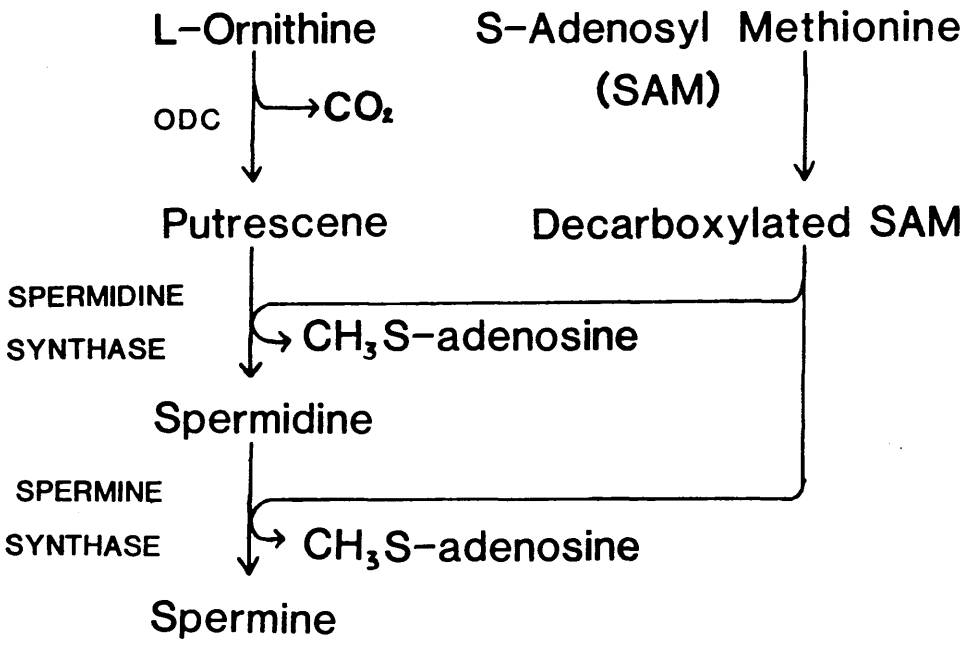
cells involves direct decarboxylation of L-ornithine by ornithine decarboxylase (ODC) to produce putrescine which is then converted to spermidine and spermine by successive donations of an aminopropyl group from decarboxylated S-adenosyl methionine (SAM) catalysed by spermidine synthase and spermine synthase respectively (Figure 13). ODC is generally recognised as the major rate-limiting enzyme of the pathway [76, 441, 733, 752] but provision of decarboxylated SAM by SAM decarboxylase may also limit synthetic rates [733].

Polyamine has been found to inhibit both cAMP-PrK [316, 339, 554] and PrKC [846] and abolish the cGMP sensitivity of cGMP-PrK [825] but crude cyclic nucleotide-independent protein kinase preparations containing casein kinases are activated by these polycations [554] and at least 2 polyamine-dependent protein kinases have now been identified [104]. The best defined of these is casein kinase G (casein kinase 2) while the other, polyamine-dependent ODC kinase, has so far only been described in detail in nuclei of the slime mould Physarum polycephalum [441, 442] and therefore will not be discussed at length in this report.

Casein kinase G (Casein Kinase 2)

Originally termed casein kinases 1 and 2 with reference to their order of elution from ion exchange columns during purification, the two enzymes from bovine adrenal cortex were renamed casein kinases A and G respectively when they were found to differ in their phosphate source requirements [107]. Casein kinase A (CKA) is only active with ATP as a phosphoryl donor but casein kinase G (CKG) will catalyse the transfer of phosphate from either ATP (K_m 8μM) or GTP (K_m 18μM) [107]. Both kinses are insensitive to cyclic nucleotides and Ca^{2+}/calmodulin and,

Figure 13 Synthesis and Molecular Structure of theThree Major Naturally Occuring Polyamines



Legend:

Putrescine $H_3N-(CH_2)_4-NH_3$

Spermidine $H_3N-(CH_2)_3-HN-(CH_2)_4-NH_3$

Spermine $H_3N-(CH_2)_3-HN-(CH_2)_4-NH-(CH_2)_3-NH_3$

ODC Ornithine Decarboxylase

therefore, had been designated messenger-independent enzymes until Cochet et al. [107] discovered that CKG activity could be stimulated 5-10 fold by millimolar concentrations of polyamine in vitro.

CKG purified from bovine adrenal cortex [108] exhibits an apparent molecular weight of 140kDa upon gel filtration and appears to be composed of two types of subunit designated α (molecular weight 38kDa) and β (molecular weight 27kDa) in an $\alpha_2\beta_2$ holoenzyme conformation [104]. Catalytic activity resides in the α-subunit but its expression is limited in the absence of β-subunit. Although the β component undergoes autophosphorylation [104], the effects, if any, of this modification on enzyme function have not been determined. Spermine appears to bind to the α-subunit and activation of the purified enzyme is characterised by a five fold increase in V_{max} with no change in K_m. However, the extent of activation depends on the nature and concentration of the substrate and is sensitive to Mg^{2+} concentrations [104]. The polyamine effect is maximal at Mg^{2+} concentrations below 5mM and is lost progressively at higher concentrations but in the absence of magnesium no polyamine stimulation of CKG activity is observed at all whereas Mg^{2+} strongly activates the enzyme in the absence of polyamine [104]. A heat-stable selective inhibitor of CKG has been isolated from various bovine tissues [369] and demonstrated to be ineffective against either CKA or cAMP-PrK. The inhibitor, termed casein kinase G inhibitor (CKG I) is competitive with casein and its inhibitory effect is antagonised in a dose-dependent manner by low (< 0.1mM) concentrations of polyamine [370]. At millimolar concentrations of spermine CKG I induced inhibition equivalent to that observed in crude adrenocortical cytosols (approximately 50-60%) is completely abolished and direct activation of

CKG proceeds resulting in a further increase in kinase activity [104, 370]. On the basis of velocity sedimentation characteristics of CKG in the presence and absence of its modulators and work with radioactively labelled polyamines, Job et al. [370] have constructed a model for the interactions between CKG, CKG I and polyamines (Figure 14) which, if shown to operate in vivo, would represent a means of extending the range of concentrations over which polyamine could effectively modulate CKG activity. The active moiety of CKG I resembles heparan sulphate [613] and, in fact, micromolar concentrations of heparin mimic CKG I inhibition of CKG in vitro [210].

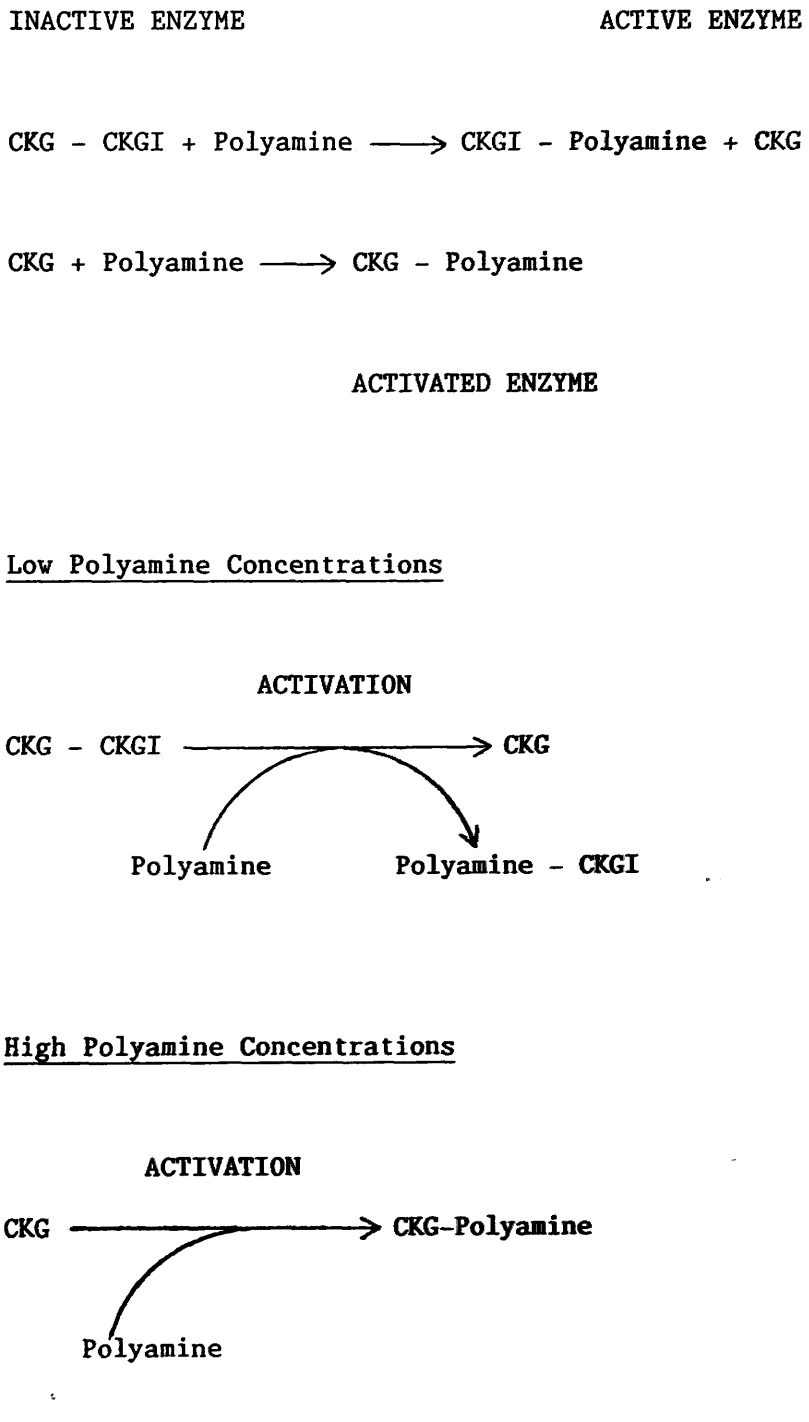
Casein kinases A and G have been described as ubiquitous in mammalian tissues [104, 298] and CKG is reported to be similar to a number of enzymes described by several laboratories [104] including casein kinase 2 from reticulocytes [297], calf thymus [141] and skeletal muscle [331], nuclear casein kinase NII [660, 661, 775], liver casein kinase TS [531] and a skeletal muscle glycogen synthase kinase termed GSK PC 0.7 [158] or GSK 5 [122]. Among the endogenous substrates identified for CKG in vitro are glycogen synthase of both muscle and liver [122, 530, 558], calsequestrin, troponin T and protein phosphatase inhibitor 1 [530], protein synthesis initiation factor eIF-2 [15,9 653], acetyl-CoA carboxylase [122] and RII of cAMP-PrK. However, the activities of enzymes such as glycogen synthase and cAMP-PrK are apparently unaffected by CKG mediated phosphorylation [122, 671] and although phosphorylation of eIF-2 has been reported to result in a slight stimulation of eIF-2-dependent met-tRNA binding to the 40s ribosomal subunit, no physiological significance has been assigned to this effect [159]. Because of the well established link between cellular polyamine levels and protein synthesis/cell growth

Figure 14

CKG = Casein Kinase G

CKGI = Casein Kinase G Inhibitor

Figure 14 Casein Kinase G Inhibitor-Mediated Modulation of Polyamine-Dependent Stimulation of Casein Kinase G



[578, 752, 843], polyamine-dependent phosphorylation of nuclear proteins has attracted a great deal of attention. Although purified cytosolic CKG has been shown to phosphorylate both RNA polymerase II [141, 142] and high mobility group (HMG) chromosomal proteins [825] *in vitro*, the polymerase activity remains unchanged by such modification and the significance of the HMG phosphorylaton *in vivo* (known to occur at the same site as that phosphorylated *in vitro* by CKG [825]) is uncertain. However, phosphorylation of polymerase II by a purified nuclear kinase very similar to CKG [104, 435] is accompanied by an increase in its activity. A homologous kinase, nuclear casein kinase NII phosphorylates RNA polymerase I [167] and II [435, 737] *in vitro* with a concomitant increase in their respective activities and the phosphorylation and activation of polymerase II has been shown to be stimulated by millimolar concentrations of spermine [355]. Indirect evidence for the involvement of CKG in the regulation of nuclear activity and RNA metabolism is provided by the observations that the enzyme is 5-fold more active in hepatoma than in normal liver nuclei [660, 661] and CKG has been found to be associated with m-RNP particles in reticulocyte extracts [653].

Recent work by Cochet *et al*. directed towards characterisation of a 50-55kDa protein found to undergo polyamine-stimulated phosphorylation in adrenal cortex cytosols [104], has led to the suggestion that ODC is itself a substrate for CKG activity [104]. If confirmed, these observations would provide an interesting parallel to the work of Kuehn and coworkers who have identified and characterised a polyamine stimulated nuclear kinase from the slime mould *Physarum polycephalum* (for review see [441]). Simply termed polyamine-dependent protein kinase, this enzyme displays some properties,

including ability to utilise ATP or GTP, insensitivity to cyclic nucleotides and susceptibility to autophosphorylation, in common with CKG but unlike the latter kinase, P. polycephalum polyamine-dependent protein kinase phosphorylates tyrosine residues only, appears to have a more restricted substrate protein specificity and is absolutely dependent on polyamine for its activity [441]. The slime mould enzyme is tightly bound to its proposed in vivo substrate, ODC and phosphorylation of this substrate by polyamine-dependent protein kinase results in a decrease in ODC activity towards ornithine but perhaps also a stimulation of its selective activation of rRNA transcription [104]. Such properties provide a mechanism for negative feedback on polyamine synthesis and may prove to be an important characteristic of polyamine metabolism in mammalian tissues also. Whether the polyamine-stimulated increase in ODC phosphorylation observed in adrenal cortex cytosols can be attributed to CKG or is an indication that an enzyme analogous to the polyamine dependent protein kinase of P. polycephalum is also present in this tissue is uncertain but there is some preliminary evidence to suggest that a kinase similar to the slime mould enzyme is indeed present in mammalian tissues [16].

1.3.8.(ii) Tyrosine Protein Kinases

Protein phosphorylation on tyrosine residues is rare compared with that on serine or threonine but the kinases responsible for tyrosine phosphorylation may prove to be equally important in the conctrol of cellular events since a major group of this type of kinase is composed of activities associated with hormone and growth factor receptors. Published evidence of soluble tyrosine kinase activity is rare, however, nuclear topoisomerase has recently been described as a

substrate for cytosolic tyrosine kinase in liver [782]. Although several oncogene-related tyrosine kinases have been described, their counterparts in normal cells have not as yet been identified and the significance, if any, of their action is unknown [436].

1.3.8.(ii)a Receptor-Associated Tyrosine Kinase Activity

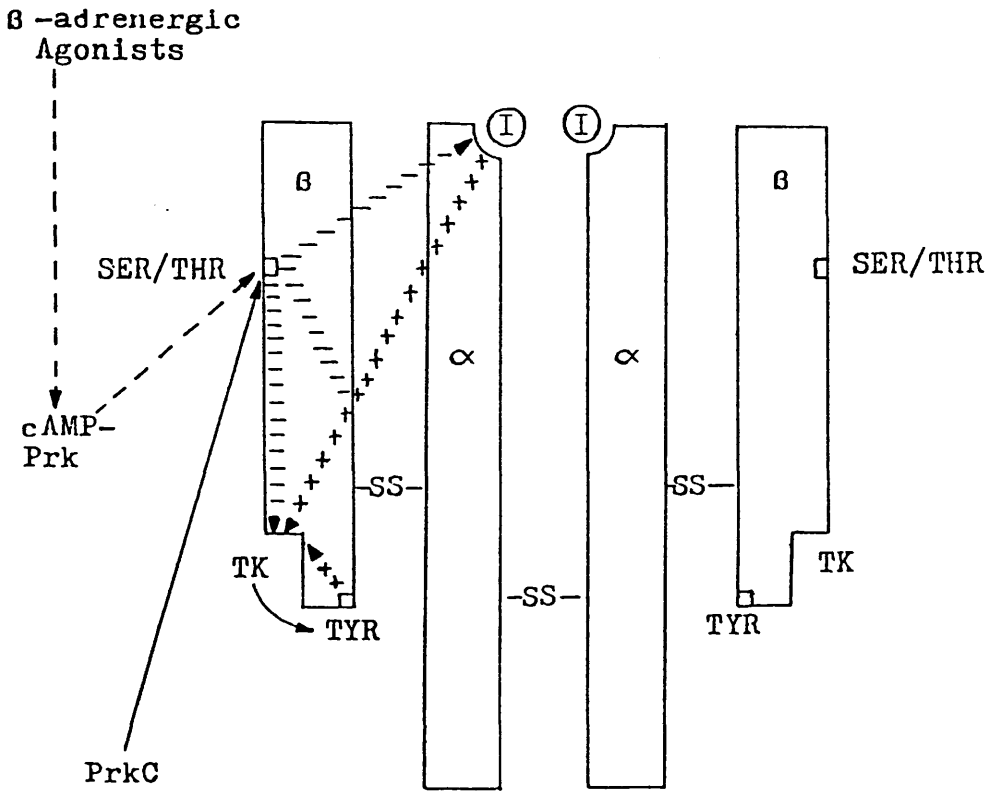
The first demonstration of receptor-associated kinase acitivity was described for the EGF receptor system ([123, for reviews of EGF receptor and other tyrosine kinases see [338, 692]) and subsequent work has revealed that receptors for platelet derived growth factor (PDGF) [177, 566], insulin [387] and insulin-like growth factor (IGF 1) [360] also possess tyrosine kinase activity [139, 338, 436]. These discoveries have stimulated research directed at elucidating the molecular mechanisms of signal transduction related to the biological actions of these receptors and particularly the insulin receptor for which the mechanism coupling hormone binding to cellular response remains elusive.

The Insulin Receptor

Stimulation of insulin receptor phosphorylation by insulin has been demonstrated in several cell types [288, 386, 388, 795]. In intact cells this phosphorylation occurs on tyrosine, serine and threonine residues of the receptor β-subunit [388, 757] but autophosphorylation of solubilised insulin receptor is solely or predominantly confined to β-subunit tyrosine residues [387]. The 2-3 fold stimulation of insulin receptor phosphorylation by insulin observed in intact cells may in fact be an underestimate of the potential in vivo effect since the interference caused by phosphatase activity can not be ignored due to

the difficulty of ensuring its elimination during receptor preparation [139]. Autophosphorylation of insulin receptors purified to varying degrees, up to near homogeneity, proceeds rapidly in the presence of [γ-^{32}P]ATP [19, 50, 384, 385, 603, 872, 875] and has been demonstrated with solubilised receptors from many different cell types including human placenta [19, 384, 603, 872], rat hepatocytes [795], 3T3-L1 adipocytes [603], cultured human lymphocytes [666] and human erythrocytes [271]. The presence of either Mn^{2+} or Co^{2+} is required in order to observe insulin receptor autophosphorylation, Co^{2+} being much less effective in supporting kinase activity than Mn^{2+}, unlike EGF receptor autophosphorylation which is supported equally well by Mg^{2+}, Co^{2+} or Mn^{2+} [19, 875]. It has been suggested that the concentration of Mn^{2+} necessary to permit optimal receptor phosphorylation indicates that, in addition to forming a complex with ATP, Mn^{2+} also acts as a receptor cofactor [139]. The marked activation of insulin receptor autophosphorylation by insulin is achieved by an increase in the apparent V_{max} of the kinase reaction rather than a decrease in its K_m for ATP [840]. Under optimal conditions insulin receptor autophosphorylation is very rapid and, like EGF receptor autophosphorylation can be readily observed at 0°C [139, 840]. Because the rate of autophosphorylation is independent of receptor concentration [702, 840] it must proceed via an intramolecular mechanism and it appears that the tetrameric holoreceptor (see Figure 15) is the most active kinase configuration since dissociation of the receptor into $\alpha\beta$ dimers induced by DTT or N-ethyl maleimide results in inhibition of insulin stimulated autophosphorylation [702]. At DTT concentrations below that required to produce dissociation, kinase activity is stimulated markedly [702] leading to detectable α-subunit

Figure 15. The Insulin Receptor; Subunit Structure and interactions between Insulin Binding and Receptor Phosphorylations.



Legend.

TK	Tyrosine Kinase catalytic site.	I	Insulin.
+ + + + +	Stimulating effect.	≡	Inhibitory effect.
——→	Direct action.	- - - →	Indirect action.

phosphorylation [139]. It has therefore been suggested that a reduced sulphydryl group at or near the kinase active site is required for maximal activity [702]. Tyrosine phosphorylation of the insulin receptor β-subunit leads to stimulation of receptor tyrosine kinase activity [662, 877] and abolition of its sensitivity to modulation by insulin [662]. Dephosphorylation of specific tyrosine residues in a single tryptic peptide of the β-subunit has been observed to be accompanied by inactivation of the receptor tyrosine kinase [872] and phosphorylation of similar sites by src kinase has also been shown to activate the insulin receptor tyrosine kinase [139].

The solubilised receptor catalyses the phosphorylation of a number of exogenous proteins including angiotensin II, tubulin, casein, histone H2B and a synthetic peptide mimicking the tyrosine phosphorylation site of Rous sarcoma virus transforming protein kinase [384, 724]. It has been reported that the src and EGF receptor protein kinases have a similar substrate specificity to that of insulin receptor kinase [724]. Despite the variety of exogenous insulin receptor kinase substrates in vitro, no endogenous proteins other than the receptor itself have been found to be rapidly tyrosine-phosphorylated in response to insulin in intact cells [139]. Although the importance of receptor tyrosine kinase activity to insulin receptor function is still a subject for debate, there is some evidence to suggest that it does not play a major role in signal transduction. This is provided by the observation that anti-insulin receptor immunoglobulin preparations mimic the biological actions of insulin without activating the receptor kinase [707, 876].

An affinity-purified and affinity-labelled receptor protein of molecular weight 45-50kDa, isolated from rat liver [358, 359] and

adipocytes [516] has been identified on electrophoretic gels and found to be derived from the insulin receptor complex. This species was later demonstrated to be a proteolytic fragment of the native 90kDa β-subunit [517]. The β-subunit is extremely sensitive to proteolytic cleavage by an endogenous lysosomal protease released during cellular disruption [139, 517]. This specific and selective proteolytic processing has been claimed to occur with remarkable uniformity and precision and is described as ubiquitous among all tissues studied [517]. While the detailed structure of the 45-50kDa β-subunit fragment (β_1) is not known, insulin receptors containing this, rather than a whole β-subunit, display characteristics of insulin binding and dose-response with respect to glucose oxidation identical to intact native receptor populations, suggesting that the β_1 fragment retains most, if not all its functional integrity. It would therefore seem reasonable to assume that the β_1 fragment contains both its serine/threonine phosphorylation site and an intact tyrosine kinase and tyrosine autophosphorylation site.

Recently it has been discovered that insulin receptor affinity is reduced by 50% in response to β-adrenergic stimulation [139, 601] or exposure to tumor promoting phorbol esters [273, 361, 757]. The β-adrenergic effect is mimicked by incubation with agents such as methylxanthines which elevate cAMP levels and is accompanied by phosphorylation of the insulin receptor or a receptor modulating protein [601]. Insulin binding is inhibited at physiological concentrations of catecholamine [601] and insulin-stimulated tyrosine autophosphorylation is decreased in insulin receptors extracted from isolated adipocytes treated with the β agonist, isoproterenol [139]. Although no change in non-insulin stimulated autophosphorylation is

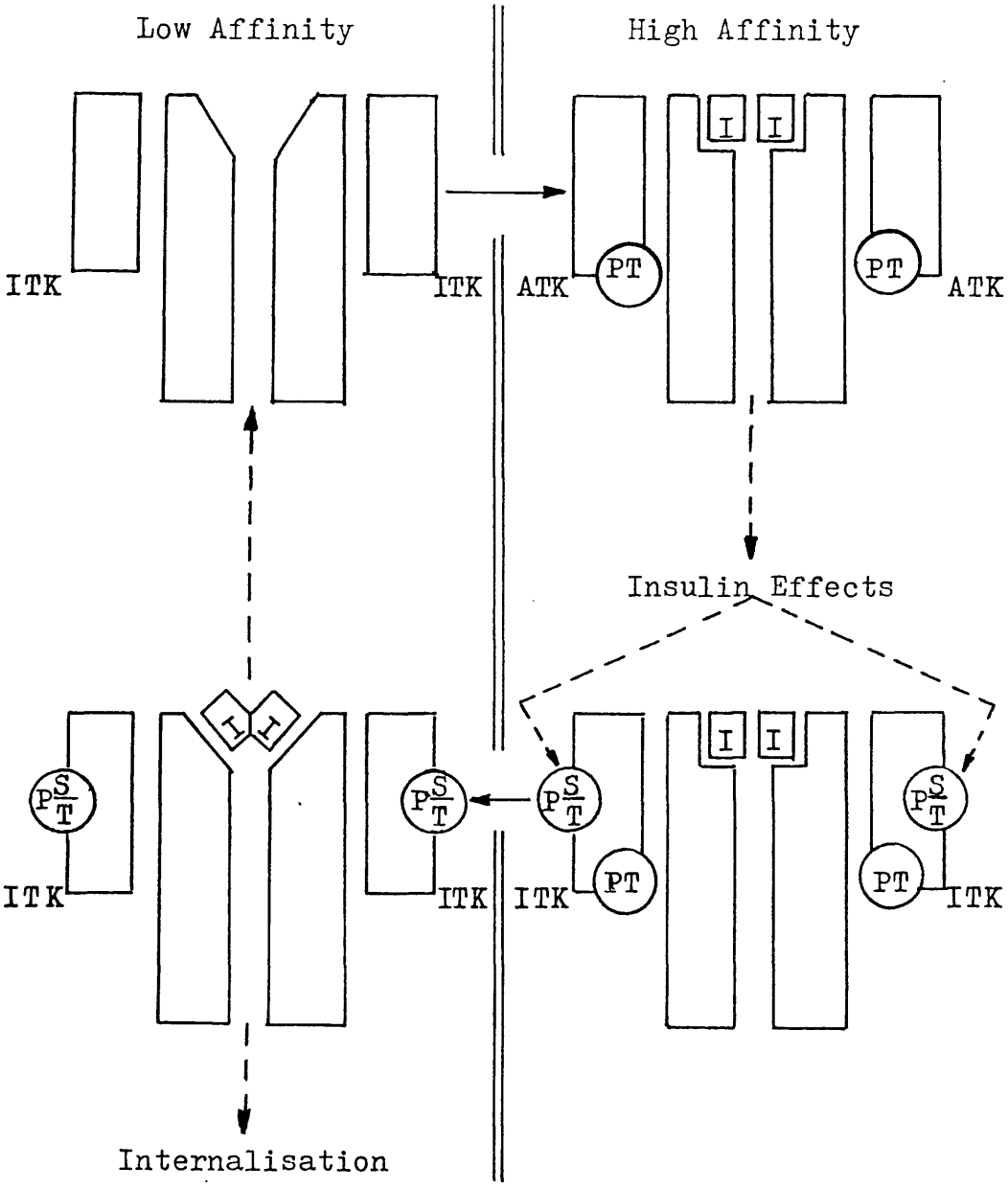
engendered by isoproterenol treatment, insulin stimulation of receptor autophosphorylation is blocked completely, even at insulin concentrations high enough to saturate the receptors [139]. Phorbol esters stimulate phosphorylation of the insulin receptor β-subunit on serine and threonine residues, presumably by activation of PrKC (see section 1.3.8.(i)b) and inhibit the ability of insulin to enhance β-subunit tyrosine phosphorylation [139, 757]. Since β-adrenergic effects on insulin binding and β-subunit autophosphorylation parallel the phorbol ester mediated effects it is tempting to conclude that raised cAMP levels also result in β-subunit phosphorylation on serine and threonine residues. However, this being so, it does not appear to be achieved by the direct action of cAMP-PrK because the insulin receptor is a very poor substrate for this kinase in vitro [139]. Nevertheless in view of the fact that insulin-stimulated serine and threonine phosphorylation of the insulin receptor β-subunit in vivo [386, 757] occurs under conditions known to provoke down-regulation of insulin receptors [239, 514, 725] it has been speculated that such modifications are important general regulators of the tyrosine kinase and hormone binding characteristics of the insulin receptor system though the phosphorylation sites favoured by the insulin-mediated and the phorbol ester-dependent effects appear to differ [757]. The proposed interactions between tyrosine and serine/threonine phosphorylation are summarised in Figure 15 and the projected effects of such a system on receptor affinity are illustrated in Figure 16. Very similar interactions between serine/threonine phosphorylation and receptor affinity/tyrosine kinase activity have been reported recently for the EGF receptor [166], suggesting that such regulatory mechanisms may be common to many receptor types.

Figure 16

Since the negative feedback control of serine/threonine phosphorylation is governed by intracellular insulin effects, at low insulin concentrations, the effect on receptor affinity will be minimal. Affinity therefore rises with increasing insulin concentration. However, the resulting increase in magnitude of insulin effects stimulates serine/threonine phosphorylation leading to increased inhibition of tyrosine phosphorylation and, consequently, reduced receptor affinity. Serine/threonine phosphorylation may also act as a "signal" for internalisation.

* The recent suggestion that depletion of cellular ATP levels precipitates a loss in insulin receptor affinity [371] is consistent with the proposal that tyrosine phosphorylation induces an increase in receptor affinity since phosphotyrosine turnover is very rapid and would therefore be extremely sensitive to kinase inhibition resulting from restrictions in ATP supply.

Figure 16. Model for the Participation of Tyrosine Phosphorylation in regulation of Insulin Receptor Affinity and Internalisation.



Legend.

ITK	Inactive Tyrosine Kinase.
ATK	Active Tyrosine Kinase.
I	Insulin.
PT	Phospho Tyrosine.
PS/T	Phospho Serine/Threonine.

Assumption. Tyrosine Phosphorylation Increases Receptor Affinity*

Models for the regulation of insulin receptor binding activity have been proposed based on interpretations of experimental results suggesting either the existence of multiple receptor affinities [164, 277, 401, 581] or a negatively cooperative response to insulin binding [151, 152]. While neither mechanism is accepted universally, it is possible to construct models with characteristics incorporating both mechanisms which also suggest important roles for tyrosine and serine/threonine phosphorylation of the insulin receptor. An example of such a model, represented in Figure 16 also allows for expression of effects mediated by receptor internalisation. The response to insulin predicted by this model is characterised by an initial increase in receptor affinity with increasing insulin concentration followed by a concentration-dependent decrease in affinity at higher insulin concentrations. Such a response, at physiological insulin concentrations, has been reported recently by Marsh et al. [513] and represents an elegant mechanism for modulating insulin action. At low insulin concentrations, receptor affinity is increased, resulting in enhanced insulin binding and, therefore, cellular response but overstimulation is prevented by a reversal of this effect as the maximally effective insulin concentration is exceeded [513].

1.3.8.(ii)b Potential Regulatory Roles of Tyrosine Kinases

A number of other tyrosine kinase activities in normal cells have been reported recently [171, 750, 854] but the nature of both the kinases themselves and their substrate proteins are unknown. One of these, termed p75 on the basis of the molecular weight of an endogenous substrate, is a soluble 70kDa cytoplasmic enzyme purified from rat liver [854], while several other proteins have been reported to be

tyrosine phosphorylated in membrane preparations from lymphoid cells [171, 750]. Phosphates on tyrosine residues turnover rapidly and, therefore, tyrosine phosphorylation potentially represents a highly sensitive control system [338]. The role of tyrosine phosphatases in such regulatory mechanisms is obviously, therefore, of equal importance to that of the tyrosine kinases but to date little information has been accumulated in this area. Whether tyrosine phosphorylation will prove to be as important in the mediation of cellular regulation as is indicated by its association with cell surface receptors remains to be seen but even if signal transduction is found to be independent of this activity, tyrosine phosphorylation may still be important in the modulation of hormone action by virtue of its ability to regulate hormone binding.

While the physiological role of tyrosine phosphorylation remains obscure, there is evidence to support a second, potentially more physiologically significant function of tyrosine kinases which might provide a valuable insight into the molecular mechanism(s) involved in propagation of intracellular signals following hormone or growth factor receptor occupation. The oncogene tyrosine kinase $p68^{v-ros}$ of avian sarcoma virus UR2 will phosphorylate itself and exogenous protein substrates on tyrosine residues [21, 211] but also catalyses the phosphorylation of phosphatidyl inositol (ptd.ins.) in vitro to give phosphatidyl inositol 4 phosphate (Ptd Ins 4P) [496]. Evidence that the latter property of $p68^{v-ros}$ is also expressed in vivo is provided by the observation that transformation of chicken embryo fibroblasts by UR2 is accompanied by an increase in phosphoinositide turnover [496]. In contrast, cAMP-PrK is not capable of catalysing phosphoinositol phosphorylation, suggesting that this property may perhaps be unique to

$p68^{v-ros}$ and other tyrosine kinases. These observations raise the possibility that the primary function of tyrosine kinase activities associated with insulin and various growth factor receptors is not in fact expressed through phosphorylation of proteins on tyrosine residues but may instead be related to the ability of such kinases to phosphorylate ptd.ins. Speculation of this kind is extremely attractive in that it provides an explanation for the observation that insulin increases phosphoinositide turnover [205] and therefore suggests a direct link between insulin binding (known to activate the insulin receptor tyrosine kinase [see earlier]) and activation of protein kinase C (see section 1.3.8.(i)b). The regulatory potential of a system capable of modifying phosphoinositide turnover is now well recognised (see reviews [45, 205] and also section 1.3.8.(i)b) and the proposed link between tyrosine kinase activity and ptd.ins. phosphorylation may therefore have far reaching implications, particularly in the elucidation of mechanisms of action for growth factors and insulin.

1.3.8.(iii) Modulator-Independent Protein Kinases

Many protein kinases have been described for which no regulatory agents or effectors have so far been identified. While this does not necessarily mean that these enzymes or the reactions they catalyse are not subject to regulation, their physiological regulatory significance is not immediately obvious and therefore has not attracted as much general interest as the kinases described above whose activities are modulated in a specific manner by factors known or thought to be important in vivo. The phosphorylations catalysed by several of the members of this diverse group of protein kinases appear to have little

or no effect on the properties of their designated protein substrates but this may simply reflect an inability to identify their physiologically important substrates. Examples of kinases whose regulatory potential was not recognised until some time after their discovery include casein kinase 2 (CKG, see section 1.3.8.(i)c) and glycogen synthase kinase 3 (phosphatase 1 activating factor, FA, see section 1.3.9.(i)). Although no effectors of the latter have yet been identified, the discovery that CKG activity is subject to modulation by polyamines has, necessarily, led to its reclassification. Bearing this in mind, it seems likely that other protein kinases, at present classified as modulator-independent will, in time, be recognised either as members of one of the established groups of effector-dependent kinases or examples of novel regulatory systems in their own right. However, since the primary object of the work described herein was to survey the contributions made by the major groups of regulatory protein kinases, particularly cAMP-PrK, to the phosphorylation of endogenous mammary proteins, a review of the numerous ill-defined kinases classified under the general heading of modulator-independent will not be presented in this report.

1.3.9. Protein Phosphatases

A comprehensive study of the protein phosphatase activities involved in regulation of the major pathways of intermediary metabolism has been undertaken by Ingebritsen and coworkers [346, 347, 352, 596, 735, 810] who have established that all of the activities directed against serine/threonine phosphate can be explained by only four enzymes with the exceptions of PDH and BCDH dephosphorylations which are governed by the activity of distinct mitochondrial phosphatases

[143, 314, 631, 644, 645]. The classification of these four enzymes is summarised in Table 1. The phosphatases which, with the exception of protein phosphatases 1 (PrP-1) and 2A (PrP-2A), are almost exclusively cytosolic [352], are divided into two classes on the basis of their subunit specificity with regard to dephosphorylation of phosphorylase kinase and sensitivity to inhibition by two thermostable proteins termed inhibitor 1 (I-1) and inhibitor 2 (I-2). Type 1 protein phosphatases (PrP-1) dephosphorylate the β subunit of phosphorylase kinase [11, 734] and are potently inhibited by the two thermostable inhibitor proteins [111, 120, 330, 562, 734] whereas Type 2 protein phosphatases (PrP-2) preferentially dephosphorylate the α subunit of phosphorylase kinase [11] and are insensitive to both I-1 and I-2 [120, 562].

1.3.9.(i) Type 1 Protein Phosphatases

Protein phosphatase 1 has a very broad substrate specificity and is the major glycogen phosphorylase, phosphorylase kinase and glycogen synthase phosphatase in skeletal muscle *in vitro* [352]. While it is also the major glycogen synthase phosphatase in liver, it accounts for only 20-50% of glycogen phosphorylase and phosphorylase kinase phosphatase activity in this tissue *in vitro* [352]. Extrapolation of these results to the situation likely to prevail in intact liver cells is complicated by the observation that PrP-1 activity is inhibited strongly in less dilute tissue extracts suggesting the presence, in these preparations, of a macromolecular phosphatase inhibitor [352]. Whether this implies that liver PrP-1 activity would be even lower *in vivo* or that the inhibitory effects are artefacts of the *in vitro* system is unknown since the nature of the factors involved has not been

Table 1 Classification of protein phosphatases in cellular regulation

Type	Protein phosphatase	Inhibition by inhibitor-1 and inhibitor-2	Specificity for phosphorylase kinase	substrate specificity	Phosphorylase phosphatase activity	Regulators
1	Protein phosphatase-1	yes	β-subunit	broad	high	inhibitor-1, inhibitor-2
2	Protein phosphatase-2A	no	α-subunit	broad	high	unknown
2	Protein phosphatase-2B	no	α-subunit	narrow	very low	calcium ions-calmodulin
2	Protein phosphatase-2C	no	α-subunit	broad	very low	magnesium ions

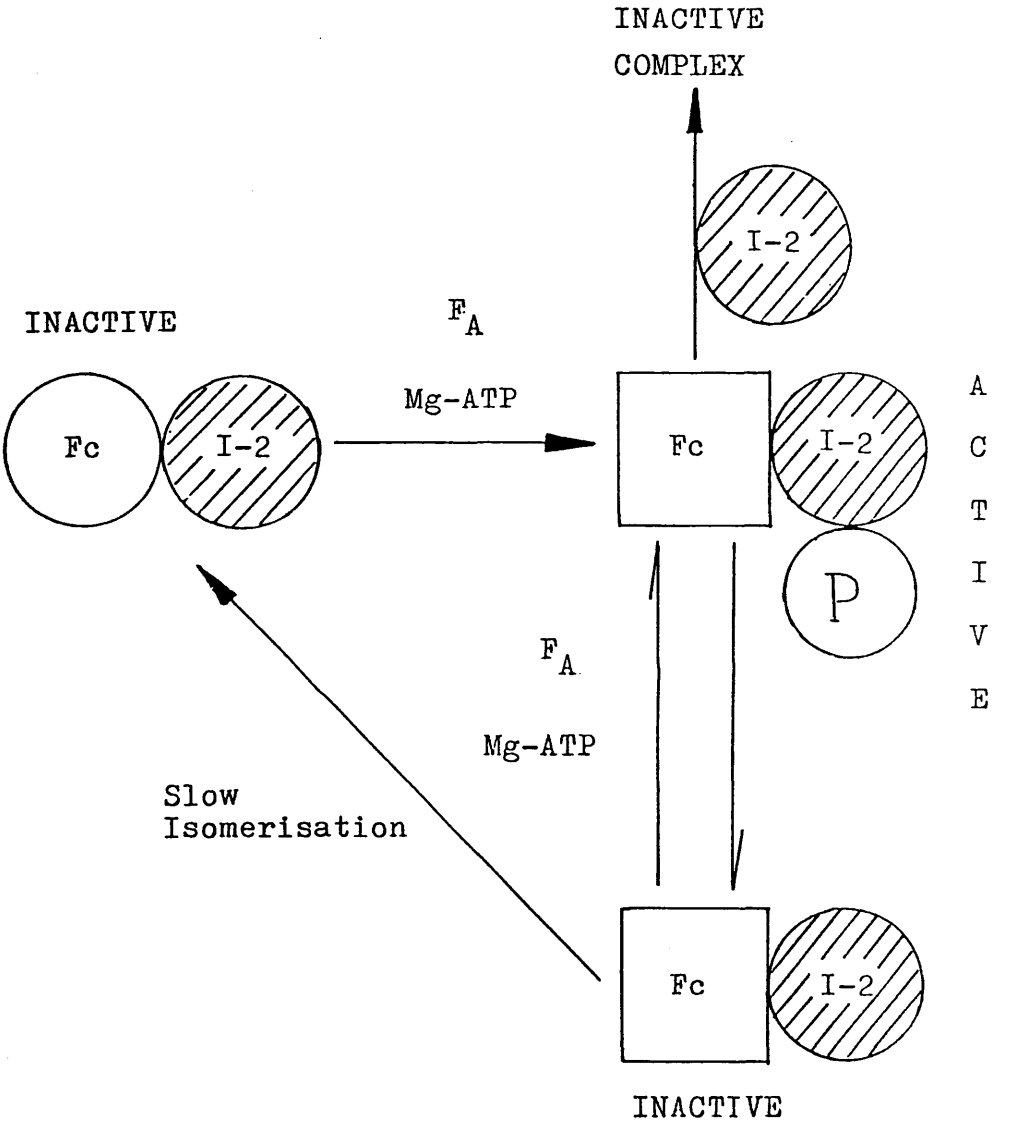
determined. However, there is good circumstantial evidence to indicate that I-1 and I-2 are not responsible for this inhibition since I-1 is essentially inactive under the conditions of assay used, the concentration of I-2 is equally high in skeletal muscle preparations where no such effects are observed and the activity of liver PrP-2A, one of the inhibitor-insensitive type 2 phosphatases (see section 1.3.9.(ii)) also shows extract concentration dependent loss of activity [352]. Protein phosphatase 1 is active in the absence of divalent cations and although dephosphorylation of certain substrate proteins by this enzyme exhibits Mn^{2+} dependence the requirement for Mn^{2+} appears to be a characteristic specific to these substrates alone and not a general property of the phosphatase itself [352].

Both I-1 and I-2 are equally effective inhibitors of type 1 PrP but I-1 is only active in this respect after phosphorylation by cAMP-PrK [121, 330, 562] on a threonine residue located 35 residues from the N-terminus of the protein [6]. Dephosphorylation of I-1 by PrP-1 is not inhibited by I-1 [115, 562] but is one of the Mn^{2+}-dependent reactions and its significance *in vivo* is therefore uncertain since physiological Mn^{2+} concentrations may not be sufficient to allow I-1 inactivation by this mechanism [352]. Inhibition of phosphatase activity occurs at nanomolar I-1 concentrations *in vitro* and since its concentration *in vivo* (1.8μM) is higher than that calculated for PrP-1 [224], I-1 is potentially a very effective regulator of phosphatase activity in intact cells. Adrenaline induces a marked increase in *in vivo* I-1 phosphorylation [223, 225, 404] and insulin is able to antagonise I-1 phosphorylation at low concentrations of β-adrenergic agonists [225, 226] suggesting that regulation of phosphatase activity by I-1 may be an important determinant of the cellular response to

hormonal stimuli. Recently it has been reported that dephosphorylation of glycogen synthase sites 3 by PrP-1 is stimulated 2.5 fold by spermine [117] which, in view of the known insulin like effects of polyamines (see section 1.3.8.(i)c), might suggest an involvement of PrP-1 in insulin induced activation of glycogen synthase.

At least two species of PrP-1 have been identified in rabbit skeletal muscle. Both the parent molecules, termed PrP-1_I and PrP-1_G and the free 37kDa catalytic (F_C)-subunit, common to both enzymes, have recently been purified to homogeneity [117]. Though not as yet confirmed, the major form of PrP-1 in skeletal muscle is reported to be PrP-1_G which consists of the 37kDa F_C-subunit associated with a 105kDa (G)-subunit in a 1:1 molar ratio [117]. The G-subunit binds to glycogen and the glycogen bound enzyme predominantes over free PrP-1_G. Phosphorylation of the G-subunit, an excellent substrate for cAMP-PrK, increases the susceptibility of PrP-1_G to inactivation by I-1. Since I-1 is itself activated by cAMP-dependent phosphorylation, this sytem represents a means of increasing the sensitivity of PrP-1_G to cAMP-dependent inhibition. Before the relative contributions of PrP-1_I and PrP-1_G to total PrP-1 activity had been described, a great deal of interest centred on the properties of Mg-ATP dependent PrP, now known as PrP-1_I, which displays the characteristics of a type 1 phosphatase only after activation involving inhibitor 2 and a protein kinase termed F_A (Figures 6 and 17). Although the activity of this phosphatase is now thought to be of secondary importance, quantitatively, in skeletal muscle [117], its subcellular distribution and mode of activation may endow PrP-1_I with regulatory significance out of proportion to its relatively minor contribution to total PrP-1 activity. In addition, the situation in skeletal muscle may not be duplicated in other tissues

Figure 17. Activation and inhibition of Protein Phosphatase 1_I mediated by inhibitor 2.



Legend.

F_c Catalytic Subunit of Protein Phosphatase 1_I

F_A Activating factor.
(Glycogen Synthase Kinase III).

I-2 Inhibitor 2

P Phosphate.

where PrP-1_I might be relatively abundant in comparison and therefore of obvious importance to the control of cellular protein phosphorylation. The activation/deactivation of PrP-1_I, as summarised in Figure 17 is complicated by the influence of I-2 which is now recognised as a regulatory subunit of the PrP-1_I complex [117, 375, 805, 865] as shown in Figure 16. The catalytic subunit (F_C) of PrP-1_I, inactive in the presence or absence of its regulatory subunit (I-2), may be activated by Mn^{2+} or Co^{2+} ions when free of inhibitor 2 [805] or by Mg-ATP and activator protein (F_A) when bound to I-2 in a specific manner [12, 115, 805]. During activation of the native PrP-1_I complex (F_C + I-2), I-2 becomes phosphorylated, inducing a conformational change in the catalytic subunit, leading to its activation [24, 305, 375, 805]. Dephosphorylation of bound I-2 results in inactivation of the enzyme complex but does not cause a concomitant reversion of F_C to its inactive conformation, demonstrating that dephosphorylated I-2 prevents expression of catalytic subunit function [805]. If released from its regulatory protein at this point, F_C displays full activity and remains active indefinitely, in contrast to F_C remaining bound to I-2 which slowly reverts to the inactive conformation of the native enzyme complex [375, 805]. The site of I-2 binding to F_C is highly specific. The site that confers Mg-ATP dependence on PrP-1_I appears to be masked on the isolated inactive catalytic subunit since I-2 binding to free inactive F_C produces a complex incapable of activation by Mg-ATP and F_A. However, a reconstituted I-2/active F_C complex exhibits the same properties as the native complex in that the active catalytic subunit reverts to its inactive conformation and may be reactivated by Mg-ATP and F_A [805]. Active native PrP-1_I appears to be susceptible to inhibition by a second I-2 molecule which behaves in

the same manner as I-2 bound to isolated inactive F_C, suggesting that there might be a second I-2 binding site on the F_C-subunit rather than that the second I-2 molecule merely replaces the phosphorylated I-2 in the active native phosphatase complex [375, 805]. The activator protein F_A exhibits protein kinase activity and is, in fact, thought to be glycogen synthase kinase 3 (GSK 3) [122, 792], surprising since it must therefore be involved in both the phosphorylation of glycogen synthase and activation of a phosphatase capable of dephosphorylating the same enzyme. GSK 3 should, then, have the potential to modulate glycogen synthase activity in a similar, if less direct manner to that demonstrated by I-2 in the regulation of PrP-1_I activity. However, since PrP-1_I is no longer thought to be the major PrP-1 in skeletal muscle [117], of equal or greater importance as a determinant of the phosphorylation state of glycogen synthase, may be the activity of PrP-1_G.

1.3.9.(ii) Type 2 Protein Phosphatases

The type 2 protein phosphatases (PrP-2) are divided into three categories, namely protein phosphatases 2A, 2B and 2C of which two (PrP-2A and PrP-2C) have broad substrate specificities similar to PrP-1, while PrP-2B displays a much higher degree of substrate specificity [347]. Besides catalysing the dephosphorylation of phosphorylase kinase at different sites, the major difference between the substrate specificities of PrP-1 and PrP-2A is that PrP-2A displays much higher myosin light chain phosphatase and ATP-citrate lyase phosphatase activities [347]. PrP-2C also has a broad substrate specificity but can be distinguished from PrP-2A by its dependence on Mg^{2+}, its extremely low phosphorylase phosphatase and histone H1

phosphatase activities, its slow dephosphorylation of glycogen synthase sites 3 relative to site 2 and its extremely high HMG CoA reductase kinase phosphatase activity [347]. PrP-2B is a Ca^{2+}/calmodulin-dependent enzyme and the only three proteins so far identified as effective substrates are the α-subunit of phosphorylase kinase, inhibitor 1 and myosin light chain. Due to its dependence on Ca^{2+}/calmodulin for activation, PrP-2B is specifically inhibited by the calmodulin inhibitor trifluoperazine [347, 735].

Enzymes classified as PrP-2A are further subdivided into three groups on the basis of their subunit structure. Designated $PrP\text{-}2A_0$, $PrP\text{-}2A_1$ and $PrP\text{-}2A_2$ they share a common 36kDa catalytic (C)-subunit [117, 346, 348, 352, 596, 735] and one other subunit of molecular weight 60kDa termed the A-subunit [117]. However, while $PrP\text{-}2A_2$ is simply a hetero dimer of A and C, $PrP\text{-}2A_0$ and $PrP\text{-}2A_1$ each possess an additional subunit, termed the B' (54kDa) and B (55kDa) subunit respectively. The B-subunits are distinct and $PrP\text{-}2A_0$ and $PrP\text{-}2A_1$, are both tetramers, each incorporating two C-subunits to give subunit compositions of $AB'C_2$ and ABC_2 respectively [117]. Dissociation of the C-subunit from the native enzymes considerably enhances its activity and all three PrP-2As can also be activated by a mechanism involving interaction with basic proteins which does not induce dissociation of the C-subunit but the physiological significance of this phenomenon is unknown [117].

Dephosphorylation of various substrates for protein phosphatases 1 and 2 is stimulated by 2mM spermine [117, 785] but the degree of stimulation is dependent both on the specific form of protein phosphatase and on the substrate towards which activity is directed [785]. PrP-2A activity is among those stimulated by spermine, the

greatest effects being observed with glycogen synthase phosphorylated at sites 3. Dephosphorylation of these sites by PrP-$2A_0$ and PrP-$2A_1$ is stimulated 8-15 fold by spermine with a half-maximal activating concentration of 0.2mM and an optimal concentration of 1-2mM [117]. This and the similar, though less dramatic effect of spermine on PrP-1 are intriguing since insulin has been shown to reduce the steady state phosphorylation of glycogen synthase sites 3 in vivo [118] and polyamines are known to mimic certain insulin effects in isolated cells in a non-additive manner with respect to insulin [485]. In contrast, the activities of protein phosphatases 1, $2A_0$, $2A_2$ and $2A_c$ towards glycogen phosphorylase are all inhibited markedly by spermine [758].

Besides its possible importance in insulin-stimulated glycogen synthase sites 3 dephosphorylation PrP-2A may also be the only effective I-1 phosphatase if PrP-1 is unable to perform this function in vivo (see section 1.3.9.(i)) and therefore PrP-2A activation, leading to antagonism of cAMP-dependent I-1 activation would be an important modulator of PrP-1 activity. Thus PrP-2A activation might stimulate increased dephosphorylation of glycogen synthase sites 3 by both direct and indirect (via decreased PrP-1 inhibition) mechanisms.

Protein phosphatase 2B (PrP-2B) appears to be composed of two subunits, termed A (approximately 60kDa) and B (approximately 15kDa) in a molar ratio of 1:1 [116, 348]. This structure is very similar to that of calcineurin which is a major calmodulin binding protein of neural tissue and is composed of A and B subunits of molecular weights 61kDa and 15kDa respectively [422]. Several lines of evidence suggest that these two calmodulin binding proteins might indeed be the same [735]. The B-subunit of PrP-2B binds Ca^{2+} and is therefore likely to be responsible for the Ca^{2+} sensitivity of this enzyme in the

absence of calmodulin while the A-subunit interacts with calmodulin and is likely to be the catalytic subunit [422, 735]. Determination of the primary structure of B-subunit has revealed similarities with calmodulin and troponin C, particularly in the region of its four Ca^{2+} binding sites which probably accounts for the sensitivity of PrP-2B to inhibition by trifluoperazine in the absence of calmodulin [116]. Because of its dependence on micromolar Ca^{2+}, it has been suggested that PrP-2B would only be active in cells whose Ca^{2+} levels had been elevated above resting levels [735]. Since PrP-2B is an extremely effective I-1 phosphatase in the presence of Ca^{2+}, it might play an important part in the regulation of PrP-1 activity by agents (neuronal or hormonal) that affect intracellular Ca^{2+} concentrations [735]. However, it is recognised that the most important physiological substrates of PrP-2B may not yet have been identified, particularly if the possible identity with calcineurin is confirmed [735].

Protein phosphatase 2C (PrP-2C) is a monomeric enzyme with a molecular weight of 40-48kDa [314, 645, 810] and, in common with PrPs 1 and 2A has been shown to be capable of *in vitro* dephosphorylation of mammary gland acetyl-CoA carboxylase phosphorylated either *in vivo* [347] or by cAMP-PrK *in vitro* [283]. However, PrP-2C activity may be of greater significance in the control of HMG-CoA reductase of which it is easily the major phosphatase *in vitro* [347]. Whether this is also the case *in vivo* is uncertain since 20% of liver PrP-1 activity is associated with the microsomal fraction and HMG-CoA reductase is a microsomal membrane bound enzyme [259, 352, 480, 657]. Thus, despite its low HMG-CoA reductase phosphatase activity *in vitro* compared with PrP-2C, PrP-1 may, nevertheless, be an important regulator of HMG-CoA reductase phosphorylation *in vivo* by virtue of close association with this substrate [352].

1.3.10. Protein Phosphorylation in Mammary Tissue

A most interesting feature of many of the enzymes involved in protein phospho-dephosphorylation is their apparent ubiquity and broad, often overlapping substrate specificities. This is evident for both kinases and phosphatases which represent the effectors of a highly complicated system allowing integrated regulation of diverse cellular processes in response to both neural and hormonal stimuli. Probably one of the most important and doubtlessly, the most extensively characterised of the multifunctional protein kinases is cAMP-PrK which appears to be common to all mammalian tissues. The activity of this enzyme is determined in vivo by intracellular cAMP concentrations and, as discussed in section 1.1., these are subject to both hormonal and neural regulation. The mechanisms of action of cAMP mediated hormones such as glucagon and β-adrenergic agonists are well characterised (see section 1.1.) but the mode of action of insulin, a hormone known to antagonise a number of cAMP-mediated events, is uncertain. This facet of insulin action is, in many cases, assumed to be a reflection of its ability to either depress prevailing intracellular cAMP concentrations or antagonise the increase normally accompanying β-adrenergic or other hormonal stimulation [617, 622]. There is good evidence that this effect of insulin may be mediated by hormone sensitive cAMP-PDE in adipose tissue and liver and stimulation of high affinity cAMP-PDE by insulin is reported here in mammary acini. However, the importance of modulation of cAMP concentrations in the regulation of mammary cell metabolism is brought into question by the observation that agents known to raise intracellular cAMP concentrations in mammary cells do not affect the steady-state phosphorylation of proteins labelled with

^{32}P in intact cells [100, 103] (see also section 3.2.). Furthermore, mammary acini have been found to lack glucagon receptors [656], raising the question of whether the intracellular mechanisms necessary for metabolic response to cAMP are in fact competent in mammary tissue or whether despite the potential ability of insulin and other agents such as β-adrenergic agonists (see section 1.1.) to regulate cAMP levels in these cells, the cAMP-PrK system is absent or defective. The major aim of the phosphorylation work described herein was to address this problem by investigating the response of protein phosphorylation observed in various sub-cellular fractions in vitro to cAMP. However, a survey of the contributions of the other major multifunctional protein kinases of particular interest in mammary gland, namely Ca^{2+}/calmodulin-dependent protein kinase, protein kinase C and polyamine dependent protein kinase to protein phosphorylation in vitro was also undertaken. Work similar in nature to that involved in achieving the first of these objectives was undertaken in 1972 by Majumder and Turkington [500] who described induction of cytosolic cAMP-PrK synthesis in response to insulin and/or prolactin in mouse mammary stem cells. However, although a number of plasma membrane and ribosomal proteins were demonstrated to be substrates for this kinases, no attempt was made either to investigate the phosphorylation of cytosolic or other membrane fraction proteins by these kinases or to determine the molecular weights or any other characteristics of those substrates which had been identified. Unlike the situation in liver, adipose tissue, brain and muscle, protein phosphorylation in mammary tissue has received little attention and the only mammary enzyme demonstrated, directly, to be regulated by reversible phosphorylation is acetyl-CoA carboxylase (see section 1.3.4.(ii))

although there is good circumstantial evidence that mammary PDH is similarly regulated [26] and work is also in progress at present to characterise the regulatory properties of mammary gland HMG-CoA reductase [715, 716], an enzyme whose activity has been shown to be regulated by phosphorylation in other tissues (see section 1.3.5.). The activity of an unspecified, quercitin-inhibited, cAMP-independent protein kinase purified from rat mammary tissue cytosols [698, 699] correlates with tissue proliferation during the normal physiological cycle of mammary gland development in pregnancy and lactation [698] and is high in DMBA-induced mammary tumors, falling to insignificantly low levels in regressing tumors [699]. In contrast, while cAMP-dependent protein kinase activity measured in the same rat mammary cytosols [698] is markedly elevated over virgin levels by mid-pregnancy, it remains at approximately the same level throughout the remainder of pregnancy and lactation and does not, therefore, correlate with tissue proliferation which continues to rise up to the end of lactation [698]. Recent reports [472, 700] by the authors of this work identifies an association between membranal tyrosine kinase activity, shown to have quercitin inhibition characteristics similar to those of the previously described cAMP-independent, quercitin-inhibited protein kinase of mammary and uterine tissue [698, 699, 701], and DMBA-induced rat mammary tumor growth. Protein kinase C activity in mouse mammary gland has been shown to vary inversely with mammary differentiation [86] and although total enzyme activity (measured under optimal Ca^{2+} and phospholipid conditions) does not correlate with tissue proliferation during the reproductive cycle, it has been suggested that a correlation may exist <u>in vivo</u> where physiological conditions might play a major role in the regulation of its activity [86]. In common with the

cAMP-independent protein kinase of rat mammary gland, mammary PrKC is inhibited by quercitin and may represent the same enzyme. Ca^{2+}/calmodulin-dependent protein kinase activity has also been identified recently in a particulate fraction isolated from rat mammary acini [56]. The enzyme appears to be associated with a 1000kDa particle composed largely of protein with a small lipid component and containing an endogenous protein substrate of molecular weight 53.6kDa [56].

While the work described above provides an indication that protein phosphorylation may be of equal importance to the control of metabolic function in mammary cells as it is in many other tissues, the profile of protein kinase activities potentially capable of contributing to such regulatory mechanisms has not been determined and a comparison with the distribution of the various well characterised effector-dependent protein kinases described in other tissues has, therefore, previously been impossible.

1.4. The Mammary Gland

Displaying remarkable synthetic capabilities, the mammary gland places enormous demands on maternal nutrition and is, therefore, necessarily subject to comprehensive regulatory controls. The nature of these regulatory mechanisms (both long and short term) is, in many instances, understood only poorly and has been the focus of numerous research projects, including the work described in this thesis. A brief introduction to the gland and its physiology is presented below.

1.4.1. Structure

The mammary gland is composed of a complex system of secretory alveoli, each connected to a network of ducts leading, ultimately, to a common chamber (cistern) beneath the nipple. The secretory alveoli are organised into discrete lobules, separated by connective tissue; during lactation they are composed primarily of secretory (glandular) epithelial cells (g in Figure 18). These are, however, associated closely with several other cell-types, identified as myoepithelial, blood capillary and fibroblast-like cells (M, bc and f, respectively, in Figure 18). In non-digested tissue, the surface of the alveoli is covered densely by collagenous fibres; a number of fat cells (F in Figure 19 [1a]) are also evident in the inter-lobular connective tissue. The intricate mesh-work of large collagenous fibres and finer reticulate fibres conceals completely the alveoli (Figure 19 [16]).

Removal of inter-lobular connective tissue by an enzyme-HCl method (see 554a) reveals the surface of the alveoli (Figures 18 and 20) which are roughly spherical and have diameters of approximately 40-50μm. As noted above, each alveolus is composed predominantly of secretory epithelial cells but up to four to six myoepithelial cells may stretch across its surface (see Figure 20) and several of the fibroblast-like cells may also be observed interspersed across the lobule (Figure 18).

Digestion of mammary tissue by the method described in Materials and Methods (section 2.2.2.) effects degradation of connective tissue and results in release of individual alveoli from the lobular matrix. The alveoli retain a degree of structural integrity and are isolated complete with remnants of their associated collecting duct. The sack-like structures (acini) so produced therefore contain a number of

Figure 18

g glandular epithelial cells

M myoepithelial cells

bc blood capillaries

f fibroblast-like cells

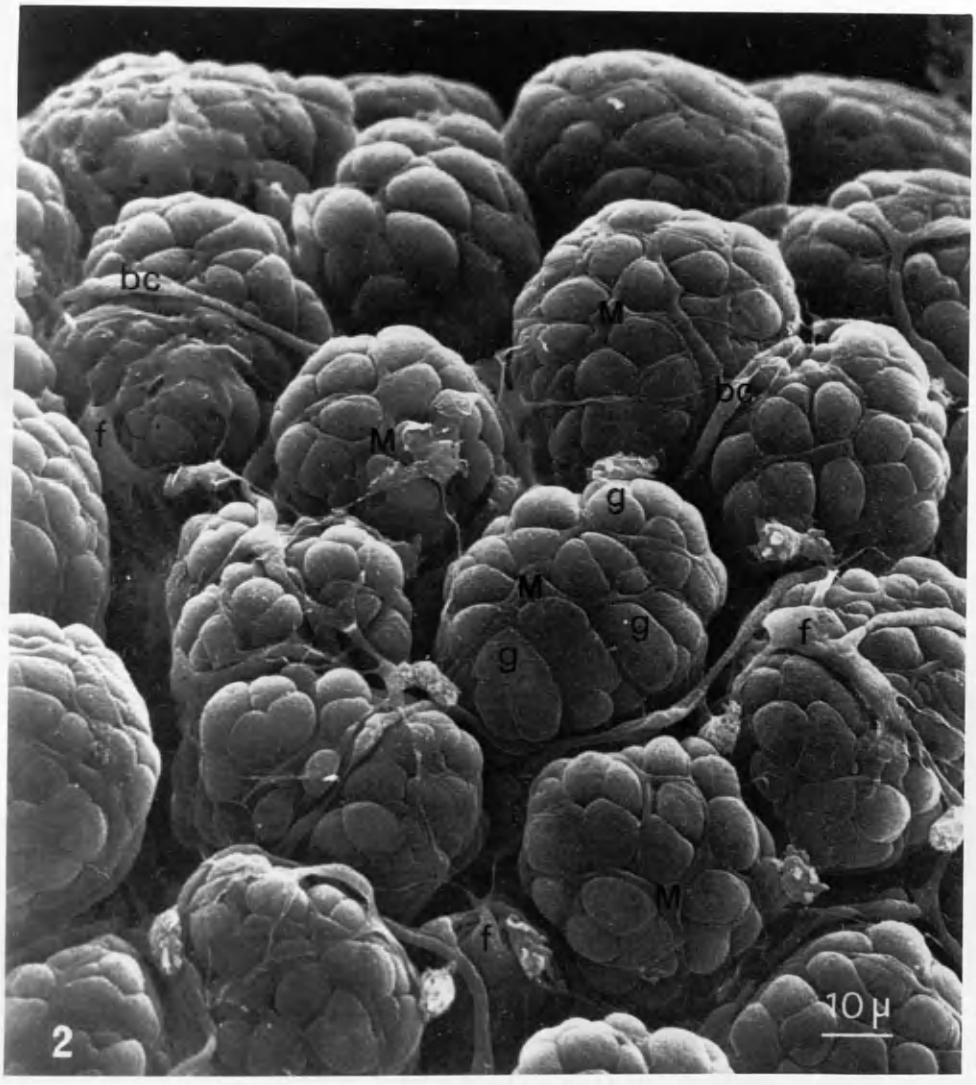


Figure 18. Scanning electron micrograph showing surface view of a lactating mammary gland in which almost all extracellular connective tissues have been removed with enzyme-HCl digestion.

Figure 19

1a. A number of fat cells (F) and a dense covering of collagenous and reticular fibres almost conceal the terminal portions (*) of the gland.

1b. A higher magnification micrograph of the area enclosed by a rectangle in 1a, showing the organisation of collagenous (C) and reticular fibres that enclose the terminal portion.

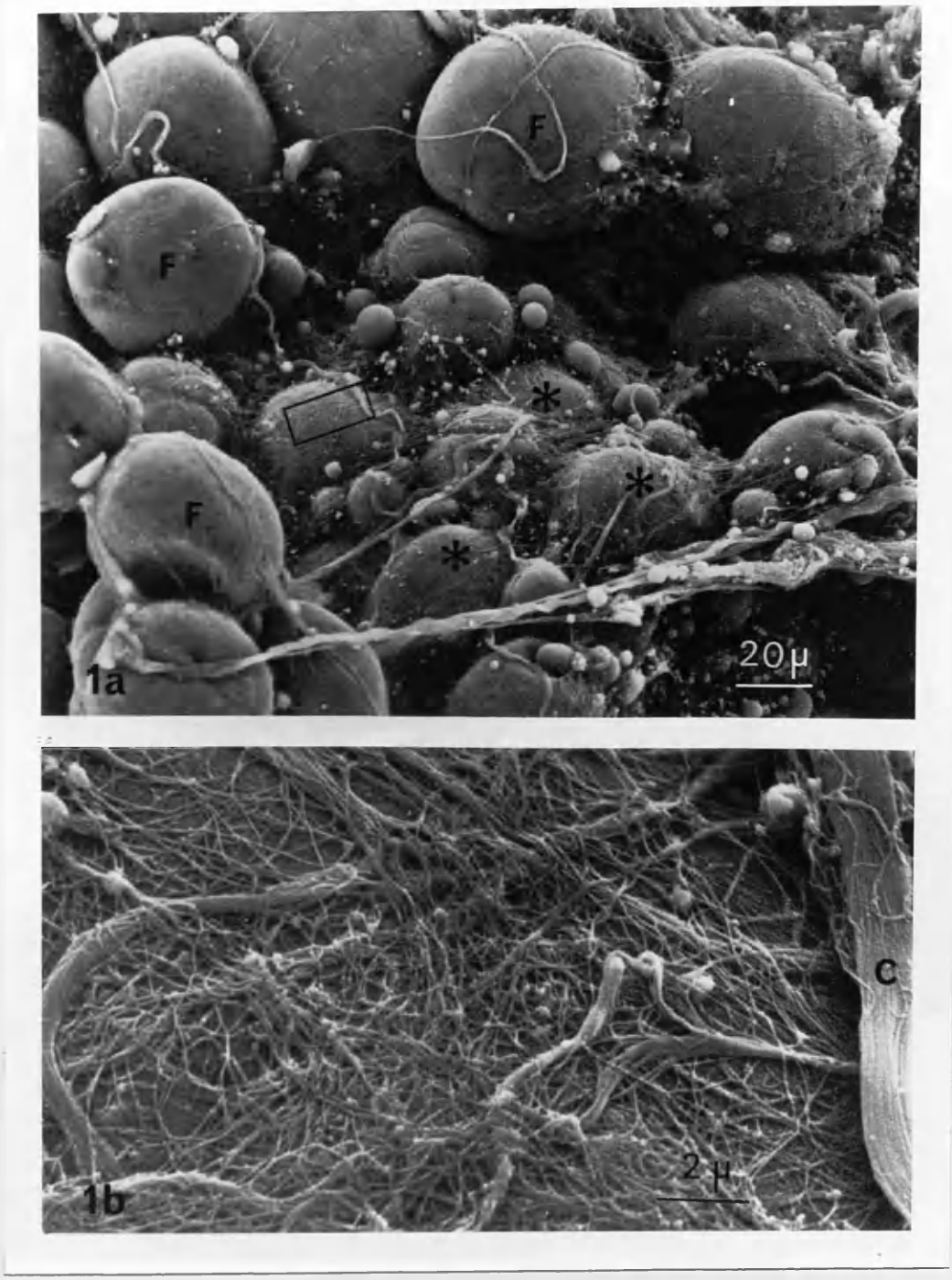


Figure 19. Scanning electron micrograph of an untreated portion of a lactating mammary gland, torn into lobules.

Figure 20

M myoepithelial cells

bc blood capillary

Large arrow indicates crossing-over of two myoepithelial cells

Small arrows show the boundaries of the glandular cells

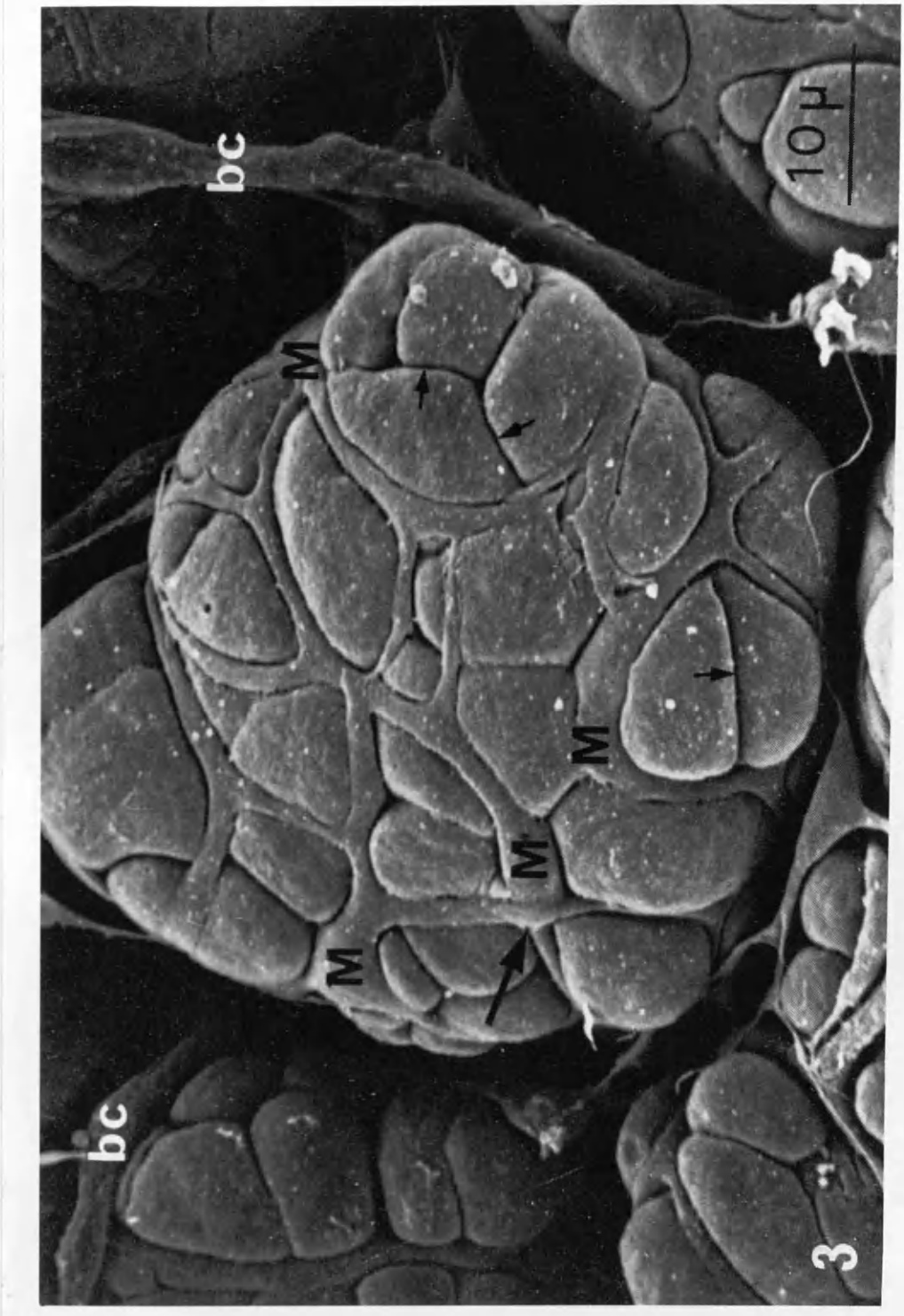


Figure 20. Scanning electron micrograph showing the terminal portions of a lactating mammary gland.

different cell types including myoepithelial, capillary and duct cells but are composed predominantly of secretory epithelial cells. Fat cells present in the interlobular connective tissue (see Figure 19 [1a]) are not sedimented by the centrifugal procedure used for harvesting of acini and are therefore removed during washing. Only secretory epithelial cells are involved directly in milk synthesis and it is therefore the metabolic regulation of these cells alone that is of interest in this study. Since acini prepared from lactating mammary gland are rich in this cell type, are easily manipulated in suspension, are responsive to hormonal stimulation and have been shown to be capable of maintaining linear rates of fatty acid synthesis for incubation periods in excess of one hour, this preparation provides an excellent system for the study of mammary gland metabolism and its regulation. Procedures are available for isolation of secretory epithelial cells from the other cell types residing in mammary acini but using such techniques, yields an insufficient quantity of these cells to allow studies of the kind conducted here and such preparations do not therefore offer a realistic alternative to the acinus preparation described above. Although the possible contribution of cell-types other than secretory epithelial to the results obtained using acinar preparations should not be ignored, in practice, this contribution is unlikely to be significant and will not therefore be considered in discussion of experimental findings.

1.4.2. Physiology

The onset of lactogenesis has been shown, in a variety of species, to be dependent on a rise in serum prolactin concentration and a fall in serum progesterone, with glucocorticoids appearing to play a

permissive role [21b, 442a]. Results obtained from various in vitro and in vivo studies suggest that prolactin, cortisol and insulin are all important in the induction of mammary enzyme synthesis at this time [21a, 21b, 276a, 778b] and insulin, glucocorticoids and prolactin have been shown to act synergistically to stimulate production of milk constituents in cultured mammary explants of mid-pregnant rats and mice [778a].

The hormonal environment required to maintain lactogenesis appears to vary between species. Both prolactin and insulin are known to be necessary for the maintenance of normal lactation and there is evidence that hormones such as thyroxine, growth hormone and corticosteroids may also be involved in the regulation of lactation (134a). Prolactin is known to act at the level of transcription but there is also evidence that this hormone may play at least a limited role in acute regulation of mammary function [1a, 213a, 529, 654b] but, as indicated by studies on mammary function during starvation/re-feeding (see below), insulin seems to be a more important hormone in this respect.

Milk production by the lactating mammary gland creates a considerable drain on available nutrients and various changes in maternal physiology are required to accommodate these demands. Both the mammary gland itself and other organs, such as liver and alimentary tract, become hypertrophied and are served by an increased blood supply [89a, 135a, 211a, 270a, 716b] which is supported by an increase in cardiac output of up to two-fold [89a]. At peak lactation, food intake may be two to three-fold higher than in the unmated animal [136a, 211a, 443a, 581a] and yet is usually accompanied by a net depletion of adipose tissue reserves [46a, 425a, 708a, 730a]. Adipose tissue lipid

reserves are mobilised to help meet the increased demands for precursors for milk lipid biosynthesis. All these changes are required in order to support the development of the mammary gland as the major site of biosynthetic activity during lactation.

In fed lactating rats, the main substrates utilised by the mammary gland are glucose, amino acids and triacylglycerol fatty acids (supplied by plasma VLDL and chylomicra). Plasma glucose has been shown to be the precursor for lactose synthesis [842a] and also for a large proportion of milk lipid [22a, 442b, 577a]. The rates of lipogenesis and lactose synthesis in the mammary gland are both very sensitive to the metabolic status of the animal and vary diurnally, depending on food intake [82a, 553a]. However, while the rate of lipogenesis at peak lactation parallels hepatic lipogenesis (maximum observed just before maximum food intake [553a]), suggesting a relationship with circulating insulin and/or glucagon levels, food withdrawal and insulin supplementation experiments have shown that the rate of lactose synthesis does not coincide with changes in circulating insulin concentrations [82a]. The rate of lactose synthesis does, nonetheless, correlate closely with food intake, implying that although lipogenesis and lactose synthesis are both governed by nutritional status, only lipogenesis shows any signs of being regulated directly by insulin. Indeed, the evidence suggests that, should such a relationship exist between circulating insulin and lipogenic rates, it is not a simple one, since plasma insulin concentrations show little diurnal variation during peak lactation (553a).

In the rat, uptake of 2-deoxyglucose by the mammary gland, measured in vivo, is inhibited 90% by 16h starvation and is restored to normal levels by 1h refeeding [776]. This close relationship between

maternal nutrition and glucose uptake by the mammary gland has been confirmed recently in a study of arteriovenous glucose differences across the mammary gland of fed, starved and re-fed lactating rats [582a]. In this study, after 18h starvation, glucose uptake had fallen to 8% of fed values but recovered substantially within 15 min of re-feeding and had returned to fed levels 1h after re-feeding.

Approximately 20% and 50%, respectively, of glucose uptake by the mammary gland is accounted for by lactose synthesis and fatty acid synthesis and in 15-18h starved rats, conversion of glucose to lactose and fatty acids is decreased to 3% and 1%, respectively, of fed values [73]. Synthesis of these milk solids is therefore shut down almost completely by 15-18h starvation and this is accompanied by a comparable fall in glucose uptake. The rapid resumption, on refeeding, of mammary activity, as indicated by recovery of glucose uptake (see above), does not appear to correlate well with concurrent changes in plasma insulin concentration [582a]. Although plasma insulin concentration also increases rapidly on re-feeding, the highly elevated levels attained do not persist. It therefore appears that the rapid peak in insulin concentration may be the signal required for resumption of mammary activity on re-feeding but that once stimulated, low insulin concentrations are sufficient to maintain this level of activity [532a,582a]. The ability to maintain elevated glucose uptake despite low plasma insulin concentrations is consistent with the need, during lactation, to direct glucose and fatty acids away from other extrahepatic tissues and towards the mammary gland [843a]. However, these observations imply that shortly after refeeding, the mammary gland acquires some independence of circulating insulin. Whether this is achieved through enhanced sensitivity to the hormone or through

by-passing of insulin-dependent controls is unknown (see Zammit [872a] for discussion).

Besides glucose, the other major substrate of dietary origin for milk production in the rat is long-chain fatty acid. Following re-esterification in the intestinal epithelium, dietary fatty acid is transported in the blood as triacylglycerol within chylomicra. However, before it can be taken up by other cells, the lipoprotein triacylglycerol must be hydrolysed to produce free fatty acids and glycerol. The majority of the fatty acid enters the cell but the glycerol produced is carried away in the circulation, to be metabolised by the liver and kidneys. This generalisation may not apply to mammary tissue since there is evidence that glycerol also may be taken up by these cells. Circulating lipoprotein triacylglycerol is hydrolysed by lipoprotein lipase on the endothelial surface of capillaries supplying the tissue. The activity of the mammary enzyme increases during lactation in contrast to that of adipose tissue which decreases around parturition and remains low throughout lactation [877a].

Another important source of fatty acids for mammary lipogenesis is plasma VLDL produced by the liver either by de novo synthesis or from plasma non-esterified fatty acids (NEFA) generated by lipolysis in adipose tissue. During lactation, lipogenesis (from circulating glucose) in adipose tissue is decreased considerably [216, 654a, 716a] and is accompanied by an increase in lipolysis [716c]. As a result, less glucose and, due to decreased lipoprotein lipase activity, less triacylglycerol fatty acid, are removed from the circulation by adipose tissue. Meanwhile, the elevated rate of adipose tissue lipolysis leads to increased release of NEFA into the bloodstream. Hence, during lactation, the mammary gland becomes the predominant site of fatty acid utilisation.

Circulating lipoprotein and NEFA are particularly important to the mammary gland during the post-absorptive period of digestion when utilisation of glucose by peripheral tissues is decreased. At this time, fatty acids from the diet (only available after a delay of several hours due to the characteristics of digestion and absorption of dietary lipid) and NEFA from adipose tissue (following conversion to hepatic VLDL) supplement the supply of fatty acids required for milk synthesis.

2. MATERIALS AND METHODS

2.1. Treatment of Animals

Wistar rats were fed pasteurised breeding diet (see section 2.9.1.) and water ad libitum, and housed at constant temperature (21°C) in a 12-hour light/dark regime (lights on, 08.00h). Males were used at 2-3 months of age and females were mated at this age. The number of pups per mother was adjusted to 8 within 24hr after birth. These rats were used 9-12 days post-partum.

Where appropriate, diabetes was induced in rats by intraperitoneal administration of streptozotocin (150mg/kg), 24hr prior to their use. The severity of diabetes was assessed by determination of blood glucose, as described previously [43] and rats were designated 'diabetic' when blood glucose concentrations exceeded 25mM. Rats deprived of food 24hr prior to use are referred to, in the text, as 24hr starved animals.

2.2. Preparation of Adipocytes and Mammary Acini

Rats were anaesthetised by intraperitoneal injection of a solution, in normal saline, of pentobarbitone (60mg/kg body weight) at 09.30h. Female rats were allowed to continue to be suckled by their young for the 10-15min period following injection, during which deep anaesthesia was being established. Male rats were simply returned to their cage for this period. All subsequent procedures were carried out in a warm room at 37°C.

2.2.1. Adipocytes

Male rats were used as the source of adipose tissue for all experiments. The epididymal fat pads were removed and adipocytes prepared from these by collagenase digestion, as described previously [216]. Adipocyte size and number were determined as in [804] except that dry weight of cells was taken as an approximation to cellular lipid content.

2.2.2. Acini

The inguinal/abdominal mammary glands were dissected rapidly and acini prepared by a modification [656] of the collagenase digestion procedure developed for adipocytes [216] as described below.

Excised mammary glands were minced, using scissors, in oxygenated (O_2/CO_2, 95%/5%) Krebs-Henseleit [440] bicarbonate-buffered saline, pH 7.4 (composition as stated in [440] except that the Ca^{2+} concentration was 1.25mM i.e. half that recommended by Krebs and Henseleit), containing 5mM glucose and transferred to a PTFE square to be chopped finely with razor blades. The resulting finely-divided tissue was rinsed twice in five volumes of the same buffered-saline before being resuspended in 30ml of digestion medium (buffered-saline, containing 2% (w/v) fatty acid-free bovine serum albumin, 5% (w/v) dialysed [against distilled water] Ficoll and 0.1% (w/v) collagenase). This suspension was placed in a 250ml plastic conical flask and incubated at 37°C in a vigorously shaking (180 strokes/min) waterbath for 60 min. The flask was gassed continuously with O_2/CO_2 (95%/5%). On completion of this incubation, the digested tissue was strained through a nylon mesh (approximately 0.15mm mesh size) which retained undigested tissue. The suspension of acini that passed through the nylon mesh was

then washed three times (in the buffered saline described above, containing 2% (w/v) dialysed Ficoll). Harvesting of acini, following each wash, was achieved by light centrifugation (400 x g for 15 sec) which also accomplished separation of the acini from adipocytes and cell-fragments. Finally, the acini were resuspended in 6.5ml of incubation medium (the buffered-saline described above containing 2% (w/v) Ficoll and 4% (w/v) fatty acid-free bovine serum albumin). Mammary acini in suspension have a tendancy to clump together, forming large agglomerates that are difficult to handle and display reduced response to hormonal challenge (as judged by lipogenic response to insulin). This clumping is ameliorated by addition of Ficoll, hence the inclusion of 2% (w/v) Ficoll in all media used for suspension of acini. The cellular integrity of acini prepared by the above procedure was verified by measurement of lactate dehydrogenase leakage as described by Christie et al. [93a] for a similar mammary cell preparation.

2.3. Incubation of Adipocytes and Acini

2.3.1. Phosphodiesterase Work

Aliquots (0.5ml) of adipocytes (suspended in the Krebs-Henseleit [440] bicarbonate-buffered saline described above, containing 5mM glucose, 4% (w/v) dialyzed, essentially fatty acid-free bovine serum albumin) or acini (suspended in a medium of similar composition to the adipocyte suspension medium but with the addition of 2% (w/v) dialyzed Ficoll) were dispensed into 25ml polycarbonate conical flasks containing 2ml appropriate incubation medium (identical composition to cell suspension medium) with or without hormones or other effectors, as indicated in the text. The flasks were gassed with an O_2/CO_2 mixture

(95%/5%), stoppered and secured in a shaking waterbath at 37°C. Incubation continued for 60min (unless otherwise stated) from time of addition of cells. The incubating flasks contained either approximately 10^5 cells/ml (adipocytes) or approximately 35-50mg (wet weight) of acini/ml. Incubation was terminated by homogenisation of the entire contents of each flask in individual glass-glass Potter-Elvehjem-type homogenisers for 45s at 0°C, either by hand (adipocytes) or with a motor-driven pestle (1000rpm). The resulting homogenates were either used directly (adipocytes or acini) for assays or (acini only) subjected to centrifugation at 178,000xg for 2 min at 4°C in a Beckman Airfuge to sediment a crude particulate fraction which was then resuspended to the original homogenate volume in 40mM Tris HCl (pH 8.0) containing 4.3mM 2-mercaptoethanol. In some experiments, the crude acinus particulate fraction suspension was incubated in the presence or absence of an inhibitor cocktail described in section 3.1.2.(ii), before assay for PDE activity. In a given experiment, each experimental condition (control, plus insulin etc.) was represented by duplicate flasks and each experiment was performed using a different preparation of adipocytes or acini as appropriate.

2.3.2. Protein Phosphorylation in Intact Acini

Acini were incubated, as described above, in phosphate deficient Krebs-Henseleit bicarbonate-buffered saline (composition as above except that the phosphate concentration was 0.24mM i.e. 1/5 that recommended by Krebs and Henseleit) containing 5mM glucose, 4% (w/v) dialyzed essentially fatty acid-free bovine serum albumin, 2% (w/v) Ficoll and carrier-free [^{32}P] P_i (40-65μCi/ml) for 1hr in order to establish steady-state labelling of phosphoproteins, as described, in

adipocytes, by Brownsey et al. [194]. At this point, hormones or other effectors (as indicated in the text) were added and incubation continued for a further 15min. On completion of the incubation period, the acini were harvested rapidly by centrifugation at 800xg for 15s. The supernatant was discarded and the acini pellets homogenised immediately in ice cold buffer (pH 7.4; 25ml per g wet weight of acini) containing 20mM Tris-HCl, 250mM sucrose, 100mM KF, 2mM EDTA, 2mM EGTA, 7.5mM glutathione (reduced), 2mM PMSF, 50μg/ml each of leupeptin, pepstatin and antipain. Homogenisation was for 30s at 20,000rpm using the PTA 10S probe of a Polytron. The homogenate was either treated immediately with an equal volume of ice cold 20% (w/v) TCA or used in the preparation of a high speed supernatant fraction, as described below. After not less than 10min on ice, the TCA precipitated protein was collected by centrifugation at 7500xg for 5min. The supernatant was discarded and the pellet was washed with ice-cold 90% (v/v) acetone in H_2O. The precipitated protein pellet was reisolated by centrifugation as before, drained and allowed to dry before being disaggregated in boiling Tris-H_3PO_4 (42.6mM) buffer, pH 6.7, containing 1% (w/v) SDS, 15% (v/v) glycerol and 2% (v/v) 2-mercaptoethanol. Samples of this preparation were loaded directly onto SDS polyacrylamide gels and subjected to electrophoresis.

Preparation of High Speed Supernatant from ^{32}P-labelled Acini

The homogenate of ^{32}P-labelled acini, prepared as described above, was centrifuged at 8000xg (av) for 10min at 4°C. After discarding the pellet, the supernatant was filtered through a plug of glass wool and centrifuged further at 105,000xg (av) for 45min at 4°C. The resulting high speed supernatant fraction was diluted with the

disaggregation buffer described above and subjected to SDS polyacrylamide gel electrophoresis.

2.4. Incubation of Crude Acinus Membrane Fractions

Acini, prepared as described above, were suspended in ice-cold buffer (3mM imidazole, pH 7.4) containing 250mM sucrose and homogenised under the conditions described above. The homogenate was subjected to centrifugation at 75xg (750rpm in an MSE bench centrifuge) for 5min at 4°C and the supernatant frozen (liquid N_2) in 1ml aliquots for storage at -80°C.

On the day of experiment, one aliquot was thawed and centrifuged at 22,500xg for 10min at 4°C. The supernatant was aspirated and discarded and, unless otherwise indicated, the pellet was resuspended immediately in 1.6ml ice cold membrane incubation buffer containing 20mM Tris-HCl, 5mM $MgCl_2$ and 100μM $CaCl_2$, pH 7.4 to give a membrane protein concentration of approximately 1.2mg/ml. Membrane incubations were initiated by addition of 200μl of this membrane suspension to a further 300μl of incubation buffer with or without appropriate additions as indicated in the text. Following vortex mixing, the incubation tubes were incubated for 5min at 30°C (unless stated otherwise). At the end of the incubation period, the tubes were cooled to 0°C and subjected to centrifugation, once again for 10min at 22500xg at 4°C. The supernatant was aspirated and, where appropriate, retained (see below) while the pellets were washed x 3 with 1mM $KHCO_3$ before being resuspended in 100μl PDE assay buffer. A 1:8 dilution of this membrane suspension was used directly for assay of PDE activity.

2.4.1. Desalting of Membrane Incubation Supernatants

Incubation supernatants (approximately 0.5ml) were diluted with 1ml incubation buffer containing dialyzed, essentially fatty acid free bovine serum albumin to a final concentration of 1mg/ml and added to Centricon 10 microconcentrator devices. These were centrifuged at 5000xg for 2hr at 4°C, achieving approximately 50-fold concentration. Fresh incubation buffer (2ml) was added to the concentrate (dead-stop volume) and centrifugation repeated. The final concentrate (approximately 30μl) was collected in weighed cups by centrifugation of the microconcentrators in an inverted position for 2min at 1000xg at 4°C and adjusted (by weight) to a volume of 500μl. These samples were assayed directly for PDE activity.

2.5. Sub-Cellular Fractionation of Mammary Acini

Acini, prepared as described above, were washed twice by repeated centrifugal harvesting and resuspension in homogenisation buffer (10mM Tris-HCl, 250mM sucrose and 0.1M KCl, pH 7.4) and finally homogenised in 2.5 x the compact acini volume of the same buffer for 1min at 10400rpm using the PTA 20N probe of a Polytron. The homogenate was centrifuged at 75xg for 5min at 4°C and the resulting supernatant subjected to further centrifugation at 1000xg for 10min at 4°C. The 1000xg supernatant was decanted and the pellet (P_1) washed once with homogenisation buffer before being held on ice pending further processing. The 1000xg supernatant was centrifuged at 3000xg for 10min at 4°C, the supernatant decanted and the pellet (P_2) washed once in homogenisation buffer before storage on ice. Finally, the 3000xg supernatant was subjected to centrifugation at 105000xg for 45min at 4°C, the resulting supernatant (Sn) was decanted and both this and the

pellet (P_3) (rinsed with homogenisation buffer) were stored on ice. All the membrane fractions were then resuspended in homogenisation buffer and, together with Sn, were adjusted to a protein concentration of 10mg/ml as determined by the dye binding method in [55]. Membrane fractions P_1, P_2 and P_3 are referred to, in the text, as the 'nuclear', 'mitochondrial' and 'microsomal' fractions, respectively, while Sn is termed the 'cytosolic' fraction.

2.6. Incubation of Subcellular Fractions to Determine Protein Phosphorylation

Samples of subcellular fractions, prepared as described above, were incubated in a buffer containing 25mM β-glycerophosphate 10mM $MgCl_2$, 1mM DTT, 2mM EGTA (unless indicated otherwise) and 60.6μM [γ-^{32}P] ATP (final radioactive concentration 500μCi/ml), pH 7.4, at a final protein concentration of 1mg/ml, in the presence or absence of additions as indicated in the text. Incubations, in a final volume of 55μl, were initiated by addition of the ATP to tubes containing the remaining incubation constituents and terminated after 30s (unless otherwise stated) at 37°C by addition of 100μl ice cold TCA (20% w/v). The precipitated protein was then isolated, washed and disaggregated, as described earlier, in preparation for SDS polyacrylamide gel electrophoresis.

Control incubations in which the TCA was added prior to addition of mammary cell fractions showed no evidence of protein phosphorylation, regardless of whether other additions such as Ca^{2+} (10μM), calmodulin (5μg/mg protein) or cAMP (1μM) (see later) were present or absent. In autoradiograms derived from such incubations, the regions corresponding to electrophoretic separations of proteins

contained in the mammary cell fractions were completely clear, indicating that, unlike neighbouring strongly exposed tracks (derived from incubations performed in the normal way i.e. TCA added to terminate the incubation), protein-associated radioactivity was not significantly greater than background. Consequently, no correction was necessary of the figures for ^{32}P-incorporation determined, as described in section 2.7., by liquid scintillation counting of excised sections of dried electrophoretic gels.

2.7. SDS Gel Electrophoresis, Autoradiography and Liquid Scintillation Counting of ^{32}P-Labelled Gel Slices

Proteins contained in samples taken from disaggregated incubation contents (from either intact cell or subcellular fraction incubations) were resolved by one dimensional SDS polyacrylamide gel electrophoresis as described in [450]. Gels were stained with Coomassie brilliant blue, destained, and dried onto Whatman No. 1 filter paper in preparation for autoradiography. Typically, autoradiographs were exposed for 10-14hr in intensifying screens at -80°C. Major phosphoprotein bands in dried gels were identified by reference to the autoradiograph and the ^{32}P content of slices of gel containing such bands was determined by liquid scintillation counting in toluene-based scintillation fluid. In various control experiments, the efficiency of ^{32}P-counting was the same whether protein-bound ^{32}P was deposited on filters, dried and counted as above or dispersed uniformly throughout the bulk phase using either dioxane-based aqueous-compatible scintillator cocktail (for example, Packard Scintillator 299) or 1ml of tissue solubiliser (for example, commercial quaternary amine-based solubilisers such as 'NCS' or 'PCS') plus 10ml

of toluene scintillator. Furthermore, no difference was noted in the count registered for an excised segment of dried electrophoretic gel whether counted as described above, simply by immersion in toluene scintillator or counted after hydration and dissolution in 0.2ml 30% (v/v) (approximately "100 volumes") H_2O_2 at 50°C overnight, followed by incorporation into 10ml toluene scintillator by the addition of 1ml NCS.

2.8. Enzyme Assays

2.8.1. Cyclic AMP Phosphodiesterase (EC 3.1.4.17)

Suitable dilutions of unfractionated adipocyte or acinus homogenates or of acinus particulate fractions were assayed in duplicate for cAMP-PDE activity at 0.2μM (adipocytes) or 1μM (acini) cAMP using the two-step procedure in [771]. In addition to sample and tritiated substrate (approximately 200,000cpm/assay), the assay mix (final volume 0.4ml) contained 10mM Tris-HCl, 1.1mM 2-mercaptoethanol and 12mM $MgCl_2$, pH 8.0. The primary reaction was initiated by addition of sample and the mix was incubated for 10min at 30°C. Termination of this reaction was achieved by immersing the assay tubes in liquid N_2 for 5s and immediately thereafter, transferring them to a boiling water bath for 45s. After cooling, on ice, the second incubation commenced on addition of 100μl of a 1mg/ml solution of snake venom rich in 5'nucleotidase activity, and continued for 10min at 30°C. On completion of this incubation period, the assay tubes were returned to ice and labelled product was subsequently separated from substrate on small columns (7mm diam. x 10mm) of Dowex 1 x 8 - 400 anion exchange resin, allowing quantitation of product by liquid scintillation counting. One unit of enzyme activity is that which generates 1μmol product/min under the defined conditions of assay.

2.8.2. Marker Enzymes

5′ Nucleotidase (EC 3.1.3.5.) was assayed at 37°C as in [99]. Lactate dehydrogenase (EC 1.1.1.27) activity was measured using a standard spectrophotometric assay [42]. Cytochrome-C oxidase (EC 1.9.3.1) was assayed as described by Brown et al. [59].

2.9. Materials

2.9.1. Animals

Rats were supplied by A. Tuck and Son, Rayleigh, Essex and were fed pasteurised breeding diet 41B produced by Oxoid, Basingstoke, Hampshire, England.

2.9.2. Radiochemicals

[8-^{3}H] Adenosine 3′,5′ cyclic monophosphate, [γ-^{32}P] Adenosine 5′ Triphosphate and [^{32}P] Pi were obtained from Amersham International PLC, Aylesbury, Buckinghamshire, England.

2.9.3. Chemicals and Equipment

The following were all purchased from Sigma Chemical Co. Ltd., Poole, Dorset, England: pentobarbital, bovine serum albumin (fraction V, essentially fatty acid-free), Ficoll type 400, tris (hydroxymethyl) aminomethane ('Trizma' base), β-glycerophosphate, imidazole, cycloheximide, 3-isobutyl-1-methyl xanthine, isoproterenol, protein kinase inhibitor type II (Walsh inhibitor), Soya bean trypsin inhibitor (type 1-S), ε-amino-n-caproic acid, benzamidine hydrochloride, phenyl methyl sulphonyl fluoride, leupeptin, pepstatin, glutathione (reduced form), adenosine, adenosine 5′-monophosphate, adenosine 3′5′-cyclic

monophosphate, adenosine 5'-diphosphate, adenosine 5'-triphosphate, guanosine 5'-triphosphate, N^6, 2'-O-dibutyryl adenosine 3'5'-cyclic monophosphate, protamine sulphate, dithiothreitol, riboflavin, 4β-phorbol, 12β-myristate, 13α-acetate, Dowex 1 X 8-400 (chloride form), cytochrome-C (type III), glycogen phosphorylase (EC 2.4.1.1), β-galactosidase (EC 3.2.1.23), catalase (EC 1.11.1.6), ovalbumin, Triton X-100, Ophiophagus hannah (King Cobra) venom and coomassie brilliant blue R. Collagenase was obtained from Worthington (CLS III, Lot. no. 415130) or Boehringer (from Clostridium histolyticum), Lewes, East Sussex, England.

Adenosine 5'-O-(3-thiotriphosphate) (ATPγS), nicotinamide adenine dinucleotide (reduced form) and pyruvate were also obtained from Boehringer. Insulin (porcine, 23.6U/mg) was a generous gift from the Boots Co. Ltd., Nottingham, Nottinghamshire, England. The Bradford dye reagent for protein determination was supplied by Bio-Rad, Watford, Hertfordshire, England. Calmodulin was a kind gift from Professor P. Cohen, Department of Biochemistry, Dundee, Scotland. Kodak X-Omatic cassette intensifying screens, X-Omat RP X-ray film and processing chemicals were from Kodak, Hemel Hempstead, Hertfordshire, England. Scintillator 299 was from United Technologies, Packard, Caversham, Berkshire, England and Forskolin was supplied by Calbiochem-Behring Diagnostics, La Jolla, California, USA. All other chemicals were of 'AnalaR' purity from BDH Ltd., Poole, Dorset, England. Centricon 10 microconcentrators were obtained from Amicon Ltd., Stonehouse, Gloucestershire, England.

The suitability of different batches of commercial collagenase for use in the preparation of mammary acini was assessed as follows: A sample of each batch available was used to prepare acini as described

in Section 2.2.2. The quality of the acini produced was then assessed by determination of the period of linearity of lipogenic rates and the degree of stimulation of these rates by insulin (as described by Robson et al. [656]). Those batches producing acini capable of demonstrating adequate linearity of lipogenic rates (i.e. linear for at least 1hr) and greatest response to insulin were chosen for future experiments. If an insulin response of consistently less than 140% of control was attained with acini prepared using a particular batch of collagenase, this batch was not used further.

3. RESULTS

3.1. High Affinity cAMP-Phosphodiesterase

3.1.1. Adipocytes

Adipocytes prepared and incubated as described in sections 2.2. and 2.3. were found to contain high affinity cAMP-PDE activity that could be stimulated by exposure of the cells to insulin.

Cyclic AMP-PDE activity measured at 0.2μM cAMP in whole cell homogenates derived from control incubations was 64.8 ± 14.0pmol/min/10^6 cells (mean ± SE of 11 observations) and the mean stimulation observed in response to insulin (1mU/ml) was 146 ± 9.3% (also mean ± SE of 11 observations) with respect to control incubations. Insulin stimulated activity was significantly greater than controls ($P < 0.0005$) as determined by Student's t-test for paired observations.

Thompson and Appleman [770] have calculated a K_m of approximately 40μM for low affinity cAMP-PDE activity of adipose tissue but a figure as low as 15μM has also been reported [506]. However, at the concentration of cAMP used for assay of PDE activity (0.2μM), even assuming a K_m of 15μM, the low affinity enzyme should be operating at only a fraction of its maximum rate whereas, with a K_m of 0.2-0.3μM [506,770,878] the activity of high affinity cAMP-PDE will be approaching half-maximal. Unfortunately, despite expressing only a fraction of its potential activity, even at 0.1μM cAMP, the low affinity enzyme shows significant activity [770] and the possibility that the calculated increase in high affinity activity in response to insulin may, therefore, have been observed against a substantial background of low affinity activity cannot be ignored.

3.1.2. Mammary Acini

3.1.2.(i) Insulin Response

Two kinetically distinguishable cAMP-PDE activities with K_m values of 21.6μM and 1.3μM have been identified recently in rat mammary tissue [549]. Heterogeneity of mammary PDE activity had previously been described by Pizarro et al. [615] and Sapag-Hagar and Greenbaum [681], the latter reporting K_m values of 6μM and 70μM for the two cAMP-PDE activities identified. The chromatographic separation of these enzyme activities performed in the most recent study [549] demonstrates that at a substrate concentration of 1μM, high affinity cAMP-PDE is the only enzyme expressing significant activity. Since PDE activity measured at a cAMP concentration of 1μM therefore reflects the activity of high affinity cAMP-PDE essentially free of interfering activities, it was this concentration rather than the 0.2μM employed for assay of adipose tissue high affinity cAMP-PDE that was chosen for the assay of the mammary enzyme. In view of the higher K_m value ascribed to mammary high affinity cAMP-PDE as compared with its counterpart in adipose tissue, the higher substrate concentration is obviously preferable in that it allows expression of greater catalytic activity. Phosphodiesterase activity measured at this concentration of cAMP was higher in a crude particulate fraction prepared as described earlier (secion 2.3.) from homogenates of acini incubated in the presence of insulin than in the same fraction of control acini homogenates derived from incubations in the absence of insulin (Table 2) but, in contrast to the behaviour of adipocyte high affinity cAMP-PDE, no such insulin-stimulated increase was observed

in unfractionated cellular homogenates. The insulin concentration used (1.68mU/ml) is that which has been shown to elicit a maximal lipogenic response in mammary acini incubated under identical conditions to those employed in this study [656].

Since incubations were of 60min duration, it was conceivable that the observed activation in response to insulin was merely an expression of generalised stimulation of protein synthesis known to be induced by insulin in several cell-types including adipocytes [670]. However, the failure of cycloheximide, an inhibitor of protein synthesis, to suppress the insulin-induced stimulation of PDE activity (Table 2) and the observation that this stimulation was evident even after an incubation period of just 15min (Table 3), demonstrate that such a mechanism was not responsible for the effects on PDE activity observed following the longer (60min) incubations. Interestingly, not only did cycloheximide fail to prevent insulin stimulation of PDE activity but its inclusion in incubations also led to the expression of an insulin-induced activation which, in contrast to incubation in its absence, was detectable even in unfractionated homogenate (Table 2). These observations remained essentially unchanged whether activities were expressed on a 'per incubation', 'per mg protein' or 'per unit of 5'nucleotidase activity' basis. Determination of a meaningful value for protein concentration in unfractionated homogenates was confounded by the high concentration of BSA present in these samples as a consequence of homogensation in incubation medium containing 4% (w/v) (40mg/ml) BSA. However, satisfactory protein determinations were possible using washed particulate fractions and expression of particulate cAMP-PDE activity was therefore possible on a 'per mg protein' basis.

Table 2 **Effect of insulin and cycloheximide on high affinity cyclic AMP phosphodiesterase activity in rat mammary acini**

Additions	Number of Experiments	High affinity cyclic AMP phosphodiesterase activity				
		Unfractionated homogenate		Particulate fraction		
		μU/ml homogenate	μU/mU 5′ nucleotidase	μU/ml homogenate	mU/mg protein	μU/mU 5′ nucleotidase
None	8	744.3±96.7	2.71±0.52	115.3±14.3	0.24±0.04	1.00±0.12
Insulin	8	750.4±96.4	2.72±0.54	146.0±15.4****	0.30±0.03**	1.37±0.16****
Cycloheximide	4	661.7±44.7	2.72±0.24	130.9±17.4	0.20±0.03	0.77±0.13
Cycloheximide plus insulin	4	825.1±68.8†††	3.43±0.32††††	175.0±19.8†	0.28±0.02†	1.00±0.10††

Acini were incubated as described in Materials and Methods (section 2.3.1), with insulin (1.68mU/ml) and/or cycloheximide (50μg/ml) as indicated. Values given are means ± SE. Statistically significant differences were determined using Student's t-test for paired observations. Asterisks denote values greater than the control (no addition) sample at $P<0.005$ (****) or $P<0.025$ (**). Daggers denote values greater than the 'plus cycloheximide' sample at $P<0.005$ (††††), $P<0.01$ (†††), $P<0.025$ (††) or $P<0.05$ (†). 5′-nucleotidase values in all homogenates, averaged 242.9 mU/ml.

Table 3 Effect of incubation time on insulin-stimulated activation of high affinity cAMP-PDE

Additions	Incubation time (min)	Number of experiments	High affinity cAMP-PDE activity				
			Unfractionated homogenate		Particulate fraction		
			μU/ml homogenate	μU/mU5′ nucleotidase	μU/ml homogenate	mU/mg protein	μU/mU 5′ nucleotidase
None	15	4	593.9±155	1.97±0.49	70.0±3.8	0.323±0.08	1.26±0.13
Insulin	15	4	648.1±198	2.01±0.50	102.1±12.9*	0.452±0.12	1.82±0.28*
None	60	4	515.3± 58	1.42±0.15	82.2± 6.1	0.265±0.04	1.16±0.08
Insulin	60	4	525.8± 69	1.36±0.12	119.9±12.5*	0.364±0.04*	1.74±0.19**
Insulin activation (% control)	15	4	106.6±5.2	101.4±2.2	144.8±19.1	140.4±22.5	142.9±6.5
	60	4	104.9±5.9	96.8±5.2	151.1±17.7	141.8±18.1	150.5±11.4

Acini were incubated as described in Materials and Methods (section 2.3.1) for 15 or 60 min, in the presence or absence of insulin (1.68 mU/ml) as indicated. Values given are means ± SE. Statistically significant differences were determined using Student's t-test for paired observations. Asterisks denote values greater than control (no additions) at $P<0.025$ (*) or $P<0.01$ (**).

3.1.2.(ii) Storage Effects

During storage at 0-4°C, particulate fraction high affinity cAMP-PDE underwent spontaneous activation. After 1½ hours the activity expressed by particulate fractions derived from control incubations was approximately 1½-fold greater than that measured in the same samples at time 0 whether results were calculated 'per ml homogenate' or 'per unit 5'nucleotidase' (Table 4). During this time, insulin activation became attenuated both in terms of absolute values and, consequently also when expressed as a percentage of control activity (Table 4). Proteolytic activation of membrane bound high affinity cAMP-PDE is well documented in adipose tissue [502, 742, 841] but the activation observed here was only partially blocked by homogensation and storage in an inhibitor cocktail containing, in final concentrations, 2mM PMSF, 0.1mg/ml leupeptin, pepstatin, antipain and trypsin inhibitor, 20mM ε amino caproate, 1mM benzamidine HCl, 2mM EDTA, 5mM EGTA and 50mM KF (after 1½ hr at 0-4°C, control high affinity cAMP-PDE activity was 132% its value at time 0 [mean of 2 experiments]). However, the percentage activation observed in response to insulin (145-150% control values, mean of 2 experiments) was preserved entirely under such conditions.

3.1.2.(iii) Effect of Physiological State on Insulin Activation

The results obtained from a single experiment comparing the insulin-induced activation displayed by high affinity cAMP-PDE of acini prepared from mammary tissue of a normal mid- lactating rat with that observed in mammary acini derived from 24hr starved or diabetic rats in mid lactation (Table 5) indicated that changes in

Table 4 Effect of storage at 0-4°C on control and insulin-stimulated high affinity cAMP-PDE activity

Incubation Additions	Storage time (hr)	Particulate high affinity cAMP-PDE activity	
		μU/min/ml homogenate	μU/min/mU 5'-nucl.
None	0	146.4±30.0	0.254±0.020
Insulin	0	198.1±43.5	0.358±0.056
None	1.5	220.5±47.1	0.383±0.032
Insulin	1.5	255.6±56.0	0.462±0.075
Insulin Activation (insulin -control)	0	51.7±15.3	0.103±0.040
	1.5	34.9±20.1	0.079±0.060
Insulin Activation (% control)	0	134.8±7.3	139.7±16.4
	1.5	116.0±15.0	120.5±16.4

Acini were incubated as described in Materials and Methods (section 2.3.1.) for 15 min, in the presence or absence of insulin (1.68mU/ml). Particulate fractions, prepared as described in the text, were assayed for PDE activity immediately and then again after storage on ice at 0-4°C as indicated. Values given are means ± SE of three separate experiments.

physiological state may have a marked effect on the magnitude of this particular response to insulin. However, tentative conclusions of this kind must be treated with considerable caution since the data on which they are based are derived from only one experiment and so may be subject to quite large errors.

In the experiment, activation of high affinity cAMP-PDE was evident even in the unfractionated homogenate, a phenomenon normally observed only in acini incubated in the presence of cycloheximide (Table 2). The differences between these results and previous ones, most likely to account for this discrepancy, are that while the high affinity cAMP-PDE activity expressed by control particulate fraction was broadly consistent with previous values, the activation observed in response to insulin was much greater and total cellular activity, represented by the value for unfractionated homogenate, was considerably lower than observed before, so that increases in the activity of insulin sensitive PDE were seen against a much reduced background activity. Since the procedures employed for preparation of samples and measurement of PDE activity were identical to those used previously, these inconsistencies are not easily explained but may arise, at least in part, from differences in the profile of enzyme activities exhibited by the collagenase preparations used for tissue digestion in the two separate experimental series. The properties of commercial collagenase, particularly, with respect to efficiency of digestion of mammary tissue are known to be highly variable and considerable differences are encountered both between the same product obtained from different suppliers and between individual batches of the same manufacturer's product. As described in section 2.9.3., the effect of such differences was minimised by

screening each batch. However, the screening process involved monitoring the linearity and insulin sensitivity/responsiveness of only one metabolic pathway (lipogenesis). Hence, it is possible that, despite appearing comparable on this basis, the different batches of collagenase did in fact differ in their effect on the insulin dependence of the acinus cAMP-PDE system.

Both diabetes and 24hr starvation had marked effects on control high affinity cAMP-PDE activity. Twenty-four hr starvation resulted in a 40% reduction in unfractionated homogenate activity and 31% reduction in activity associated with the particulate fraction, while the diabetic animal displayed 45% and 54% reductions respectively. The insulin-induced activation of high affinity cAMP-PDE assayed in both unfractionated homogenate and particulate fraction was greater in starved or diabetic rats than in control animals when expressed as a percentage but only the unfractionated homogenate derived from diabetic rat mammary acini showed an increase in the absolute amount of activation (Table 5).

3.1.3. Membrane Incubations

Addition of insulin (1.68mU/ml or 3.36mU/ml) to membrane incubations performed as described in Materials and Methods had no effect on the high affinity cAMP-PDE activity expressed by membranes incubated with ATP (3mM) or ATP (3mM) and cAMP (100μM) in the presence or absence of NaCl (9mM) or KF (60mM) whether conducted at pH 7.4 or pH 4.6 (mean activity of insulin-treated membranes expressed as a percentage of appropriate controls was 102 ± 3%).

During the course of this series of experiments, it was noticed that KF, a potent inhibitor of protein phosphatase activity,

Table 5 Effect of physiological status on insulin stimulation of high affinity cAMP-PDE activity in rat mammary acini

Physiological State	Incubation Additions	High affinity cAMP-PDE activity (μU/ml homogenate)		Insulin stimulation (% control)	
		Unfractionated homogenate (ufh)	Particulate fraction (pf)	ufh.	pf.
Normal	None	218.6	74.5	134	166
	Insulin	293.8	123.6		
24hr starved	None	130.1	51.3	156	174
	Insulin	203.5	89.5		
Diabetic	None	119.5	34.2	178	203
	Insulin	212.8	69.2		

Acini, prepared from untreated, 24hr starved or diabetic (streptozotocin induced) rats, were incubated (as described in section 2.3.1) for 60 min, in the presence or absence of insulin (1.68 mU/ml). Values given are means of duplicate incubations from single experiments.

Table 6 Effect of KF, ATP and EDTA on high affinity cAMP-PDE activity in a crude membrane fraction from rat mammary acini

Incubation Additions	Number of Experiments	High affinity cAMP PDE activity (μU/mg protein)
None	14	23.9±1.1
KF (100mM)	14	45.6±2.2*
KF (100mM) + ATP (5mM)	5	23.4±3.7
KF (100mM) + EDTA (5mM)	3	31.0±5.5
ATP (3mM)	2	20.3
EDTA (5mM)	1	32.7

Samples of mammary acinus particulate fraction were prepared, incubated in the presence or absence of additions (final incubation concentrations as indicated; all incubations also contained 5mM Mg^{2+} and 100μM Ca^{2+}) and assayed for high affinity cAMP-PDE activity as described in section 2.4. Values given represent means ± SE (where appropriate). Statistically significant differences were determined using Student's t-test for paired observations. An asterisk denotes value greater than control (no additions) at $P<0.0005$.

was capable of enhancing markedly the high affinity cAMP-PDE activity expressed by membranes isolated from control incubations but not those derived from incubations performed in the presence of ATP (Table 6). In this and the following series of experiments, the high affinity cAMP-PDE activities measured in control incubations were several-fold lower than those recorded in particulate fractions isolated from acinus incubations (Tables 2 and 3). This discrepancy is likely to have arisen as a consequence of the difference in procedures employed for preparation of the two particulate fractions, the major contributory factors being differences in homogenisation and centrifugation conditions (see sections 2.3.1. and 2.4.). In contrast, another significant difference in methodologies, storage of homogenate in liquid N_2 (as described in Section 2.4.), has no discernable effect on high affinity cAMP-PDE activity observed in membrane fraction incubations and can probably therefore be eliminated from the list of possible contributory factor. That the KF-induced increase was also dependent on divalent cation was demonstrated by the discovery that addition of EDTA to incubations abolished the ability of KF to elevate PDE activity measured in the membrane fraction. Although both ATP and EDTA were equally effective inhibitors of the KF-stimulated increase in particulate high affinity cAMP-PDE activity if added prior to KF, when added to incubations already treated with KF, only EDTA was able to return activity expressed by the membrane fraction to approximately control levels (Table 7). This suggests that the mechanisms of action of ATP and EDTA are distinct, an inferrence that is supported by the observation that marked reduction in particulate high affinity cAMP-PDE activity occurs at ATP concentrations below 1mM (Figure 22). Since the

Table 7 Effect of delayed addition of ATP or EDTA on KF-induced enhancement of high affinity cAMP-PDE activity in a crude membrane fraction from rat mammary acini

Incubation	Additions		High affinity cAMP-PDE activity (μU/mg protein)
Time 0	5min	10 min	
None	None	None	23.04
None	100mM KF	None	37.16
10mM EDTA	100mM KF	None	23.31
None	100mM KF	10mM EDTA	26.96
5mM ATP	100mM KF	None	22.09
None	100mM KF	5mM ATP	33.83

Samples of particulate fraction were incubated, as described in Materials and Methods (section 2.4) for a total of 15 min and were treated, during this time, with KF and/or ATP or EDTA in the sequences indicated. Values given are means of duplicate incubations from a single experiment only.

concentration of Mg^{2+} added exogenously was 5mM in all incubations, chelation of Mg^{2+} by ATP, at a maximum concentration of only 1mM, is unlikely to have depressed free Mg^{2+} concentrations sufficiently to have had a significant effect on particulate cAMP-PDE activity.

The effect observed with KF was not reproduced in incubations exposed to an equal concentration of KCl but, under the same conditions, NaF was capable of mimicking at least partially the action of KF (Table 8). The concentration dependence of particulate PDE activity enhancement by KF was of a markedly biphasic nature with a maximally effective KF concentration of between 30 and 100mM (Figure 21), however, the precipitous decline in activity observed at concentrations above 300mM is probably partially artifactual, reflecting inhibition of the PDE assay by KF not removed during the membrane wash procedure. Potassium fluoride in the PDE assay at a concentration of 5mM in the first incubation of the two-step procedure (see section 2.8.1.) resulted in a 28% decrease in the measured activity of high affinity cAMP-PDE in control membranes, rising to 35% at 10mM and 76% at 100mM (all figures represent means of 2 experiments). At KF concentrations below 5mM, interference with the PDE assay was undetectable and, therefore, at most membrane incubation concentrations, including the standard 100mM, the concentration of KF to which PDE assays were themselves exposed as a result of incomplete removal during the wash procedure would probably have been insignificant in terms of inhibition of the assay but at higher concentrations, carry-over of KF might well have been sufficient to interfere with the assay and give rise to an exaggerated fall in the measured PDE activity.

Table 8 Effect of KF, NaF and KCl on high affinity cAMP-PDE activity associated with a crude membrane fraction from rat mammary acini

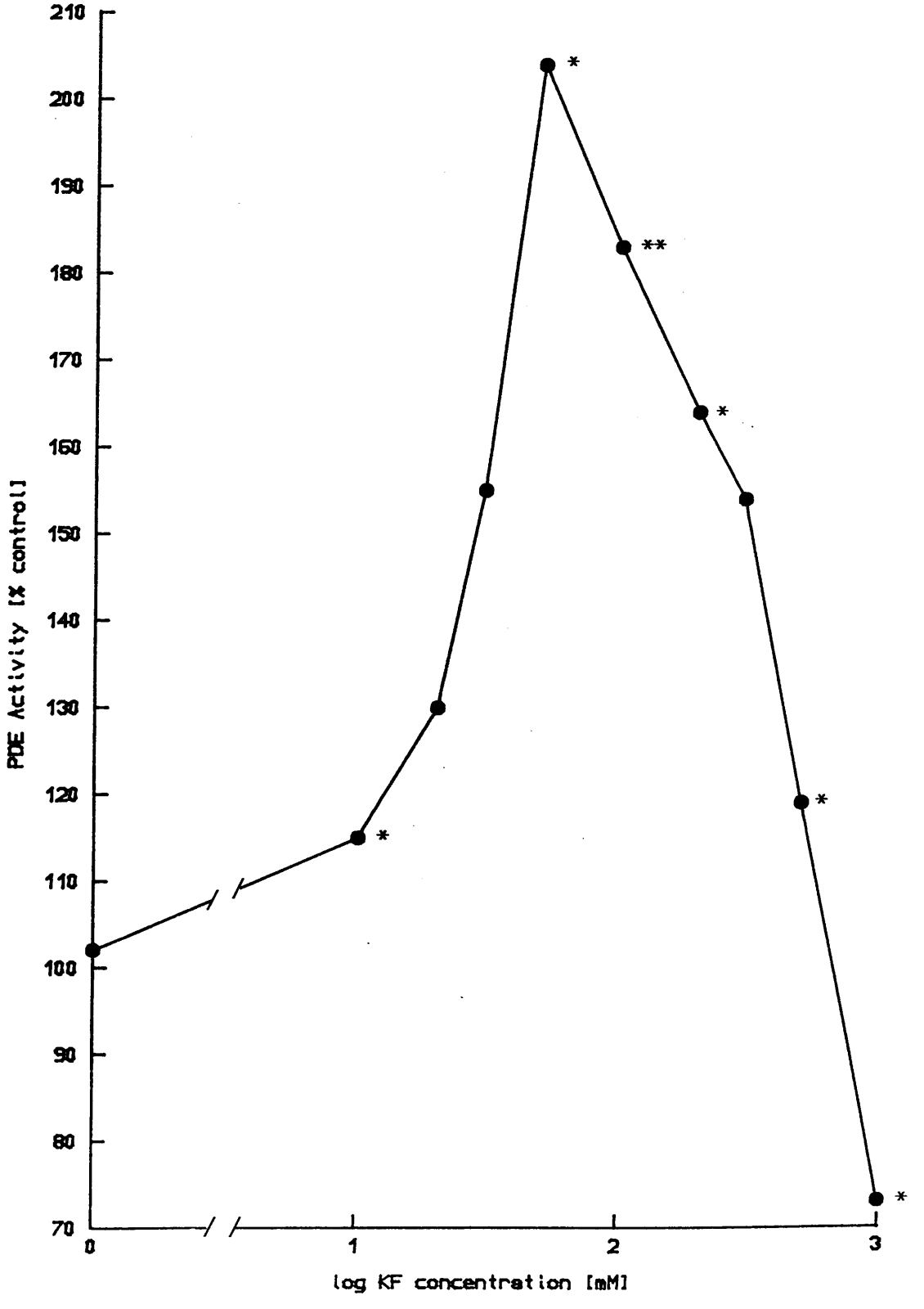
Incubation Additions	Number of Experiments	Particulate high affinity cAMP-PDE activity (% control value)
None	8	100
KF	6	148.6±6.7 ††
NaF	6	118.4±5.9*†
KCl	2	95.3±18.2

Samples of particulate fraction were incubated, as described in Materials and Methods (section 2.4) in the presence or absence of KF, NaF or KCl, all at a final concentration of 100mM. Values given are means (±SE) of the number of experiments indicated. Statistically significant differences (calculated from PDE activities expressed in U/mg protein) were determined using Student's t-test for paired observations. An asterisk denotes a value less than the 'plus KF' sample at $P<0.005$. Daggers denote values greater than control at $P<0.01$ (†) or $P<0.005$ (††).

Figure 21

Samples of mammary acinar membranes were incubated, as described in section 2.4, in the presence of increasing concentrations of KF. Points represent figures from one experiment only or mean of two experiments (*) except the point for PDE activity at 100mM KF (**) which represents the mean of four experiments. The latter served as a reference by which all other data were standardised.

Figure 21. High Affinity cAMP-PDE Activity Associated with a Crude Mammary Acinus Membrane Fraction as a Function of KF Concentration



The increase in particulate PDE activity provoked by KF was dependent on the presence of divalent cation in the incubation medium but an accurate assessment of the relative importance of each of the three physiologically important metal ions investigated here is confounded by the high variability of data arising from incubations in the presence of BAPTA and, to a lesser extent, EDTA (Table 9). However, it appears that approximately physiological concentrations of either Ca^{2+} or Mg^{2+} but not Mn^{2+} were capable of supporting the effect of KF on these membrane preparations, as evidenced by the activity observed in incubations containing BAPTA (a potent chelator of divalent cations with particularly high affinity for Mn^{2+}), supplemented with excess Ca^{2+} or Mg^{2+} as appropriate. Qualitatively identical results were obtained in an experiment where EDTA was substituted for BAPTA (1,2-bis (2-aminophenoxy)ethane-NNN'N'-tetra-acetic acid). In this same experiment, while, once again, Mn^{2+} (1mM) on its own showed no evidence of an ability to facilitate the KF effect, its inclusion in incubations already containing Ca^{2+} and Mg^{2+} resulted in an enhanced increase in particulate PDE activity following KF treatment (increase in the presence of Mg^{2+} and Ca^{2+} alone was 156% but in the presence of Mg^{2+}, Ca^{2+} and Mn^{2+}, this rose to 216%).

Effective antagonism of the increase in particulate PDE activity normally associated with KF treatment was achieved by the inclusion of ATP, ADP, GTP or pyrophosphate (all at a final concentration of 5mM) in the incubation buffer. However, the non-hydrolysable analogue of ATP, ATPγS was not as effective in this respect, adenosine was completely ineffective and inclusion of either AMP or KH_2PO_4 in the incubations was not only ineffective but

Table 9 Effect of three physiologically relevant divalent cations on the ability of KF to enhance high affinity cAMP-PDE activity in a crude membrane fraction from rat mammary acini

Incubation additions	High affinity cAMP-PDE activity	
	μU/mg protein	% control (Ca^{2+}/Mg^{2+})
Ca^{2+}/Mg^{2+}	24.6	100
Ca^{2+}/Mg^{2+}/KF	58.0	236
EDTA/KF	34.8	142
BAPTA/Mg^{2+}*/KF	69.0	281
BAPTA*/Ca^{2+}*/KF	74.0	301
Mn^{2+}/KF	25.7	105

Samples of particulate fraction were incubated, as described in Materials and Methods (section 2.4), except that the incubation buffer contained no Ca^{2+} or Mg^{2+} unless otherwise indicated. Values given are means of 2 separate experiments. Incubation addition concentrations were: Ca^{2+}, 100μM; or * 1mM; Mg^{2+}, 5mM or *, 10mM; Mn^{2+}, 1mM; EDTA, 5mM; BAPTA, 5mM or * 0.5mM and KF, 100mM.

resulted in a significant enhancement of the increase in particulate activity observed in response to KF (Table 10). The effect of protamine sulphate, an organic polyanion, on this system was indeterminate since, although a significant reduction in the KF-induced increase was observed in its presence, protamine sulphate also appeared to enhance particulate PDE activity in the absence of KF. The concentration-dependence of antagonism was the same for ATP and GTP with maximal inhibition of the KF effect at a concentration greater than 1mM (Figure 22).

The increase in particulate high affinity cAMP-PDE activity observed following incubation of membranes with KF was accompanied by a dramatic fall in the activity contained in supernatants derived from such incubations (Figure 23). The loss of supernatant activity was not due to inhibition of the PDE assay by KF remaining in the samples after desalting (conducted as described in Materials and Methods, section 2.4.1.) since recoveries of PDE activity were identical (approximately 100%) whether or not KF (final concentration 100mM) was added to samples of control supernatant before the desalting procedure. This apparent redistribution of PDE activity from supernatant to particulate fractions following incubation with KF was completely blocked by ATP (final incubation concentration 5mM, Figure 21) and at least partially blocked by pyrophosphate (also 5mM) (data not shown), as would be expected from previous results (Table 10). The phenomenon was also characterised by a concomitant decrease in total PDE activity (Figures 23, 24 and 25) suggesting that redistribution from soluble to particulate fractions involved inhibition of the enzyme's catalytic activity.

Membrane incubations were performed as described in materials and methods (section 2.4). Values given are means of experimental results ± SE, where appropriate. Statistically significant differences (calculated from PDE activities expressed in μU/mg protein) were determined using Student's t-test for paired observations. Asterisks denote values greater than that for incubations in the presence of KF (100mM) alone at $P<0.025$ (*) or $P<0.005$ (**). Daggers denote values less than that for incubations in the presence of KF (100mM) alone at $P<0.005$ (†) or $P<0.0005$ (††).

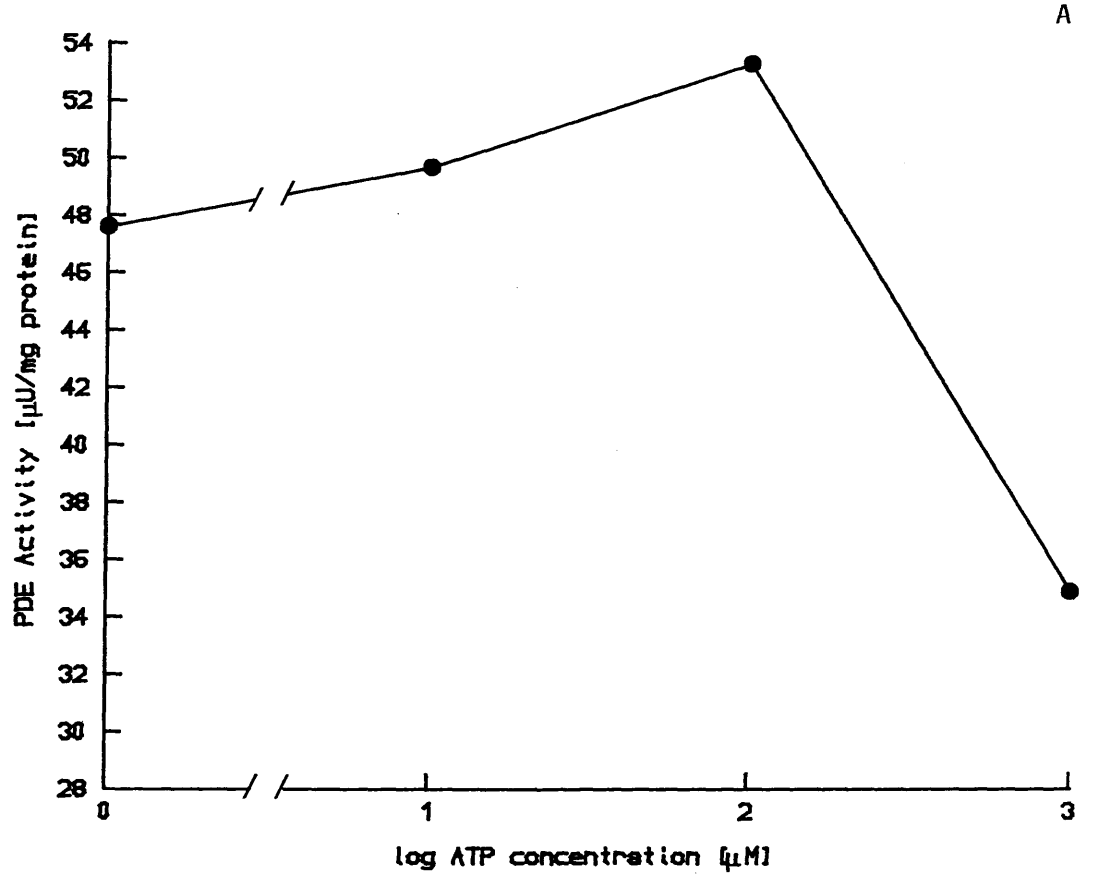
Table 10 Effect of various agents on the ability of KF to enhance PDE activity associated with a crude membrane fraction from rat mammary acini

Incubation additions	Number of experiments	High affinity cAMP-PDE activity (% control)
None	6	100
KF (100mM)	6	150±7
KF (100mM), ATP (5mM)	6	82±5††
KF (100mM), ATP S (5mM)	1	136
KF (100mM), ADP (5mM)	1	78
KF (100mM), AMP (5mM)	5	167±9**
KF (100mM), Adenosine (5mM)	4	155±11
KF (100mM), GTP (5mM)	1	86
PPi (5mM)	2	91±2
KF (100mM), PPi (5mM)	4	84±1†
Protamine sulphate (1mg/ml)	2	115±1
KF (100mM), protamine sulphate (1mg/ml)	4	136±9†
KF (100mM), protamine sulphate (5mg/ml)	2	139±25
KF (100mM), KH_2PO_4 (5mM)	4	164± 7*

Figure 22

Membrane incubations were performed, as described in section 2.4, at various concentrations of ATP (A) or GTP (B) in the presence of 100mM KF. High affinity cAMP-PDE activity in the absence of KF, ATP or GTP was 28.5μU/mg protein (A) or 23.6μU/mg protein (B). Each plot represents data from one experiment only.

Figure 22. High Affinity cAMP-PDE Activity Associated with KF-Treated Crude Mammary Acinus Membrane Fractions as a Function of ATP (A) or GTP (B) Concentrations



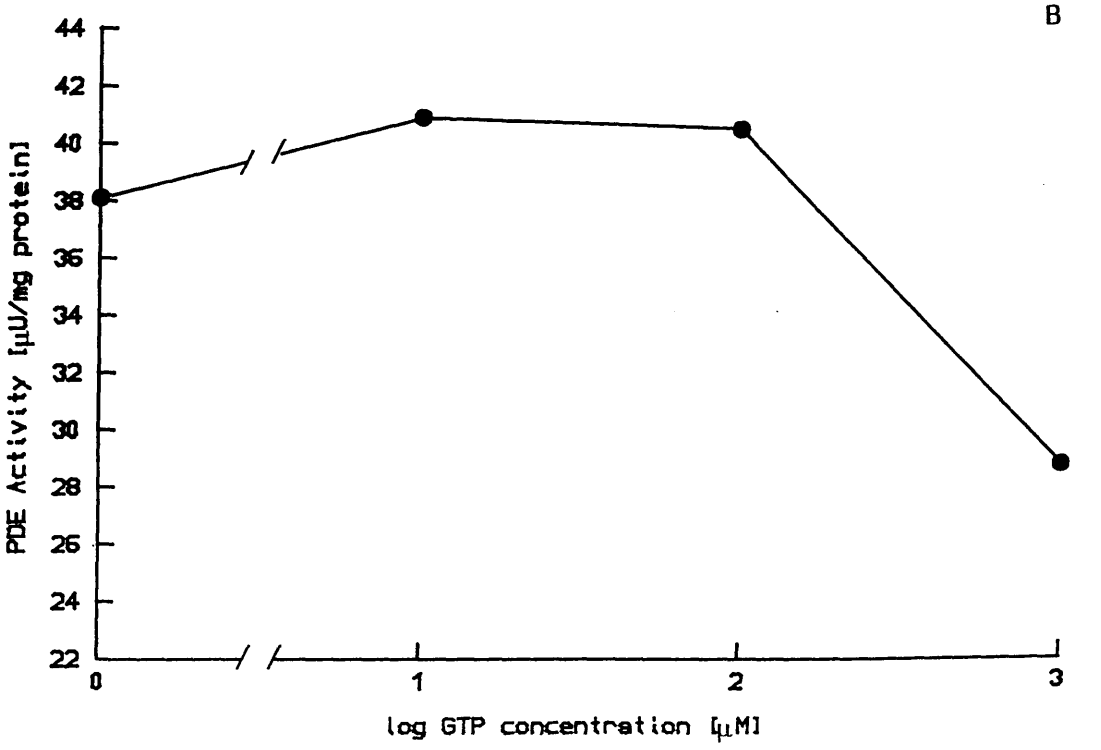


Figure 23

Samples of acinar membrane preparation were incubated, as described in section 2.4, in the presence or absence of KF at two final incubation concentrations, as indicated. The membranes were reisolated and the resulting supernatants treated, as described in section 2.4.1, to remove KF. Columns represent means of duplicate incubations from a single experiment. Black shading denotes whole incubation, red shading denotes particulate fraction and blue shading denotes soluble fraction. Dotted lines represent the sum of particulate and soluble activities.

Figure 23. Effect of KF on the Activity of High Affinity cAMP-PDE in Soluble and Particulate Fractions of Mammary Acinus Membrane Incubations

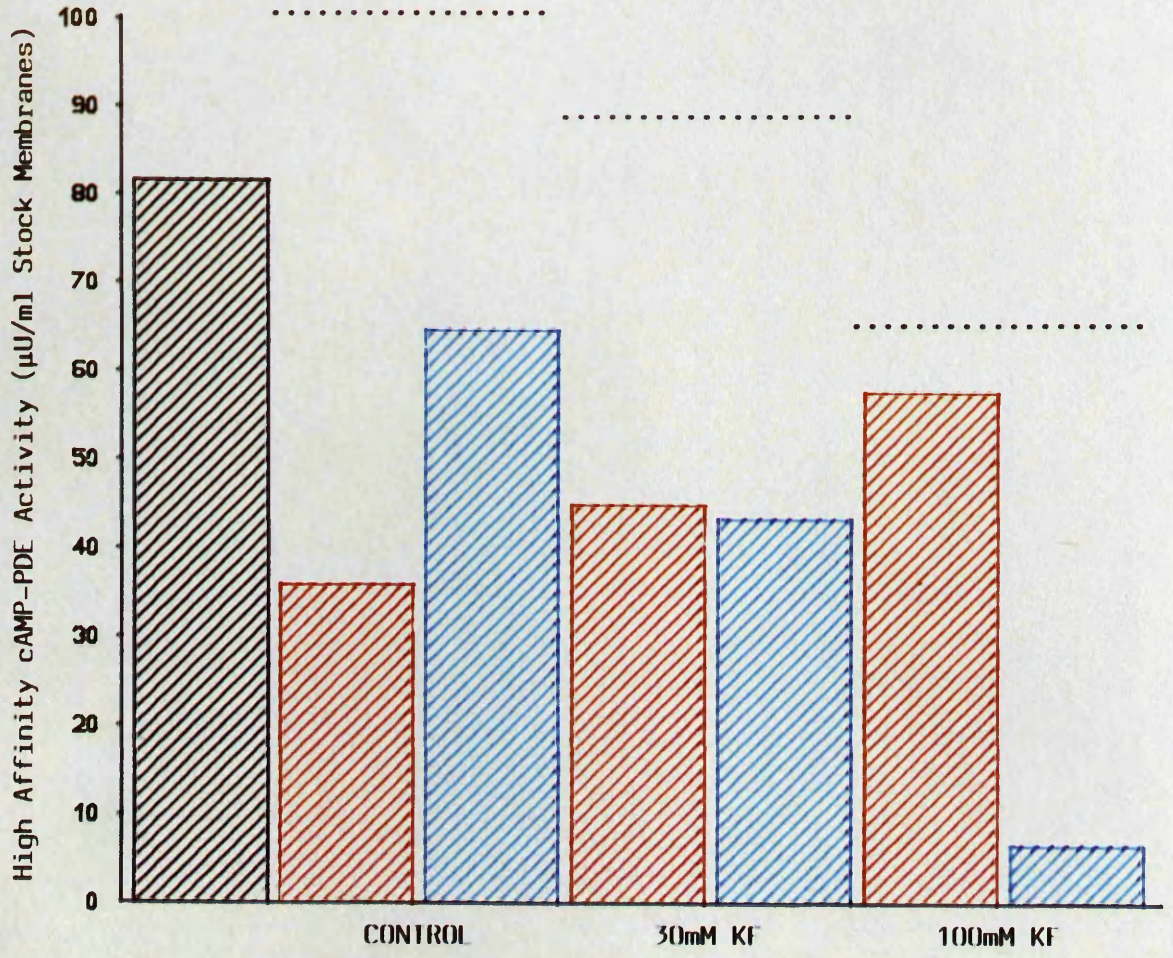


Figure 24

Samples of acinar membrane preparation were incubated, as described in section 2.4, in the presence or absence of KF or KF plus ATP as indicated. The membranes were reisolated and the resulting supernatants treated, as described in section 2.4.1, to remove KF. Columns represent means of duplicate incubations from a single experiment. Black shading denotes whole incubation, red shading denotes particulate fraction and blue shading denotes soluble fraction. Dotted lines represent the sum of particulate and soluble activities.

Figure 24. Effect of KF and KF plus ATP on the Activity of High Affinity cAMP-PDE in Soluble and Particulate Fractions of Mammary Acinus Membrane Incubations

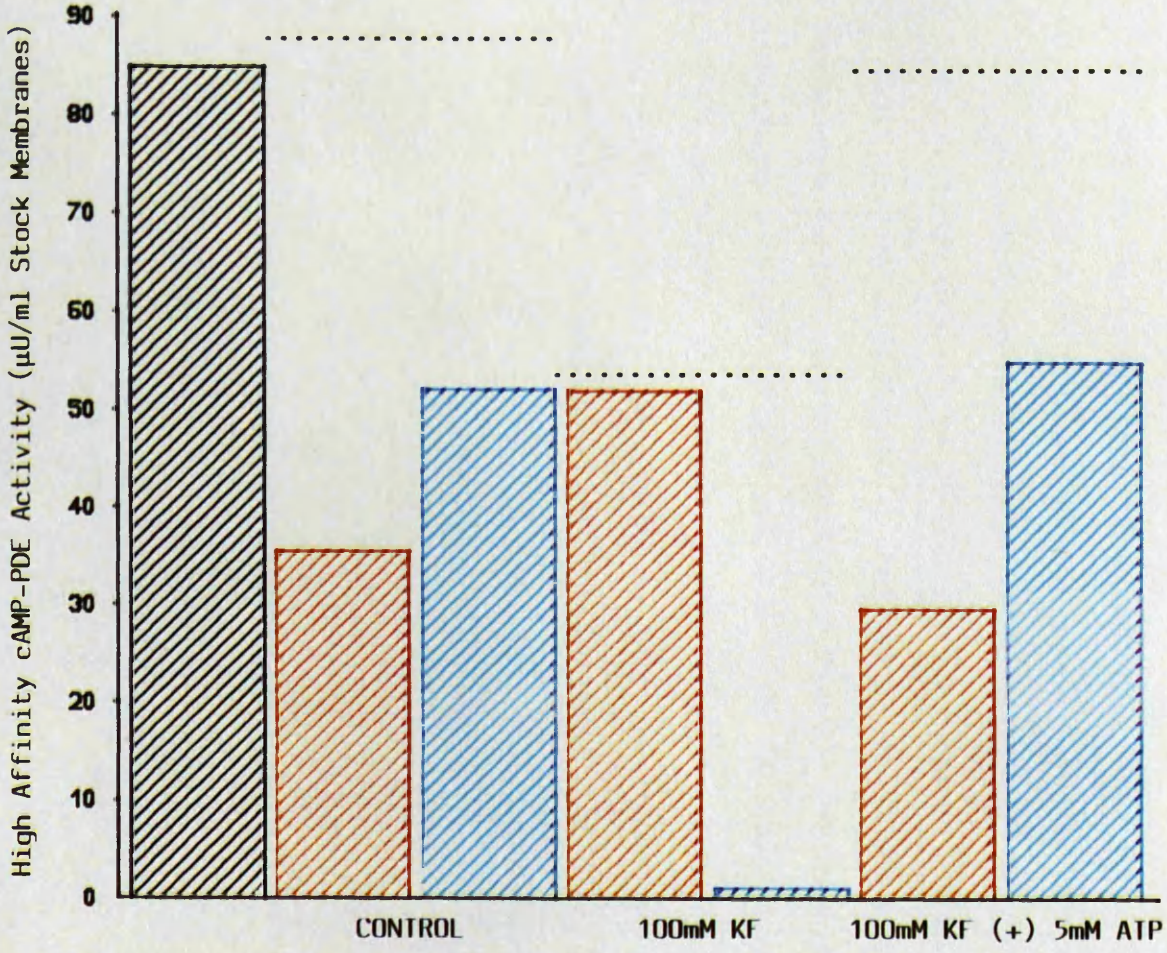
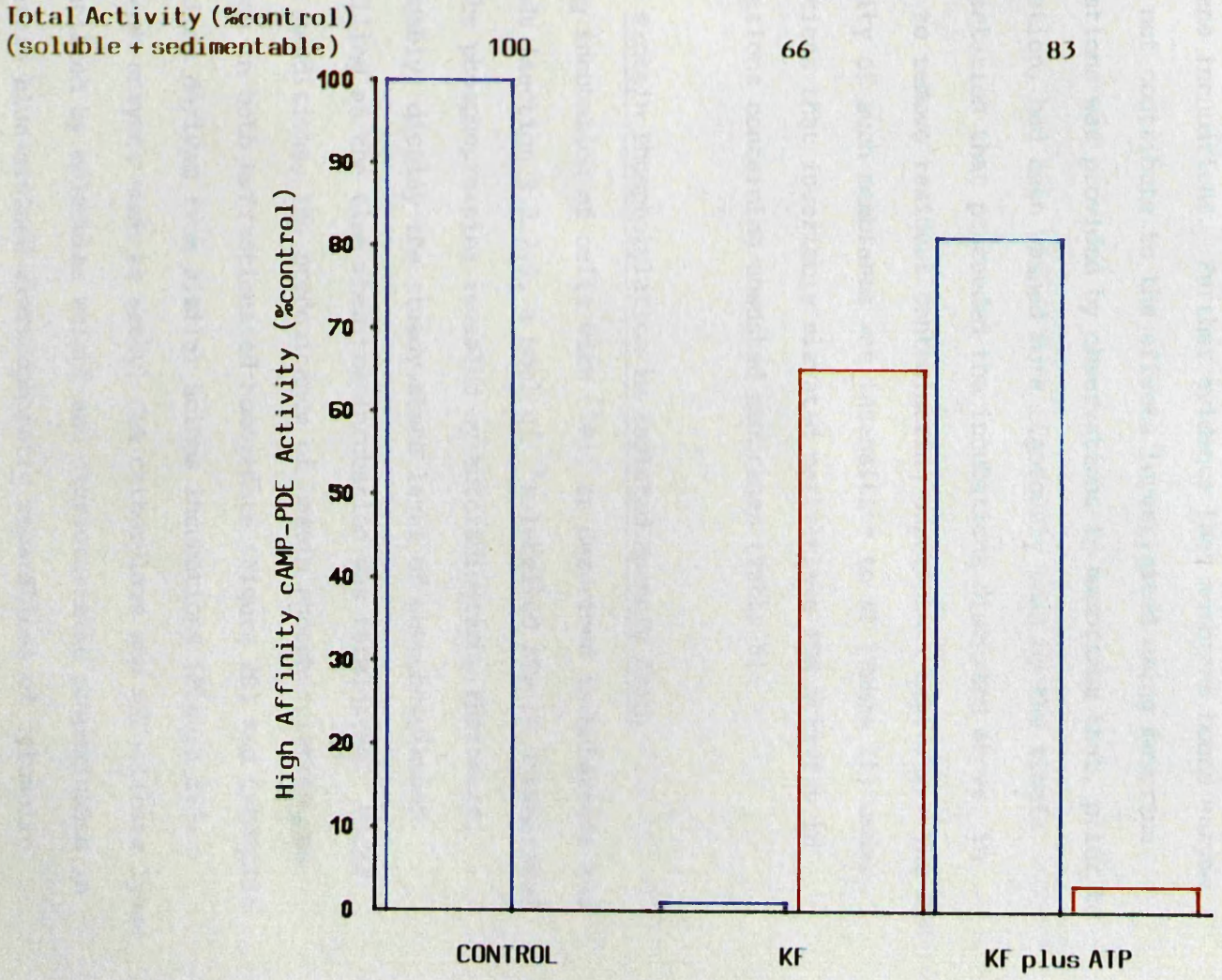


Figure 25

Supernatants from membrane incubations, performed as described in section 2.4, were treated with KF and both these and control (no additions) supernatants were subjected to centrifugation under the conditions employed for reisolation of the membrane fraction. The 'new' supernatant was treated, as described in section 2.4.1, to remove KF and any pellets were resuspended to the original supernatant volume. Columns represent means of values from two separate experiments. Red columns denote activity sedimented under the conditions stated above and blue columns denote soluble activity. Total activity (soluble plus sedimentable), expressed as % control is indicated above relevant columns.

Figure 25. Effect of KF on the Solubility of High Affinity cAMP-PDE Activity in Supernatants from Mammary Acinus Membrane Incubations



That the effects described above were manifestations of properties belonging to a soluble PDE was confirmed by the ability of KF to precipitate activity from supernatants in the apparent absence of any particulate material. As demonstrated in Figure 25, this process was also blocked by ATP and therefore displays the major distinguishing features characterising the effects observed in membrane incubations. Further evidence that membrane bound enzyme could not contribute to the effects investigated using membrane incubations was provided by observations in membranes that, prior to incubation, had been washed more rigorously than by the single sedimentation that preceeded the incubations discussed above, in order to remove residual contaminating supernatant activity. The PDE activity of such membranes was insensitive to KF (Table 11) under conditions that invariably elevated particulate PDE activity in incubations containing unwashed membranes (Table 6).

3.2. Protein Phosphorylation in Isolated Mammary Acini

During incubation of cells with ^{32}Pi, as described in Materials and Methods (section 2.3.2.), a pool of ^{32}P-labelled ATP is established and the phosphoproteins revealed by autoradiography therefore, presumably, display the steady-state level of phosphorylation prevailing at the time when the incubation was terminated. Under these conditions, the predominance of casein phosphorylation was obvious in both unfractionated homogenate (Figure 26) and cytosolic fractions derived from similar acinus incubations (Figure 27). Although enzymes such as acetyl-CoA carboxylase and ATP-citrate lyase (identified by molecular weight and characteristic predominance in Coomassie blue-stained electrophoretic separations of cytosolic

Table 11 Effect of KF and ATP on high affinity cAMP-PDE activity associated with a crude membrane fraction (washed prior to incubation) from rat mammary acini

Incubation additions	Particulate high affinity cAMP-PDE activity (μU/ml membrane suspension)	(μU/mg protein)
None	14.91	15.8
KF (100mM)	16.22	17.9
KF (100mM), ATP (5mM)	14.51	16.6

Acinus membranes, prepared, as described in section 2.4, were washed by suspension in $KHCO_3$ (1mM) and reisolated by centrifugation at 22500 xg for 10 min prior to use as a source of membranes for incubations. Incubations were then conducted as described in materials and methods. Values presented are means of duplicate incubations from a single experiment.

Figure 26

The proteins contained in mammary acini, incubated with ^{32}Pi (as described in section 2.3.2), in the presence or absence of additions as indicated, were resolved electrophoretically on 7.5% (w/v) polyacrylamide SDS gels. An autoradiogram representative of two separate experiments is shown.

Figure 26. Effect of Insulin and Agents Capable of Elevating Intracellular cAMP Concentrations on Protein Phosphorylation in Intact Mammary Acini

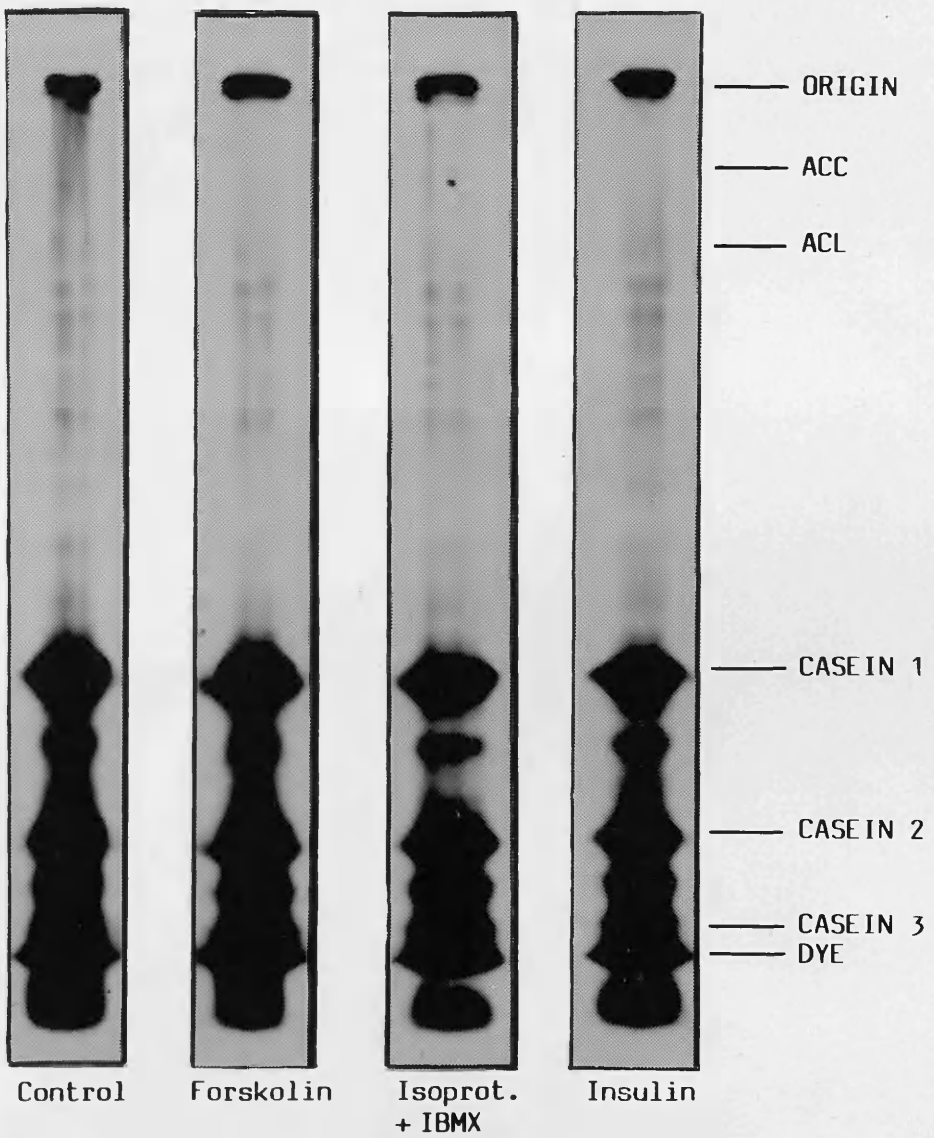
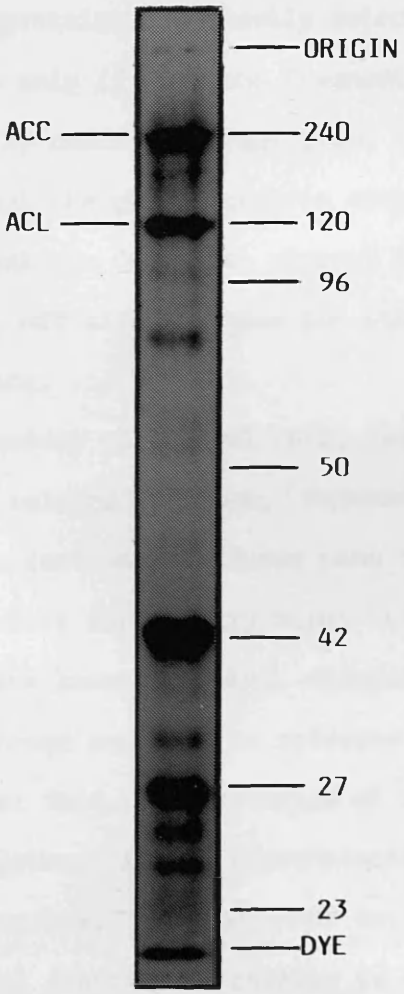


Figure 27

Acini were incubated in the presence of ^{32}Pi, as described in section 2.3.2 and a cytosolic fraction prepared, as described later in the same section. Phosphoproteins present in the electrophoretic separations (on 8% (w/v) polyacrylamide SDS gels) of these samples were identified by autoradiography. A separation representative of two separate experiments is shown.

Figure 27. Protein Phosphorylation in Cytosols from Mammary Acini Equilibrated with ^{32}Pi



mammary proteins) are major phosphoprotein components of mammary cytosols (Figure 27), they did not give rise to prominent bands in autoradiogrpahs of unfractionated cellular homogenates following SDS polyacrylamide gel electrophoresis, even when the photographic plate was deliberately over-exposed in the region of the caseins in order to reveal less extensively phosphorylated or less abundant phosphoproteins of higher molecular weights (Figure 26). The fact that these (acetyl-CoA carboxylase and ATP-citrate lyase) and other major cytosolic phosphoproteins were easily detectable in SDS-polyacrylamide gels only if membrane fragments and casein micelles were removed (by centrifugation) prior to electrophoretic separation, suggests that the phosphoprotein complement of those structures served to mask the otherwise obvious phosphorylations of acetyl-CoA carboxylase, ATP-citrate lyase and other prominent cytosolic phosphoproteins.

In the intact mammary epithelial cell, caseins are confined to the lumen of Golgi and related vesicles. However, under the relatively harsh homogenisation procedures used to obtain the cytosols characterised here for mammary acini, it is likely that these vesicles would have been disrupted, releasing their contents into the cytosol. Although much of the released casein would initially be in micellar form, sequestration of Ca^{2+} by the Ca^{2+}-chelating agents present in the homogenisation buffer would have rendered it non-sedimentable. This accounts for the presence of caseins in these cytosol fractions. Because of the intensity of casein labelling, all autoradiograms exposed to reveal phosphoproteins labelled less intensely will be over-exposed to saturation in the region of the caseins. Hence, although the

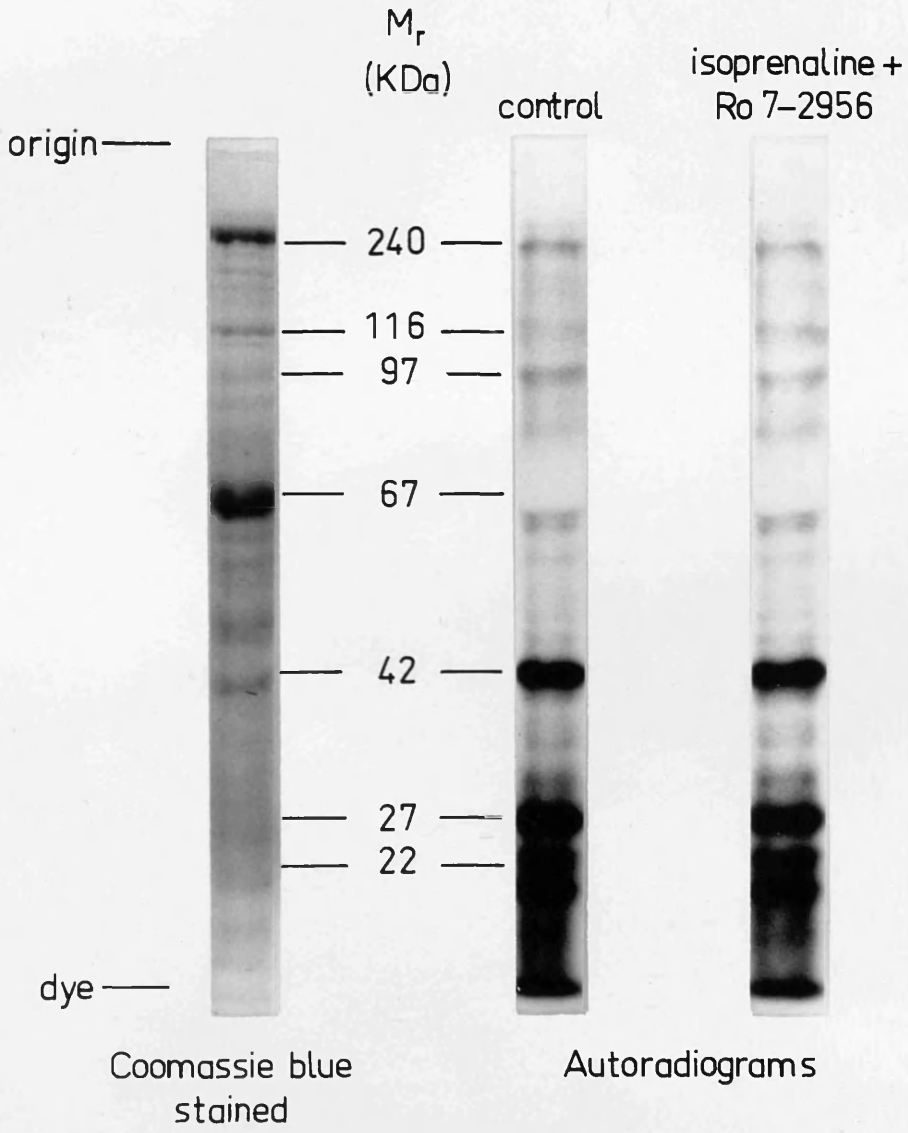
exposure of caseins appears approximately the same in Figures 27 and 28, the difference in photographic intensity of other bands, such as those of acetyl-CoA carboxylase and ATP-citrate lyase, between the two Figures, is probably due, predominantly, to differences in exposure time (since the caseins are saturated in both autoradiograms, exposure time has little effect on their observed photographic intensity). Another possible contributory factor is a difference in the degree of carry-over of 105000xg pellet (containing casein-loaded vesicles) into the supernatant fraction.

The pattern of phosphorylation observed in both unfractionated homogenates and cytosol preparations following incubation of mammary acini under the conditions described above was unchanged whether the final 15 minutes of the incubation period were conducted in the presence or absence of insulin or agents known to raise intracellular cAMP concentrations (Ro7-2956, isoprenaline, forskolin, IBMX) [102] (see Figures 26 and 28). While changes in the phosphorylation state of many proteins with molecular weights between about 20kDa and 45kDa would have been obscured completely by the extremely high density of casein associated labelling, higher molecular weight proteins displaying susceptibility to cAMP or insulin dependent changes in phosphorylation state should have been readily identifiable, particularly in autoradiographs of 4% (w/v) total acrylamide gels, since caseins run at the dye-front in such gels. However, experiments of this type using both unfractionated homogenate (data not shown) and cytosols prepared from the acinus incubations (100a) failed to reveal anything that was not also discernable in the 8% gels shown in Figures 26 and 28.

Figure 28

Acini were incubated as described in section 2.3.2., in the presence and absence of isoprenaline and Ro7-2956 as indicated and a cytosolic fraction prepared as described later in the same section. Phosphoproteins present in the electrophoretic separations (on 8% (w/v) polyacrylamide SDS gels) of these samples were identified by autoradiography. A separation representative of two separate experiments is shown.

Figure 28. Effect on Cytosolic Protein Phosphorylation of Incubating Mammary Acini with a Combination of Isoprenaline and Ro 7-2956



Consistent with work of a similar nature conducted by Clegg et al. [100], slices of gels used to separate phosphoproteins labelled with ^{32}P in intact cells showed no changes in the level of phosphorylation, as determined by liquid scintillation counting, of acetyl-CoA carboxylase isolated from homogenates (Table 12) or cytosols (Table 13) of cells incubated in the presence or absence of either insulin or agents that raise intracellular cAMP levels. This supports the visual evidence provided by autoradiographs such as the one shown in Figures 26, that steady state acetyl-CoA carboxylase phosphorylation was not affected either by exposure of intact acini to insulin or by elevation of their intracellular cAMP concentrations.

Allred et al. [9a] have shown that, in rat liver, acetyl-CoA carboxylase may associate with mitochondria and that the extent of association varies with nutritional status. It might therefore be reasoned that the explanation for an inability to observe effects of hormone and other agents on acetyl-CoA phosphorylation in acinus cytosols is that the hormone-sensitive acetyl-CoA carboxylase population is associated predominantly, if not totally with those cellular fractions removed during preparation of the cytosol. That this was not in fact the answer was demonstrated by experiments using unfractionated homogenates in which no change in the extent of phophorylation of acetyl-CoA carboxylase was observed following incubation of acini with insulin or agents known to raise intracellular cAMP concentration (data not shown). The strength of this evidence is perhaps undermined, to a certain extent, by the proposed obscuring of acetyl-CoA carboxylase phosphorylation by other membrane-associated phosphoproteins. However, it seems unlikely that

Table 12 Effect of insulin and forskolin treatment of rat mammary acini on the steady state level of phosphorylation of acetyl-CoA carboxylase

Incubation additions	Radioactivity associated with gel slice (CPM x 10^{-2})		ACC Associated radioactivity (% of counts in control band)
	ACC band	Control band	
None	9.9	7.5	132
Forskolin	9.6	6.9	139
Insulin	9.3	6.6	141

Acini were incubated as described in Materials and Methods (section 2.3.2) with forskolin (10μM) or insulin (1.68 mU/ml) as indicated. Five replicate incubations were performed for each condition and samples of the disaggregated homogenate derived from each incubation were run on 4% (w/v) total acrylamide SDS-PAGE gels. Phosphoproteins were detected and ^{32}P content quantitated as described in Section 2.7. Values given (CPM x 10^{-2}) are means of 5 replicate observations obtained from 1 experiment only; individual determinations differed by not more than ± 12% of the means tabulated.

Table 13 Phosphorylation of selected proteins in mammary cytosol in the presence of dibutyryl cyclic AMP or of agents that increase intracellular cyclic AMP

Phosphoprotein		^{32}P incorporation relative to control incubation (%)		
10^{-3} x M_r	Identity	1mM-dibutyryl cyclic AMP	1μM-isoprenaline plus 1mM-Ro 7-2956	100μM-Forskolin
240	Acetyl-CoA carboxylase	94.7± 6.6	101.0± 2.6	101.2± 4.7
116	ATP-citrate lyase	100.2± 4.8	108.8± 9.9	100.1± 7.6
55	unknown	104.0± 8.2	96.3± 8.1	96.5± 7.4
42	casein	108.1±12.2	112.9±14.7	102.3±13.3
27	casein	89.9±11.1	107.4± 5.2	94.8± 9.1

Acini were incubated in the presence of $^{32}P_i$ as described in Section 2.3.3.; agents were added after 45 min, with control incubations receiving no additions. Cytosol fractions were prepared as described later in the same section and phosphoproteins were separated by SDS-polyacrylamide gel electrophoresis. Phosphoproteins were detected by autoradiography and their ^{32}P content quantitated as described in Section 2.7. ^{32}P-radioactivity of each band was expressed per unit of lactate dehydrogenase activity in the corresponding cytosol fraction to normalise differences in concentration among these samples. Normalised values were then compared between control and experimental samples and expressed as a percentage of the former. The radioactivity incorporated into the phosphoprotein tabulated was in the approximate proportion (in descending order of M_r) 2:2:1:5:5. Values given are means ± SEM for 5

significant changes in the phosphorylation of such an abundant protein could be masked in this manner.

3.3. Protein Phosphorylation in Mammary Cell Fractions

A survey was conducted of the contributions made by four major effector-dependent protein kinases (cAMP-PrK, CC-PrK, PrK-C and polyamine-PrK see section 1.3.8.(i)) to the endogenous protein phosphorylations observed in each of four subcellular fractions prepared from mammary cells as described in Materials and Methods (section 2.5.). Differential centrifugation allowed the isolation of three membrane fractions and a high speed supernatant fraction with consistently reproducible protein separation patterns on SDS-polyacrylamide gel electrophoresis (Figure 29). The fractions correspond to a nuclear and large membrane fragment fraction (1000xg pellet), a mitochondrial fraction (3000xg pellet), a microsomal fraction (105000xg pellet) and a post-microsomal supernatant (cytosolic) fraction (105000xg supernatant). Marker enzyme assays, performed as described in Materials and Methods (section 2.8.2.) provided a quantitative characterisation of the general composition of these cellular fractions and once more demonstrated the reproducibility of the fractionation technique (Table 14).

The rate of incorporation of ^{32}P into proteins present in mammary cell supernatant fractions incubated with [γ-^{32}P] ATP, as described earlier (section 2.6.), was approximately linear for at least 60 sec. at 37°C for most of the major phosphoproteins revealed by autoradiography of SDS polyacrylamide gels containing proteins labelled with ^{32}P during such incubations (Figure 30). Incorporations determined after 30 sec. incubation therefore probably

Table 14 Activities of enzyme markers, specific to mitochondria, plasma membrane and cytosol, expressed by the subcellular fractions used in investigation of mammary protein phosphorylations

Fraction	ENZYME ACTIVITY					
	5'nucleotidase†		Cytochrome-c oxidase*		LDH*	
	(U/mg protein)	(% total)	(U/mg protein)	(% total)	(U/mg protein)	(% total)
Nuclear	1.15±0.05	34±1	5.1±2.2	30±5	52.8±17.9	7±1
Mitochondrial	1.07±0.13	31±2	11.2±3.4	67±2	31.6± 7.8	4±1
Microsomal	0.90±0.11	26±2	0.6±0.7	3±4	74.5± 8.0	10±1
Cytosolic	0.31±0.02	9±1	0.0±0.0	0±0	613.9±121.0	79±2

Assays for 5'nucleotidase, cytochrome-c-oxidase and lactate dehydrogenase (marker enzymes for plasma membrane, mitochondrial membrane and cytosol respectively) were performed as described in Materials and Methods (section 2.8.2). Values given are means ± SE of data from 5 (†) or 3 (*) separate fractionations.

Figure 29A

Mammary cell fractions were prepared by differential centrifugation, as described in section 2.5 and samples were resolved electrophoretically on a 5% (w/v) polyacrylamide SDS gel. Protein bands were detected by staining with Coomassie brilliant blue. The stained gel is shown. Each track within a particular fraction represents a sample taken from a separate fractionation performed on acini prepared from a separate animal. N denotes 'nuclear' fraction and M denotes 'mitochondrial' fraction.

Figure 29 A. Electrophoretic Separations of Proteins Contained in Mammary Cell "Nuclear" and "Mitochondrial" Fractions

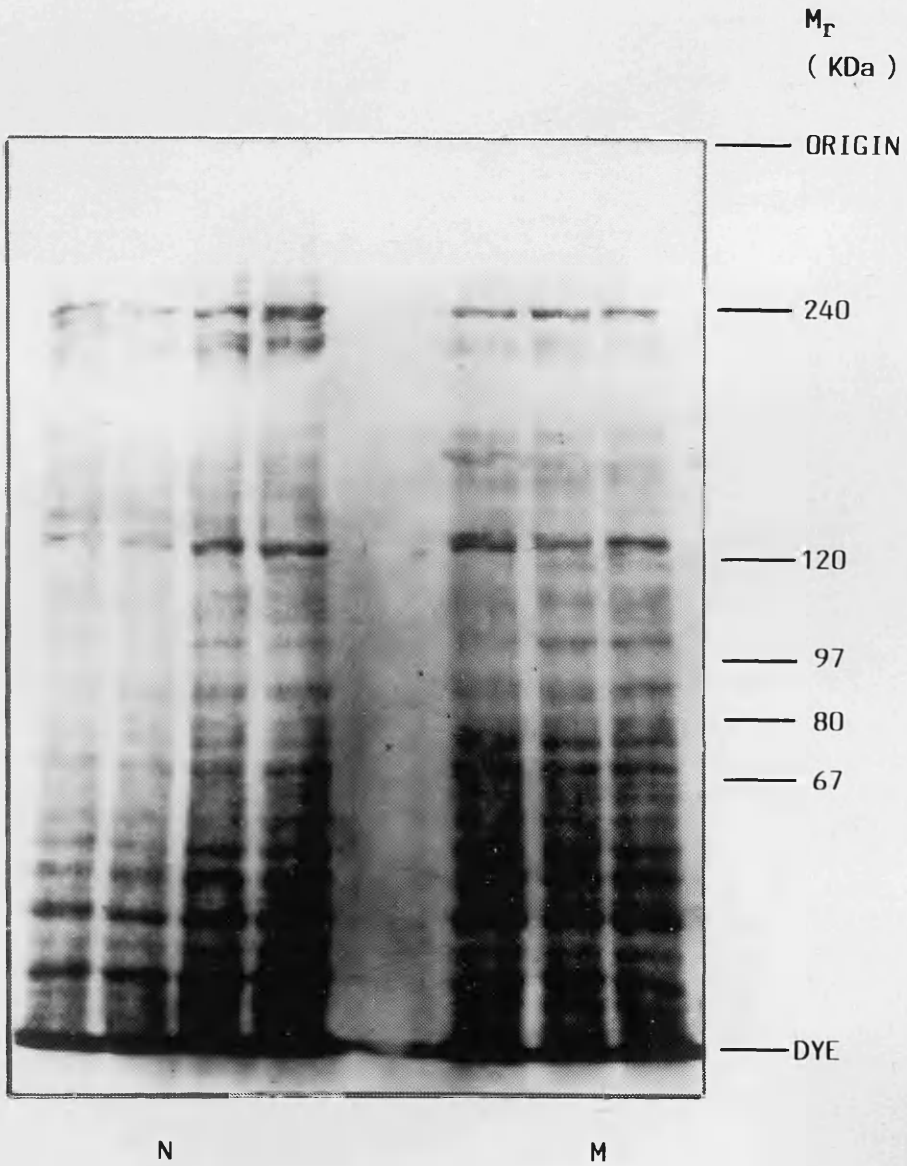


Figure 29B

Mammary cell fractions were prepared by differential centrifugation, as described in section 2.5 and samples were resolved electrophoretically on a 5% (w/v) polyacrylamide SDS gel. Protein bands were detected by staining with Coomassie brilliant blue. The stained gel is shown. Each track within a particular fraction represents a sample taken from a separate fractionation performed on acini prepared from a separate animal. M denotes 'microsomal' fraction and C denotes 'cytosolic' fraction.

Figure 29 B. Electrophoretic Separations of Proteins Contained in Mammary Cell "Microsomal" and "Cytosolic" Fractions

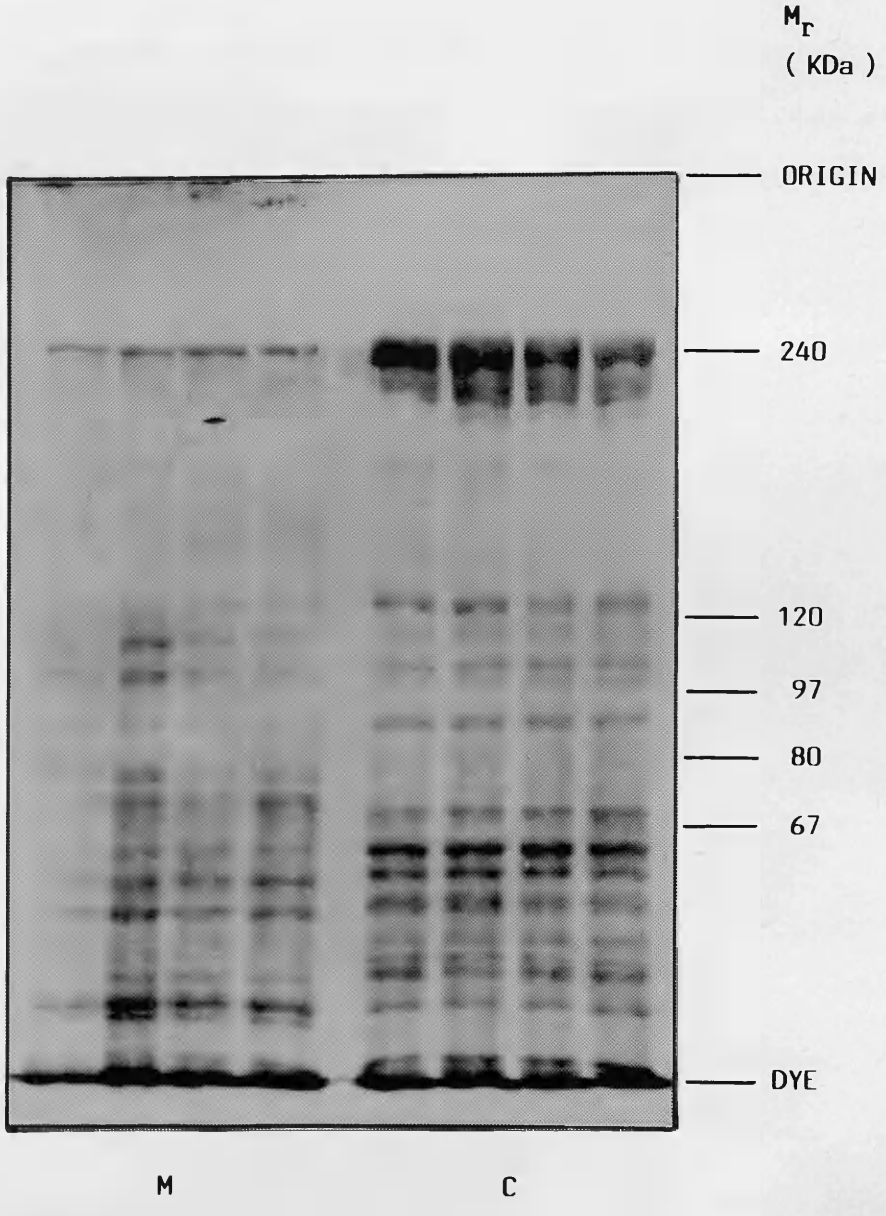
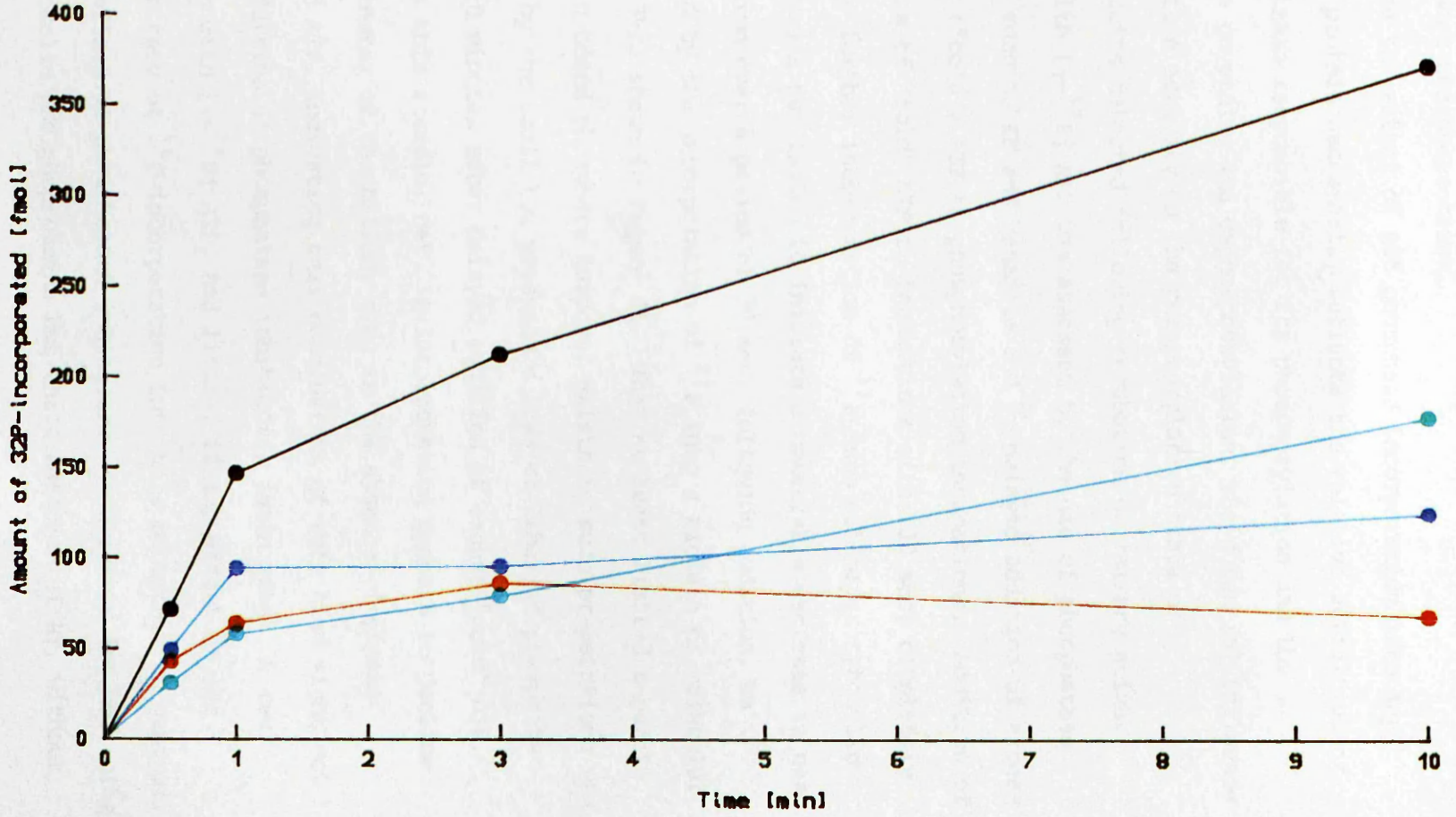


Figure 30

Acinus 'cytosols', prepared as described in section 2.5, were incubated for 0.5, 1.0, 3.0 or 10.0 minutes under the conditions described in section 2.6. Data points represent means of duplicate incubations from a single experiment representative of a pair of separate experiments. Results are presented for ^{32}P-incorporation (determined by liquid scintillation counting, as described in section 2.7) into bands in electrophoretic gels corresponding to proteins of molecular weights 57.3kDa (red), 47kDa (blue), 100.3kDa (green) and 29.5kDa (black).

Figure 30. ^{32}P-Incorporation into Four of the Major Phosphoproteins Identified in Rat Mammary Acinus Cytosols as a Function of Time



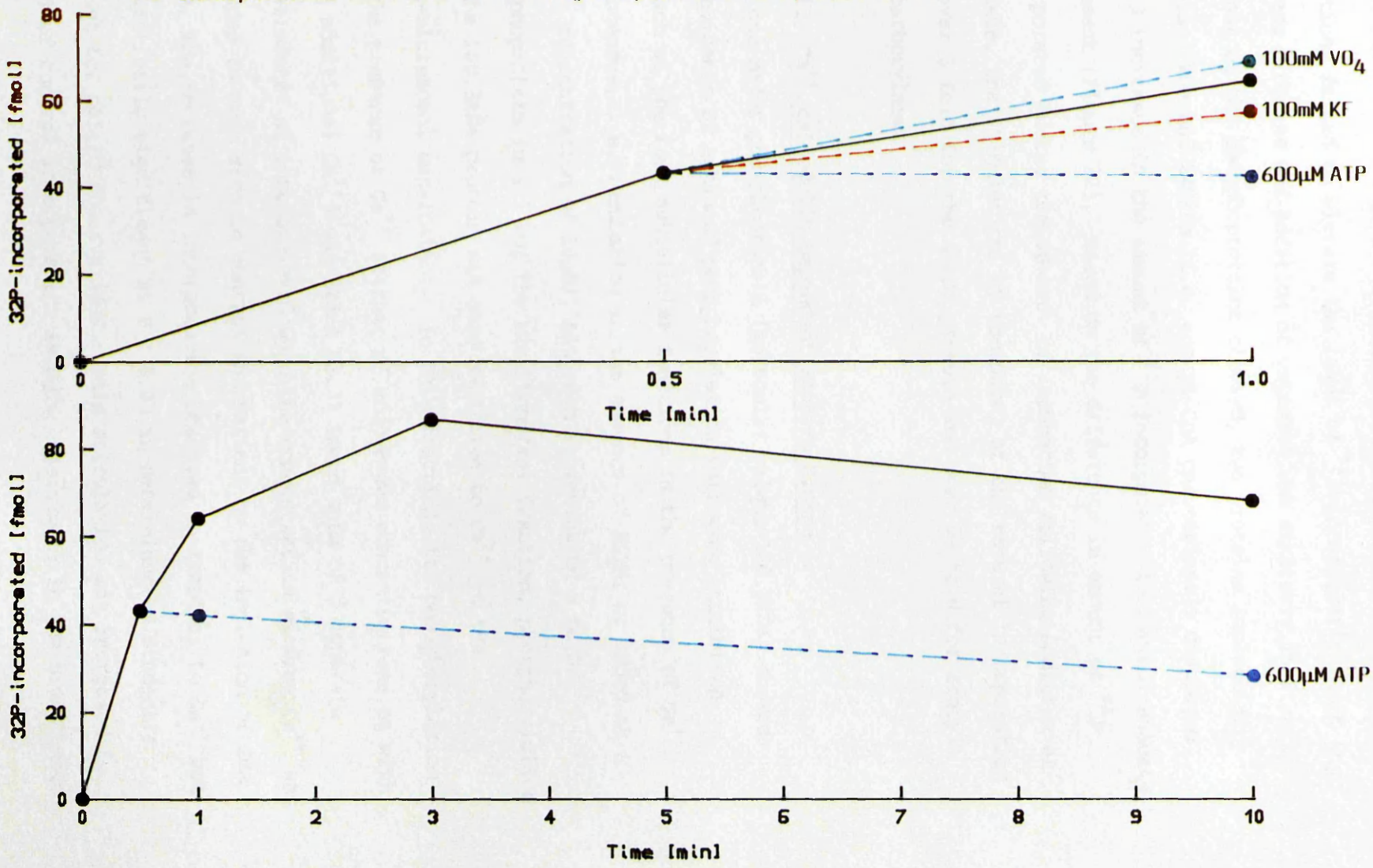
approximated to measurements of initial rates of net incorporation of ^{32}P into those phosphoproteins.

Since the extent of net phosphate incorporation into any particular protein necessarily reflects the relative activities of both the kinase responsible for its phosphorylation and the phosphatase governing its dephosphorylation, the potential influence of phosphatase activity on the phosphorylation state of phosphoproteins detected following incubation of mammary acinar cytosols with [γ-^{32}P] ATP was assessed by the use of phosphatase inhibitors such as KF and vanadate and by delayed addition of excess unlabelled ("cold") ATP to phosphorylation incubations. Addition of large excess of "cold" ATP to incubations after 30 sec. completely blocked any further incorporation of ^{32}P into all major cytosolic phosphoproteins but failed to initiate a measurable decrease in net incorporation over a period of 30 sec. following addition, as exemplified by the incorporation of ^{32}P into a protein of molecular weight 57.3kDa shown in Figure 31. That at least limited capacity for protein bound phosphate turnover exists in such preparations was suggested by the small but measurable loss of labelled phosphate observed 10 minutes after delayed addition of excess "cold" ATP, however in this example, net ^{32}P-incorporation appears to decline after 3 minutes of incubation, even in the absence of excess unlabelled ATP, indicating that conclusions of this kind might not be valid. Addition of phosphatase inhibitors, again after 30 sec. incubation with [γ-^{32}P] ATP, had little, if any effect on the subsequent rate of ^{32}P-incorporation into this protein. In general, the same effect of phosphatase inhibitors was observed for most other major cytosolic phosphoproteins but while addition of KF, without

Figure 31

Samples of acinus cytosol, prepared as described in section 2.5, were incubated, under the conditions described in section 2.6, for 0.5 minute before addition of phosphatase inhibitors (A) or a large excess of unlabelled ATP (A and B). Changes in incorporation of ^{32}P into the 57.3kDa protein, contained in these samples, were assessed by liquid scintillation counting, as described in section 2.7, after a further incubation period of either 0.5 min or 9.5 min as indicated. Data points represent mean values of duplicate incubations from a single experiment.

Figure 31. Effect of Delayed Addition of Phosphatase Inhibitors or a Large Excess of Unlabelled ATP on ^{32}P-Incorporation into one of the Major Cytosolic Phosphoproteins



exception, failed to elevate the level of ^{32}P-incorporation into any of these proteins and addition of vanadate was similarly ineffective for most of the phosphoproteins studied, two proteins (molecular weights 47kDa and 240kDa [i.e. acetyl-CoA carboxylase]) displayed marked increases in the amount of ^{32}P incorporated following vanadate treatment (Figure 32). Based on the difference in amount of ^{32}P incorporated during the 30 sec. of incubation following addition of vanadate, the stimulation, by vanadate, of the rate of incorporation was over 3 fold for the 47kDa protein and over 5½-fold for acetyl-CoA carboxylase.

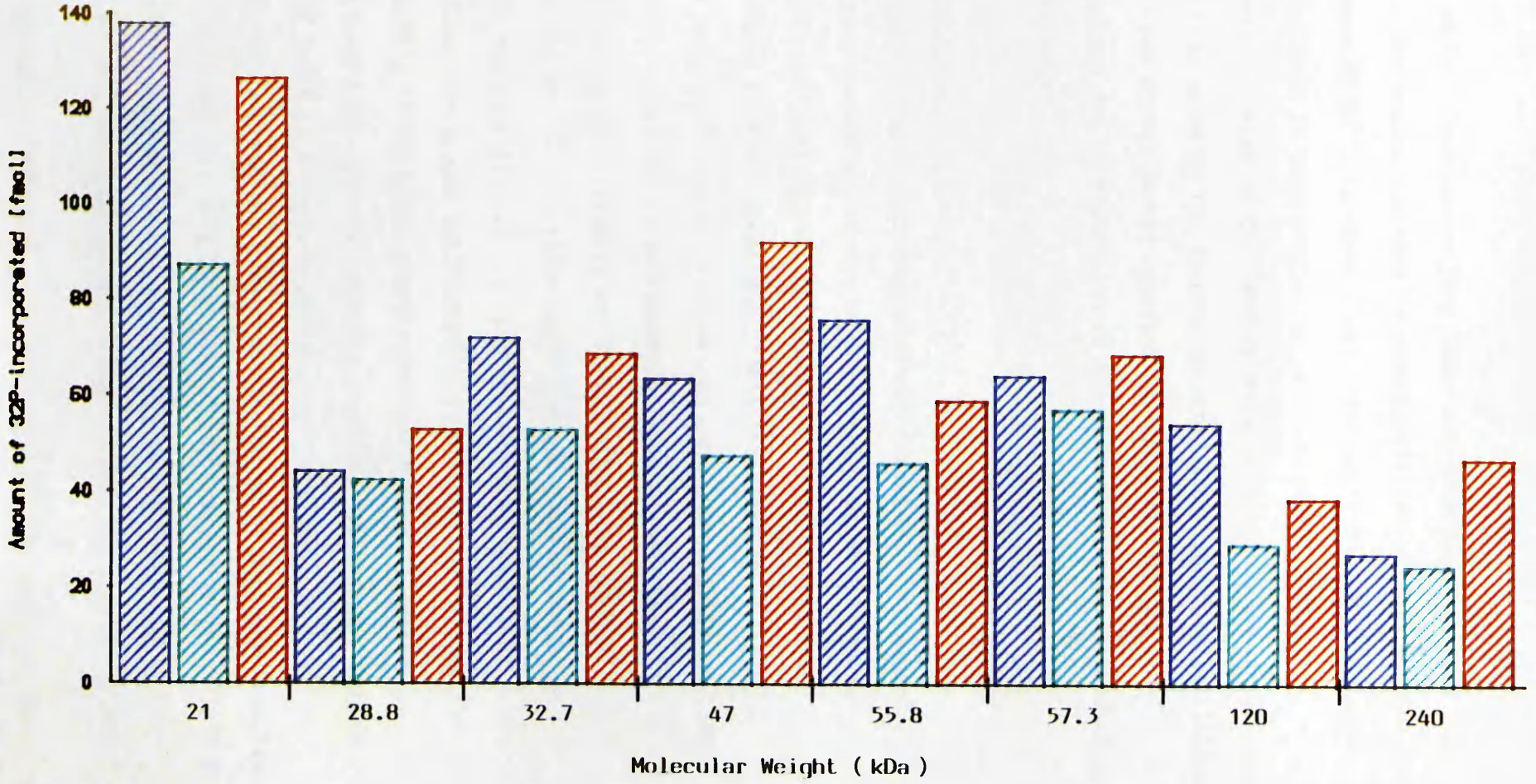
3.3.1. Ca^{2+}/calmodulin-Dependent Protein Kinase

Only one major phosphoprotein (molecular weight 100.3kDa) showed any evidence of enhanced phosphorylation following incubation of each of the four subcellular fractions in the presence of Ca^{2+} (endogenous, i.e. incubation in the absence of EGTA, or added at a final concentration of 10μM). Although representing a major phosphoprotein in all but the mitochondrial fraction, phosphorylation of the 100.3kDa protein was only sensitive to Ca^{2+} in the post-microsomal supernatant. In this fraction, its phosphorylation in the presence of Ca^{2+} (either at endogenous concentrations or with 10μM additional Ca^{2+}) was 323 ± 62.3% (mean ± SE of 4 separate experiments) of that achieved when the concentration of free Ca^{2+} was reduced to near zero in control incubations by the inclusion of 2mM EGTA. The increase in incorporation observed in response to Ca^{2+} was statistically significant at $P < 0.05$ as determined by Student's t-test for paired observations. This stimulation was increased to 929% of control incorporation (single experiment) in the simultaneous

Figure 32

Samples of acinus cytosols, prepared as described in section 2.5, were incubated as described in section 2.6, for 0.5 minute before addition of phosphatase inhibitors. Changes in ^{32}P-incorporation into proteins contained in the samples were assessed, as described in section 2.7, after a further incubation period of 0.5 minute. Data represent means of duplicate incubations from a single experiment. Blue shading denotes control, green shading denotes KF (100mM) and red shading denotes VO_4^{3-} (100mM).

Figure 32. Effect of Phosphatase Inhibitors on ^{32}P-Incorporation into Proteins Contained in Rat Mammary Acinus Cytosols



presence of Ca^{2+} (endogenous plus 10μM) and calmodulin (5μg/mg protein). The marked increase in phosphorylation of this protein, in the presence of Ca^{2+}, is shown clearly in Figure 33. Although some further increase in phosphorylation is discernible in the simultaneous presence of Ca^{2+} and calmodulin, the full extent of this increase (indicated by the figures obtained from liquid scintillation counting, see above) is not apparent by inspection of the autoradiogram, due to saturation of the photographic emulsion during autoradiography.

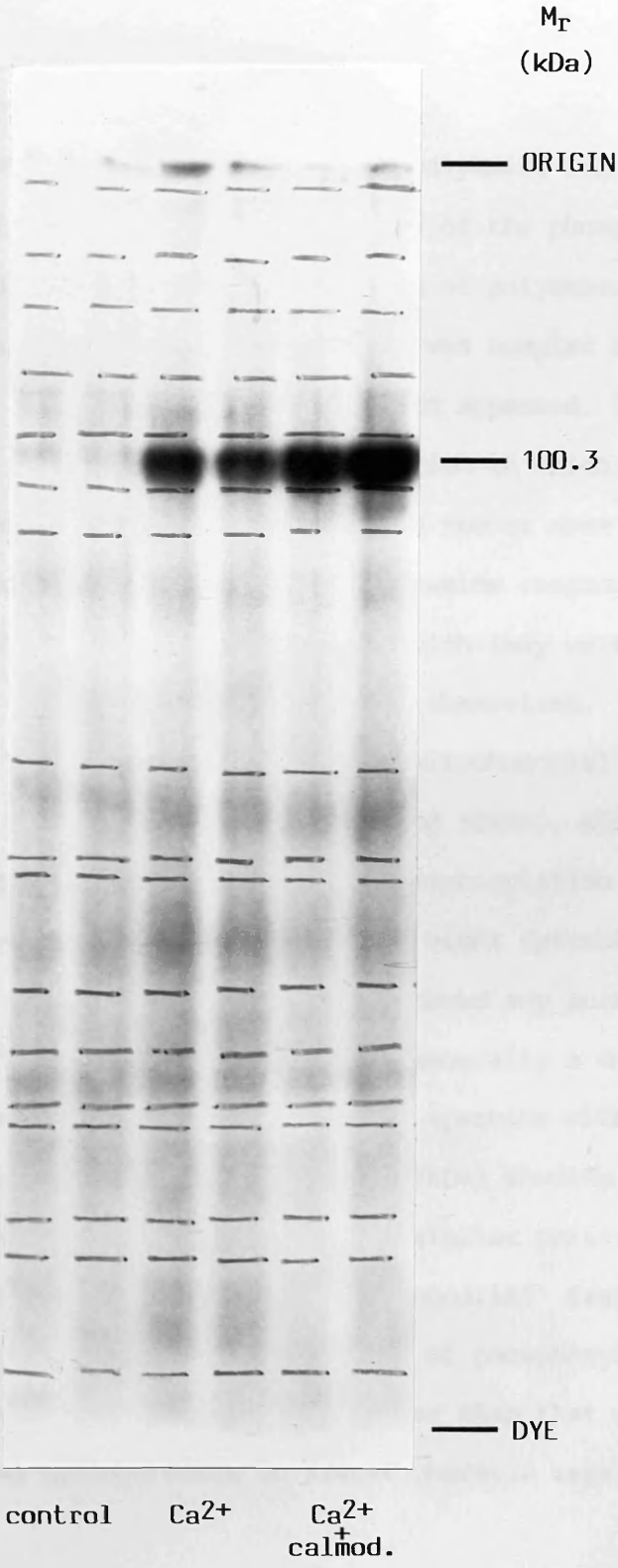
3.3.2. Protein Kinase C

Once again, only the 100.3kDa protein of acinar post-microsomal supernatants showed any change in extent of phosphorylation in response to Ca^{2+} and neither phosphatidyl serine (incubation concentration 20μg/ml) alone nor phosphatidyl serine (20μg/ml) together with phorbol ester (incubation concentration 100ng/ml PMA) affected the level of ^{32}P-incorporation into any of the major phosphoproteins visualised by autoradiography. The lack of phospholipid and phorbol ester sensitive phosphorylation in any of the three membrane fractions or post-microsomal supernatant fraction was confirmed by liquid scintillation counting of gel slices corresponding to the major phosphoproteins separated by SDS polyacrylamide gel electrophoresis. Phosphatidyl serine is known to be one of the most effective phospholipid activators of PrK-C [377,420,755] and PMA has been demonstrated to be a potent stimulator of PrK-C activity [84,567] by virtue of its ability to substitute for diacyl glycerol (see section 1.3.8(i)b). Since these agents were without effect, the implication is that either PrK-C activity was

Figure 33

The proteins contained in samples of mammary acinus 'cytosol', prepared as described in section 2.5. and incubated with [γ-^{32}P] ATP, as described in section 2.6., in the presence and absence of Ca^{2+} or Ca^{2+}/ calmodulin, as indicated, were resolved electrophoretically on 8% (w/v) polyacrylamide SDS gels. Control incubations contained EGTA (2mM). All other incubations were performed in the absence of EGTA and contained $CaCl_2$ (final concentration 10μM). Ca^{2+}/calmodulin incubations contained calmodulin added at a final concentration of 5μg/mg protein. The control and plus Ca^{2+} incubations are representative of 2 separate experiments and the autoradiogram presented is that obtained in the single experiment in which an incubation in the simultaneous presence of Ca^{2+} and calmodulin was included. The horizontal lines visible at intervals throughout the tracks were drawn during the experiment as an aid to subsequent slicing of the gel for liquid scintillation counting.

Figure 33. Effect of Ca^{2+} and Ca^{2+}-calmodulin on Protein Phosphorylation in Mammary Acinar Cytosols



extremely low in all fractions tested or that the kinase was predominantly confined to one fraction while its substrate(s) resided in another.

3.3.3. Polyamine-Dependent Protein Kinase

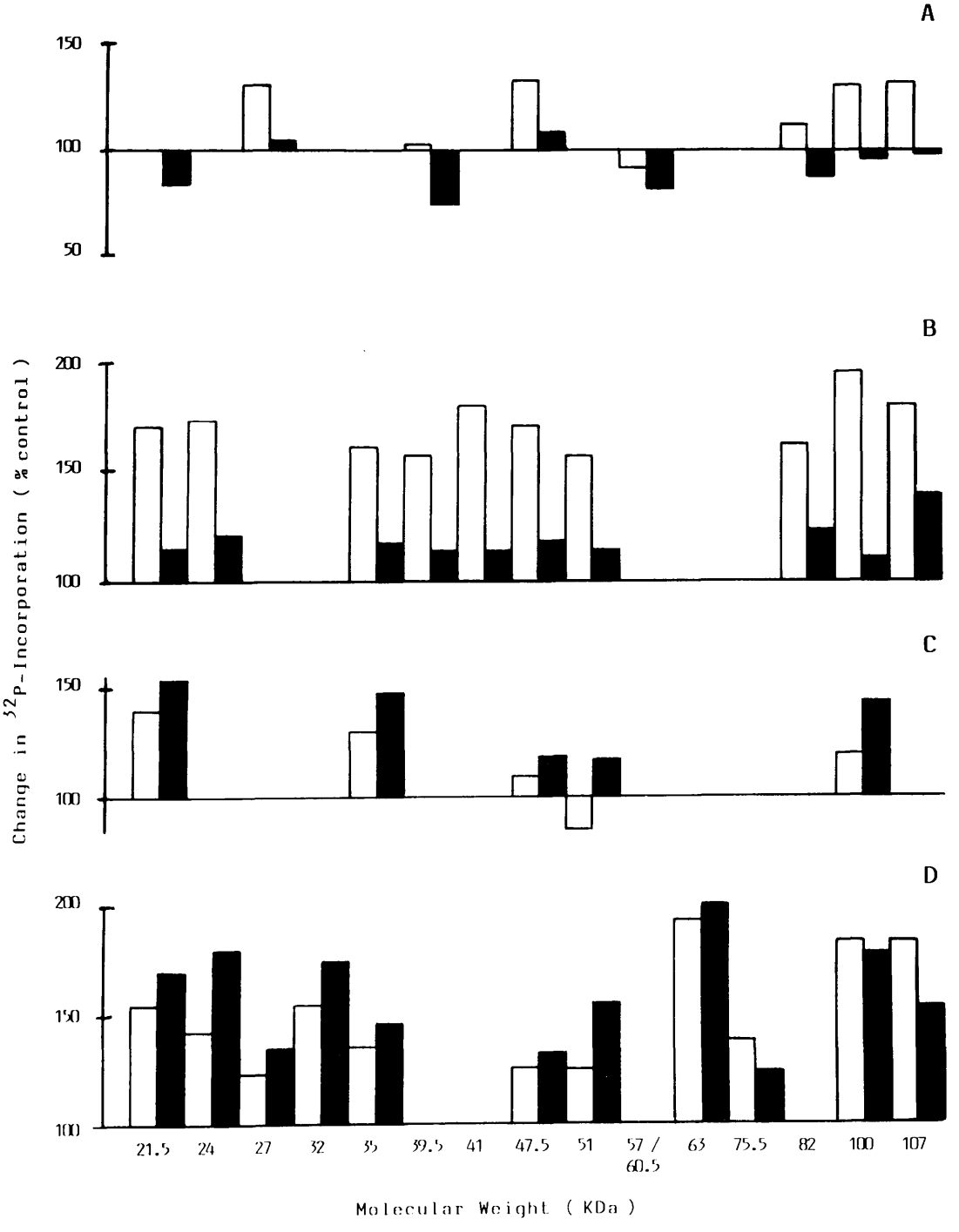
Incubation of mammary cell fractions with polyamine led to changes in the amount of ^{32}P incorporated into many of the phosphoproteins identified by autoradiography. The pattern of polyamine associated changes in mammary protein phosphorylation was complex and the major phosphoproteins of each subcellular fraction appeared, in general, to show properties characteristic of the fraction in which they resided (Figure 34). Indeed all proteins common to two or more different fractions displayed characteristics of polyamine response apparently much more dependent on the fraction with which they were associated than the nature of the individual proteins themselves.

With the possible exception of two mitochondrial fraction phosphoproteins (molecular weights 47kDa and 51kDa), all membrane proteins exhibited obvious increases in phosphorylation in the presence of polyamine but only four of the eight cytosolic phosphoproteins analysed for ^{32}P content showed any such response. In the 'nuclear' fraction, spermidine was generally a more effective stimulator of protein phosphorylation than spermine with only two proteins (molecular weights 75.5kDa and 107kDa) showing convincing reversal of this ranking (Figure 34A). A similar pattern was evident in the major phosphoproteins of the 'mitochondrial' fraction (Figure 34B) except that the degree of enhancement of phosphorylation was, with the exception of a 35kDa protein, lower than that displayed by the corresponding protein bands in electrophoretic separations of the

Figure 34

Samples of mammary cell 'cytosolic' (A), 'microsomal' (B), 'mitochondrial' (C) and 'nuclear' (D) fractions, prepared as described in section 2.5, were incubated, under the conditions described in section 2.6, in the presence or absence of spermine (unshaded columns) or spermidine (shaded columns), both at final concentrations of 1mM. Changes in ^{32}P-incorporation into proteins contained in the samples were assessed as described in section 2.7. Proteins corresponding in M_r to the subunits of acetyl-CoA carboxylase (240kDa) and ATP-citrate lyase (116 kDa) underwent only a very small degree of phosphorylation that could not accurately be quantitated in these kind of experiments. Data are means of duplicate incubations from a single experiment and are representative of a pair of separate experiments.

Figure 34. Effect of Polyamines on Protein Phosphorylation in Subcellular Fractions Prepared from Rat Mammary Acini



'nuclear' fraction. Two of the proteins associated with this fraction (molecular weights 47kDa and 51kDa) showed no increase in phosphorylation as a result of exposure to spermine and only a marginal increase in response to spermidine. In complete contrast, phosphorylation of all the major phosphoproteins residing in the 'microsomal' fraction was much more sensitive to spermine than spermidine (Figure 34C). While the increase in phosphorylation induced by spermine ranged in magnitude from 157-196% control, that engendered by incubation with spermidine was generally less than about 120% and reached a maximum of only 139% control. The major 'cytosolic' phosphoproteins may be divided readily into two groups; those whose phosphorylation was stimulated by spermine and those whose phosphorylation showed little or no response to spermine (Figure 34D). The degree of stimulation within the former group was remarkably consistent, presenting a mean increase ± SE of 131 ± 1% control. The lack of effect of spermidine on ^{32}P-incorporation into this group of proteins was equally consistent, displaying a mean ± SE of 101 ± 3% control. Not only did polyamine fail to stimulate phosphorylation of the second group of cytosolic proteins but spermidine actually elicited a modest reduction in control ^{32}P-incorporation into these proteins.

3.3.4. cAMP-Dependent Protein Kinase

No significant effects of cAMP on protein phosphorylation in any of the three membrane fractions were elicited by the inclusion of Bt_2cAMP (final incubation concentration 1μM) in incubations performed as described in section 2.6 but at least two cytosolic phosphoproteins (molecular weights 47kDa and 57.3kDa) showed enhanced

incorporation of ^{32}P following incubation under such conditions (Figure 35). Although small increases in phosphorylation, in the presence of Bt_2cAMP (or cAMP/IBMX), were observed for all the protein bands studied in cytosolic fractions (e.g. 24kDa "control" band in Table 15), only the 47kDa and 57.3kDa proteins consistently showed marked (usually over 2-fold) increases in phosphorylation under these conditions. Cyclic AMP itself was an equally effective stimulator of ^{32}P-incorporation into these proteins both in the presence and absence of IBMX (added to inhibit endogenous cAMP-PDE activity) (Table 15). The Bt_2cAMP dose-response relationships for phosphorylation of the 47kDa and 57.3kDa proteins both in the presence and absence of EGTA, are shown in Figure 36. As noted above, these two phosphoproteins were the only ones whose phosphorylation was consistently enhanced markedly by inclusion of Bt_2cAMP in mammary acinar cytosol incubations, however, a third phosphoprotein (molecular weight 38.4kDa), displaying similar Bt_2cAMP dose response characteristics was occasionally revealed in electrophoretic separations obtained from incubations in the absence of EGTA (Figure 37). While a 38.4kDa phosphoprotein was also readily identifiable in electrophoretic separations of cytosols incubated in the presence of EGTA (i.e. in the absence of endogenous Ca^{2+} and Mn^{2+}), under these conditions there was no indication of its phosphorylation being cAMP-dependent. Although the apparent absolute requirement for endogenous divalent cation characterising cAMP-dependent phosphorylation of the 38.4kDa protein was not displayed by ^{32}P-incorporation into the 47kDa and 57.3kDa protein, the responsiveness of phosphorylation of the latter two cytosolic proteins to stimulation by Bt_2cAMP was enhanced markedly when EGTA was omitted from the incubation buffers (Figure 36).

Table 15 Comparison of the effects of cAMP (in the presence or absence of IBMX) and Bt_2 cAMP on ^{32}P-incorporation into three cytosolic proteins

Incubation additions	^{32}P-incorporation (% control ie no additions) 24kDa	47kDa	57.3kDa
None	100	100	100
Bt_2 cAMP (1μM)	115	178	224
Bt_2 cAMP (10μM)	102	204	277
cAMP (1μM)	93	158	218
cAMP (10μM)	115	297	266
cAMP (1μM) plus IBMX (1mM)	120	204	238
cAMP (10μM) plus IBMX (1mM)	134	224	285

Rat mammary acinar cytosols were incubated, in the absence of EGTA, as described in Materials and Methods (section 2.6). Incubation additions (final concentrations in parentheses) were as indicated and values given are means of duplicate incubations from a single experiment. Values for ^{32}P-incorporation into a cytosolic protein of molecular weight 24kDa are presented as an example of cAMP-independent phosphorylation.

Figure 35

The proteins contained in samples of mammary acinus 'cytosol', prepared as described in section 2.5 and incubated with [γ-^{32}P]ATP as described in section 2.6, in the presence (+cAMP) or absence (-cAMP) of Bt_2cAMP (1μM) as indicated, were resolved electrophoretically on 8% (w/v) polyacrylamide SDS gels. Autoradiograms representative of 4 separate experiments are shown alongside a corresponding Coomassie blue-stained gel.

Figure 35. Effect of Dibutyryl cAMP on Protein Phosphorylation in Mammary Acinar Cytosols

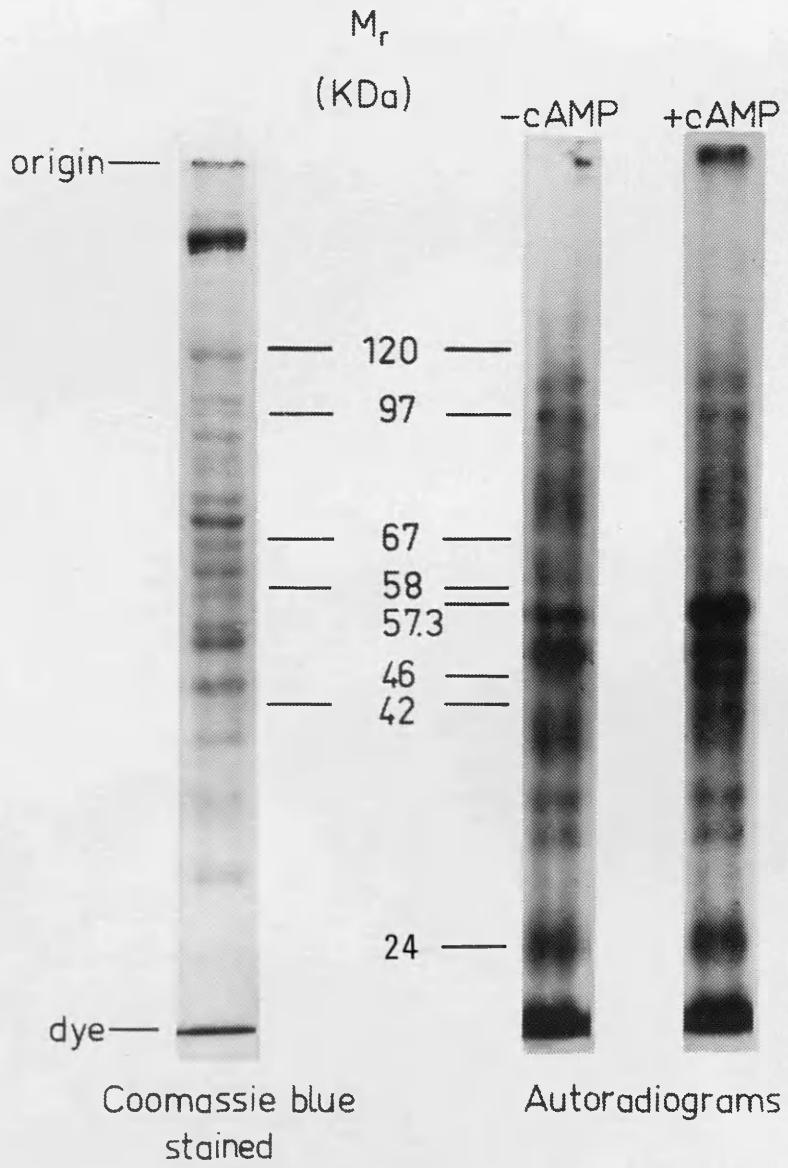


Figure 36

Samples of mammary acinus 'cytosols' prepared as described in section 2.5, were incubated under the conditions described in section 2.6, at a range of Bt_2cAMP concentrations, in the presence (A) or absence (B) of EGTA (2mM). Cyclic AMP-dependent phosphorylations were quantified as described in section 2.7. Data represent means of duplicate incubations from two separate experiments and relate to proteins of molecular weights 57.3kDa (circles) and 47kDa (squares).

Figure 36. Change in ^{32}P-Incorporation into Two Cytosolic Proteins as a Function of dibutyryl cAMP Concentration , in the Presence (A) or Absence (B) of EGTA

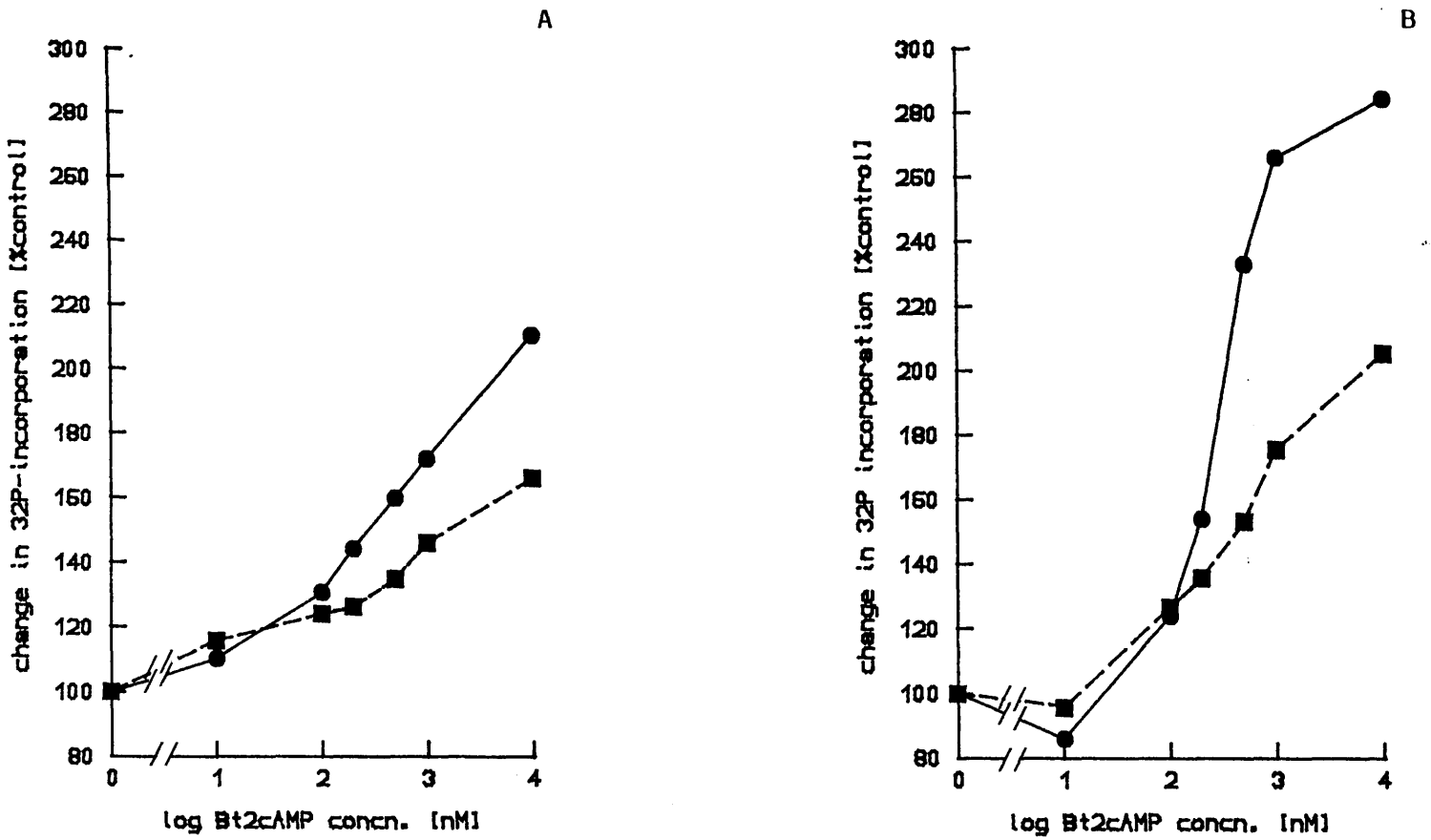
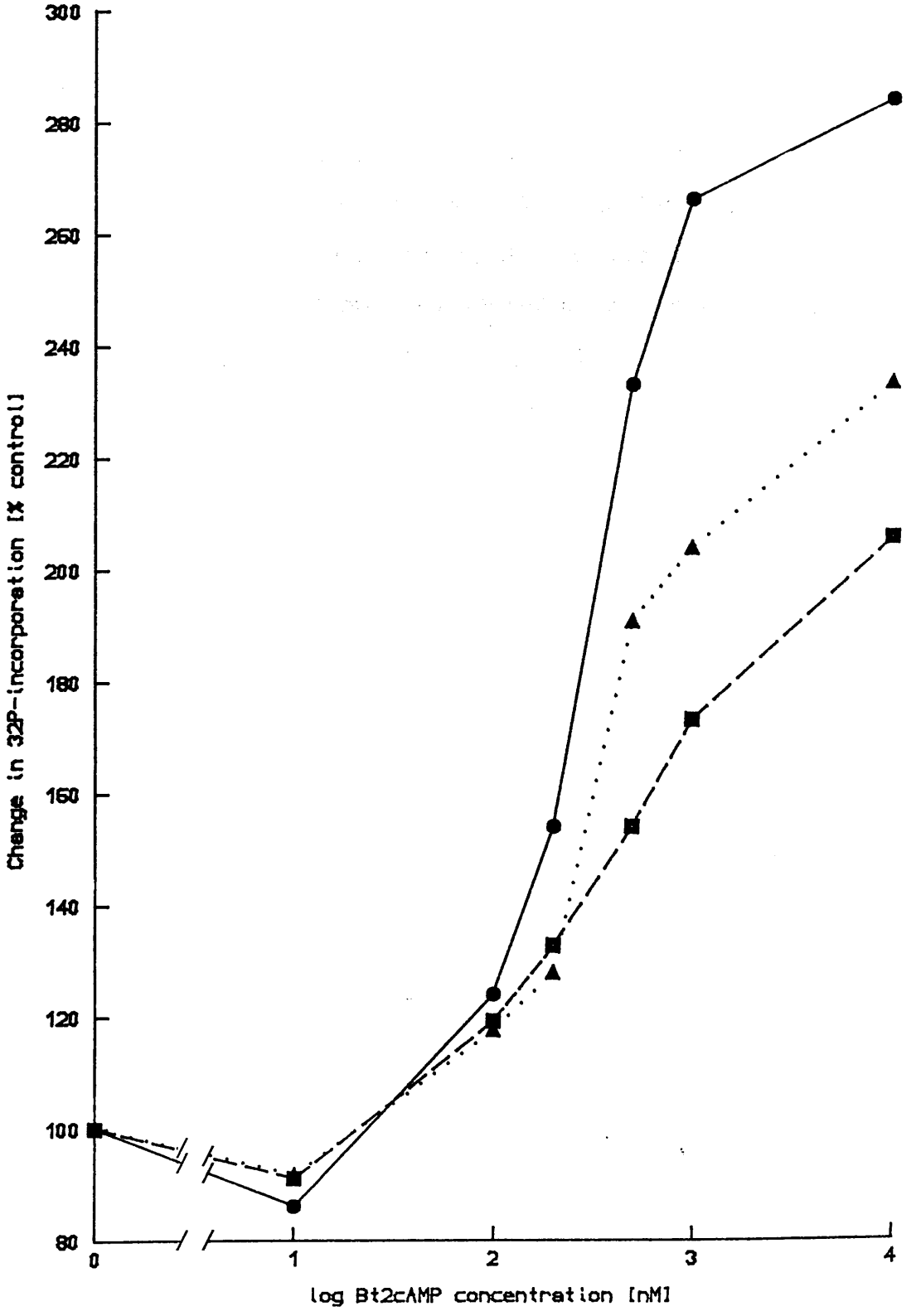


Figure 37

Samples of mammary acinus 'cytosols' prepared as described in section 2.5, were incubated, under the conditions described in section 2.6, at a range of Bt_2cAMP concentrations, in the absence of EGTA. Data points represent means of duplicate incubations from a single experiment and relate to a protein of molecular weight 38.4kDa (triangles). Data for proteins of molecular weights 57.3kDa (circles) and 47kDa (squares) are redrawn from Figure 32, for comparison.

Figure 37. Change in ^{32}P Incorporation into Three Cytosolic Proteins as a Function of dibutyryl cAMP Concentration



In view of the pronounced reduction in background labelling observed in electrophoretic gels derived from Bt_2cAMP dose-response incubations in the absence of EGTA (Figure 38) and the associated substantial decrease in radioactivity measured in specific protein containing bands of such gels, including the 38.4, 47 and 57.3kDa proteins (Figure 38), it is probable that the apparent increase in responsiveness of cAMP-dependent phosphorylation expressed in the presence of endogenous divalent cation reflects an increase in the sensitivity of the experimental techniques to changes in the phosphorylation of specific proteins, rather than necessarily implying an additional (Ca^{2+}- or Mn^{2+}-dependent) regulatory property of the cAMP dependent phosphorylation system(s).

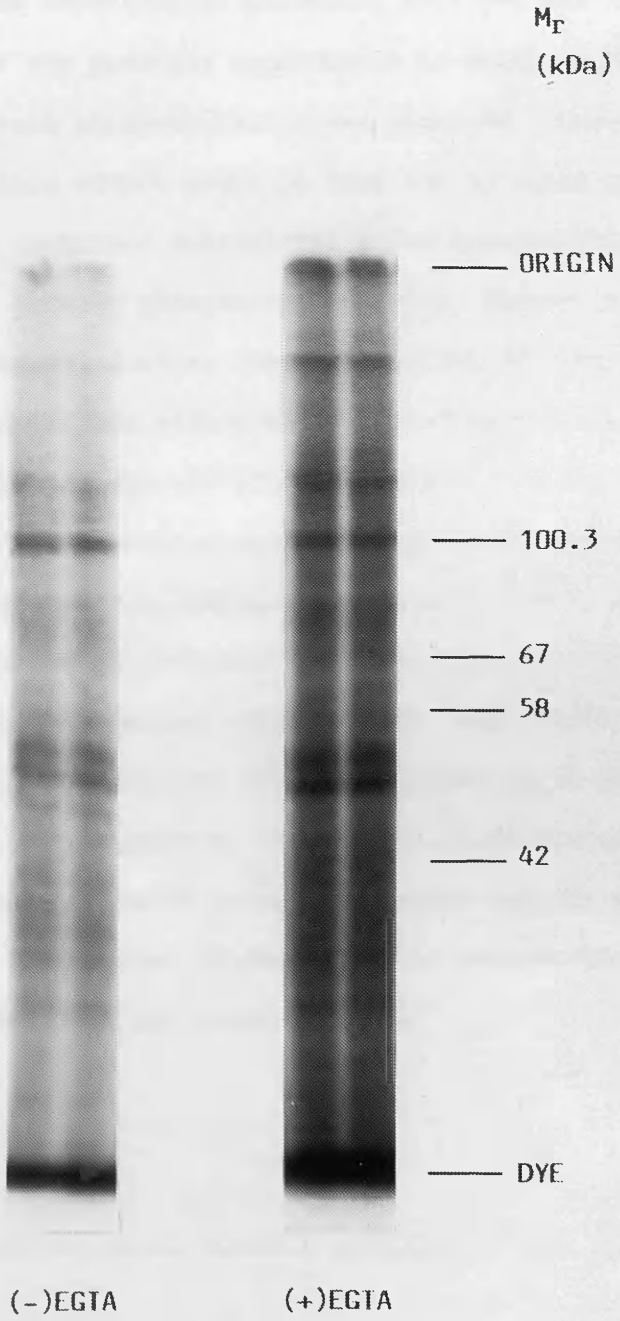
The half maximally effective concentration for the stimulation of ^{32}P-incorporation by Bt_2cAMP observed with these three phosphoproteins is difficult to assess due to the lack of a reliable figure for the maximally effective Bt_2 cAMP concentration. However, all the dose-response curves showed maximum sensitivity to changes in Bt_2cAMP concentration between the values of 0.1 and 1μM and were apparently unaffected by the presence of endogenous divalent cations (except, as noted above, for the 38.4kDa protein) (Figures 36 and 37).

Unlike previous experiments in which omission of EGTA from incubation buffers resulted in marked increases in the phosphorylation of a 100.3kDa protein (see section 3.3.1. and Figure 33), in these experiments, phosphorylation of the 100.3kDa protein appeared, if anything, to decrease marginally in the absence of EGTA (Figure 38). However, bearing in mind the profound decrease in phosphorylation observed for all other proteins in cytosol fractions

Figure 38

The proteins contained in samples of mammary acinus 'cytosol', prepared as described in section 2.5 and incubated under the conditions described in section 2.6, in the presence and absence of EGTA (2mM) as indicated, were resolved electrophoretically on 8% (w/v) polyacrylamide SDS gels. Autoradiograms representative of two separate experiments are shown.

Figure 38. Effect of EGTA on Protein Phosphorylation in Mammary Acinar Cytosols



under these conditions, the much less marked effect, on phosphorylation of the 100.3kDa protein (Figure 34) is, in effect qualitatively consistent with previous results. Why the absence of EGTA should have had such a pervasive effect in later experiments is unclear, since the experimental procedure employed was identical to that followed for the previous experiments in which no discernable reduction in protein phosphorylation was observed. One possible explanation for this effect might be that the cytosols prepared in the later series contained substantial broad specificity divalent cation-dependent protein phosphatase activity, absent in previous preparations, however, further characterisation of this putative divalent cation-dependent effect was hampered by its poor repeatability. Further speculation along this or other lines could not therefore be substantiated and is arguably unnecessary since the important finding was that, whether in the presence or absence of EGTA, phosphorylation of at least two cytosolic proteins varied, in a classic dose-dependent manner, with Bt_2cAMP concentrations. Furthermore, while the results for Ca^{2+}-dependence of phosphorylation of the 100kDa protein appear at face value, to be contradictory, as noted above, when considered in context, these results are in fact consistent with the earlier finding that the phosphorylation of this protein was enhanced in the presence of Ca^{2+}.

4. DISCUSSION

4.1. Insulin Sensitive High Affinity cAMP-phosphodiesterase

4.1.1. Adipose Tissue

High affinity cAMP-PDE activity measured in adipose tissue at substrate concentrations of 10μM [491] and 0.45μM [878] has been reported to be 4420pmol/min/g dry wt. and 1153.6pmol/min/g lipid respectively. Expressing the activity observed in adipocyte homogenates in this study in terms of adipocyte dry weight which, for such cells, is also a reasonable approximation to lipid weight gives a value of 476.3pmol/min/g dry wt. While, at first sight, this figure appears low in comparison, it represents PDE activity at a cAMP concentration of only 0.2μM and is therefore probably in reasonable agreement with the value of 1153.6pmol/min/g lipid reported by Zinman and Hollenburg [878]. Comparison with the higher value reported by Loten and Sneyd at a cAMP concentration of 10μM [491] is difficult because of the increasingly large, indeterminate contribution made by low affinity activity as substrate concentration is increased but, once again, allowing for such factors, these results may also be considered broadly comparable. Under various incubation, homogenisation and assay conditions, insulin stimulation of adipocyte high affinity cAMP-PDE activity in whole cell preparations has been reported to be 121% control [491], 140% control [506] and 140-150% control [878] at insulin concentrations of 800 U/ml, 1mU/ml and 145μU/ml respectively. The figure of 146% increase over control activity reported here in adipocytes exposed to 1mU/ml insulin is therefore consistent with the upper end of the range of expected values. Although activations of this order may seem modest, it must be remembered that they are

observed under conditions where the activity of insulin insensitive enzyme is likely to make a substantial contribution to the measured high affinity cAMP-PDE activity irrespective of the substrate concentration at which it is assayed. Simply by preparing a crude membrane fraction, much of the interfering activity (confined, as it is, largely to the cytosol) may be selectively removed, allowing detection of a stimulation approaching 2-fold in response to insulin [878] though Kono et al have been able to demonstrate a 2-3 fold activation in both unfractionated homogenates and crude endoplasmic reticulum preparations [429,502,803].

The compatibility of the results presented in this report with previously documented hormonal effects on the activity of adipose tissue high affinity cAMP-PDE validates the procedures employed and allows extension of the investigation to tissues such as mammary gland whose characteristics, with respect to such effects, have not previously been determined.

4.1.2. Mammary Gland

Techniques used to study the effect of insulin on adipose tissue high affinity cAMP-PDE were successfully applied to mammary gland but while the results obtained demonstrated that the mammary enzyme displays similar insulin-dependent regulatory properties to the corresponding PDE systems in adipose tissue and liver, neither tissue appears to provide a comprehensively consistent model for insulin-dependent high affinity cAMP-PDE activity in mammary cells.

4.1.2.(i) Sulphydryl and Proteolytic Effects

Activation, by dithiothreitol (DTT), of endogenous protease activity, apparently analagous to lysosomal thiol protease activity observed in liver [490,673] and kidney [742], has been demonstrated in a membrane fraction isolated from adipocytes [502,748]. Proteolytic activation of high affinity cAMP-PDE either by trypsin or endogenous protease(s) has been described in a number of tissues including rat kidney cortex [742,743], rat uterus [193], adipose tissue [501,502] and liver [490]. Since the buffer in which particulate fractions were resuspended in this study contained the thiol reagent β-mercaptoethanol (1.1mM), an activation of endogenous protease similar to that observed in other tissues might be expected to occur during storage of the mammary membrane fraction, leading to proteolytic activation of the mammary high affinity cAMP-PDE. Proteolytic activation therefore provides an attractive explanation for the increase in high affinity cAMP-PDE activity observed during storage of the mammary acinar particulate fraction used in this study. However, activation of the mammary enzyme appears to be more rapid than that observed for the membrane bound high affinity cAMP-PDE of adipose tissue and less susceptible to inhibition by protease inhibitors such as antipain and leupeptin [502].

The loss of insulin-stimulated activity seen here during storage of particulate fractions at 0-4°C in the presence of β-mercaptoethanol is similar in nature to that observed in adipocyte membranes exposed to DTT ([501,502], see section 3.1.2.(ii)) and might be supposed to arise from reduction of S-S groups necessary for stability of insulin-stimulated activity as proposed by Kono and co-workers [501]. However, the percentage activation was preserved (absolute activation

increased) during storage when particulate fractions were suspended in an inhibitor cocktail containing PMSF, leupeptin, pepstatin, antipain, trypsin inhibitor, ε-amino caproate, benzamidine, EDTA, EGTA and KF. Besides preventing DTT-stimulated activation of membrane bound high affinity cAMP-PDE, protease inhibitors such as antipain, leupeptin, pepstatin, chymostatin, E-64, DFP and TLCK have been shown to alleviate, partially, the suppression of insulin-induced activation imposed by the presence of DTT during storage of adipocyte particulate fractions at 4°C [502]. Such interactions, which involve an increase in both absolute and percentage insulin activations with respect to their values in the presence of DTT alone, are broadly consistent with the above observation in mammary tissue that protease inhibitors maintained per cent activation while provoking an increase in the absolute values for insulin-induced stimulation of high affinity cAMP-PDE activity. With respect to their influence on the activation of non-insulin-stimulated PDE observed during storage at 0-4°C in the presence of reducing agents (DTT or β-mercaptoethanol), however, these inhibitors were less effective in the mammary membrane system than in the analogous adipocyte system. Whether this implies that other, non-proteolytic processes are involved, or whether the endogenous mammary protease is simply less sensitive to these inhibitors is a matter for conjecture.

It has been suggested that limited proteolysis, resulting in solubilisation and activation of membrane bound PDE catalysed by an endogenous protease may represent an important mechanism for regulation of PDE activity [227]. This being so, the putative protease of mammary tissue which appears to be much more active than its counterpart in adipose tissue, would seem, potentially, to provide an effective mediator of such a regulatory mechanism.

4.1.2.(ii) Effects of Cycloheximide, 24hour Starvation and Diabetes

Rather than inhibiting the stimulation, by insulin, of mammary high affinity cAMP-PDE activity, cycloheximide actually enhanced this response. The major inference from this observation was that insulin stimulated synthesis of PDE enzyme molecules could not account for the increase in PDE activity detected in response to insulin. However, it does not necessarily follow that the enhancement of insulin activation provoked by exposure to cycloheximide is, therefore, entirely independent of its ability to block protein synthesis. Nevertheless, in order to propose an explanation based on such a premise, it would be necessary to construct mechanisms for which there is apparently little supporting evidence in the literature. As an illustration, one possible explanation would be that cycloheximide inhibits synthesis of a protein-antagonist of PDE activation. Under Houslay's scheme for PDE regulation via G-proteins analogous to those of the adenylate cyclase system (see Section 1.1.), a possible identity for this putative protein inhibitor might be envisaged to be the PDE G-protein corresponding to G_i. However, this suggestion would require a disparity in either regulatory potency or synthetic turnover rate between stimulatory and inhibitory G-proteins. Furthermore, if the action of these putative G-proteins was also analogous to their adenylate cyclase counterparts, diminution of the effect of the G_i analogue relative to the G_s analogue would result in an increase in control (unstimulated) high affinity cAMP-PDE activity. This was not observed (Table 2) and there is apparently little evidence in the literature to suggest the existence of any other system capable of modulating the hormone sensitivity of PDE in a manner consistent with

these proposals. The alternative, i.e. that this effect of cycloheximide is not expressed via its well-documented ability to inhibit protein synthesis, is therefore perhaps more promising. The enhanced response to insulin apparently displayed by PDE in mammary cells from diabetic or 24 hr starved rats (preliminary results, Table 5) is, in common with the effect of cycloheximide treatment of acini, unlikely to result from synthesis of extra enzyme molecules. Bearing in mind this and other similarities between the observed effects of cycloheximide and those revealed by preliminary results with manipulation of physiological state, it is tempting to speculate that the mechanisms involved might be related. However, in the absence of corroborative data for the latter results, it would be premature to discuss their implications further.

Treatment with cycloheximide appears to allow expression of an insulin-induced activation of PDE in unfractionated homogenates otherwise devoid of detectable insulin-sensitive activity. The obvious conclusion is that insulin-sensitive activity is not confined to the crude particulate fraction isolated in these experiments and that cycloheximide potentiates the effect of insulin on either a soluble high affinity cAMP-PDE or one associated with a less rapidly-sedimenting membrane fraction than that collected under the centrifugation conditions employed in this study. In view of the lack of supporting evidence in the literature for soluble insulin-sensitive PDE activity, a light microsomal membrane fraction might be supposed to represent the most likely source of latent insulin-sensitive PDE activity of this kind. While this model for the effect of cycloheximide on the observed distribution of insulin-sensitive PDE activity in mammary acini provides a convenient explanation for

insulin-stimulated activity not directly attributable to the activation observed in particulate fractions, it does not represent the only possible interpretation of the available information. Perhaps a more attractive proposition would be to assume that all insulin-sensitive cAMP-PDE activity is confined largely to membranes constituting the particulate fraction but that activation of this enzyme renders it more susceptible to dissociation, releasing a fully active soluble catalytic domain from its membrane-associated regulatory domain, in accordance with the molecular arrangement proposed for insulin-sensitive PDE by Francis et al ([227, 490] see section 1.2.2.(ii)). In support of this, the insulin-stimulated form of high affinity cAMP-PDE in liver endoplasmic reticulum appears to be more sensitive than the basal form to solubilisation by an endogenous protease [490]. The corresponding effect in mammary tissue might therefore be envisaged to be mediated by the endogenous protease activity suggested to reside in mammary acinus particulate fractions (see section 4.1.2.(i)).

Inclusion of cycloheximide in the acinus incubation medium reveals an insulin-dependent stimulation of PDE activity in unfractionated homogenate which cannot be accounted for entirely by the activation expressed by the particulate fraction alone. Indeed, the particulate fraction activation may represent as little as 27% of the absolute increase observed in unfractionated homogenate under these conditions. That cycloheximide does not simply activate the putative endogenous protease is demonstrated, in cycloheximide-treated acini, by the lack of a decrease in insulin-stimulated activation of particulate fraction PDE if not by the fact that this effect would result, predominantly, in a redistribution, rather than overall activation of

PDE activity. The latter statement would not necessarily hold true if a significant proportion of unstimulated PDE molecules were solubilised by the putative protease since such solubilisation has been reported to induce up to 4-fold activation of the adipocyte insulin-sensitive PDE [502]. However, under these circumstances, an increase in the PDE activity of non-insulin-treated acini would be expected following incubation with cycloheximide: this effect is not observed. To account for the lack of such a response it would be necessary to propose a secondary effect of cycloheximide capable of suppressing this type of activation in control but not insulin-stimulated acini. Basically the same arguments may be used to dismiss the possibility that changes in the sensitivity to proteolytic attack of the insulin-stimulated form are responsible for the observed effects. Instead, it must be supposed that cycloheximide is capable of augmenting the insulin-dependent stimulation of membrane-bound PDE and that it is the increase in amount of activated enzyme that stimulates increased solubilisation rather than a direct effect of cycloheximide on the proteases, proposed to govern solubilisation. To account for the results presented here, the cycloheximide-stimulated increase in insulin-sensitive activation of particulate fraction PDE must then be assumed to be accompanied by an increase in proteolytic solubilisation of activated PDE sufficient to limit severely if not to obscure completely the cycloheximide-induced enchancement of particulate enzyme activation in response to insulin. The tendency towards a residual cycloheximide-dependent enhancement of the insulin response in the particulate fraction, suggested by the values in Table 2, both in terms of absolute values and per cent control values (except when expressed per Unit 5'nucleotidase activity) is therefore consistent with these proposals.

It is perhaps premature to present detailed proposals outlining possible in vivo justifications for the existence of an arrangement capable of influencing the subcelluar distribution of high affinity cAMP-PDE in the manner described above. However such a regulatory system might be imagined to represent either a means of limiting the amount of PDE activity expressed at the membrane in order to prevent excessive deactivation of membrane associated cAMP-dependent processes or a mechanism capable of allowing dissemination of a membrane associated effect throughout the cell.

Protein synthesis inhibitors such as cycloheximide and puromycin have been shown to block hormone-induced increases in phosphatidyl inositols but not diacylglycerol [206]. This effect is thought to be a consequence of rapid enzymic hydrolysis of phospholipids induced by cycloheximide, particularly in hormone-stimulated tissues but while this leads to a decrease in the mass of phosphatidyl inositol, it is followed by a secondary increase in phosphate incorporation into phospholipid so that, in effect, cycloheximide treatment precipitates an increase in phospholipid turnover [205]. Since changes in phosphatidyl inositol metabolism have been implicated in the mechanism of action of insulin [205, 325, 412, 494, 495], it is not surprising to find evidence for interaction between cycloheximide and insulin in the activation of high affinity cAMP-PDE. Phosphatidyl serine (PS) and, to a lesser extent, phosphatidic acid (PA) have been shown to activate microsomal high affinity cAMP-PDE in rat adipocytes, while phosphatidyl inositol 4 phosphate (diphospho inositide, Ptd Ins 4P or PIP) inhibits the activity of this enzyme [494, 495]. Insulin has been reported to raise cellular levels of phosphatidyl inositol (PI), PIP, phosphatidyl inositol 4,5 bisphosphate (triphosphoinositide, Ptd Ins 4,5 P_2 or

PIP_2), PS and phosphatidyl ethanolamine [205] and therefore generates two potential stimulators of PDE activity (PA and PS). However, the synthesis of PIP, an inhibitor of PDE activity, is also increased by insulin and therefore any stimulatory effects of insulin on high affinity cAMP-PDE activity mediated by this mechanism will be at least partially antagonised. Perhaps the observation that cycloheximide, which blocks hormonally-stimulated increased in a number of phosphatidyl inositols (including PIP), PS and PA but cannot depress their levels to below control [205], appears to potentiate insulin activation of mammary high affinity cAMP-PDE, might be construed to imply that the mammary enzyme is more sensitive to inhibition by PIP than activation by PS or PA. In the context of the proteolytic solubilisation model proposed earlier, the fact that enhanced insulin activation is only apparent in unfractionated homogenates (when this reasoning would seem to suggest that the degree of activation should also be elevated in the particulate fraction) might be explained by assuming that under normal circumstances, the effect of insulin on mammary membrane-bound PDE is to a certain extent, self limiting, by virtue of the inhibition imposed by PIP. In the presence of cycloheximide, this inhibition would be abolished and insulin-induced activation of membrane-bound PDE would be enhanced as a result. However, the increased activation effectively represents an increase in the proposed preferred substrate of the endogenous protease and would therefore lead to an increase in proteolytic solubilisation. The resulting increased loss of membrane bound PDE would then limit the increase in activation apparent in the particulate fraction, while adding to that expressed in the cytosol, which would then be registered as an increase in insulin-dependent activation of PDE observed in unfractionated homogenate.

The observation that cycloheximide either completely inhibits insulin receptor down-regulation [602] or, at least, markedly decreases the rate and extent of such insulin-induced down-regulation [431, 643] may also have some bearing on the apparent synergism between insulin and cycloheximide. It has been reported, recently, that cycloheximide does not inhibit insulin receptor cycling but does block receptor inactivation [426], perhaps, therefore allowing an enhanced or prolonged response to insulin and conceivably contributing to the potentiation of insulin-induced activation of mammary high affinity cAMP-PDE observed here in the presence of this agent.

4.1.2. (iii) Membrane Incubations

Having tentatively identified membrane-bound high affinity cAMP-PDE as the major source of insulin-dependent PDE activity in mammary acini, in order to characterise the hormone sensitivity of this enzyme further, a broken cell system was developed, similar to that used previously in rat hepatocytes for investigation of the corresponding effect of insulin on liver PDE [511]. The failure to demonstrate any increase in membrane-bound high affinity cAMP-PDE activity following insulin-treatment of a crude membrane fraction *in vitro* under conditions known to elicit such a response in rat hepatocyte membranes [511] seems to suggest that the mammary high affinity cAMP-PDE system is more closely related to that of adipose tissue in which it has not yet proved possible to demonstrate hormone-induced activation in anything other than in intact cell preparations. Assuming the mammary and adipose PDE systems are indeed comparable and that, as in adipose tissue, mammary insulin-sensitive

PDE resides in membranes of the endoplasmic reticulum, it seems unlikely that the relatively direct activation mechanism, via a unique GTP-binding modulator protein (G- or N-protein) proposed by Houslay and coworkers [310, 311, 312] to mediate in the activation of the plasma membrane-bound insulin sensitive PDE of liver (see section 1.2.2.(ii)) is involved in activation of the mammary enzyme. Insulin-dependent activation of liver plasma membrane-bound PDE appears to be achieved, ultimately, by phosphorylation of the enzyme [511] and while the intermediate mechanisms of signal transduction involved in activation of mammary PDE may differ from those expressed in hepatocytes, the possibility that mammary PDE activity is also influenced by phosphorylation should not be ignored. Therefore in order to optimise any phosphorylation-mediated effects involved in insulin-dependent activation of mammary PDE, ATP and the phosphatase inhibitor, KF were added to the mammary cell-free incubation system and comparison of ATP- and/or KF-containing incubations with appropriate controls revealed an interesting relationship between these agents and the observed PDE activity. The initial observation that addition of KF to membrane incubations resulted in an apparent divalent cation-dependent activation of membrane-associated PDE and that simulataneous addition of ATP would prevent expression of this effect was intriguing and seemed to point to the possibility of a regulatory mechanism involving protein phosphorylation, though not, perhaps, in a straightforward, conventional manner since ATP would not normally be expected to antagonise the effect of a phosphatase inhibitor (but see later). That this effect was not simply due to chelation of divalent cation by ATP was demonstrated by the dose response relationship for antagonism of the KF effect. As discussed earlier

(Section 3.1.3.), ATP was effective in this respect at concentrations well below those required for significant chelation of the divalent cation present in the incubations. The discovery that GTP, ADP and even pyrophosphate are all effective substitutes for ATP would seem to preclude an explanation for antagonism of the KF effect involving increases in protein kinase activity. Therefore, in order to preserve the notion of a mechanism involving phosphorylation, it would be necessary to propose that mammary tissue contains a protein phosphatase with unusual regulatory properties, whether it is assumed that the apparent activation is provoked by phosphorylation, or conversely, by dephosphorylation. The possibility that the observed effects are mediated by a 'conventional', KF-inhibited protein phosphatase and that the apparent activation is therefore stimulated by phosphorylation may, at first sight, appear to provide a plausible explanation but this proposal would necessitate acceptance of regulatory characteristics not normally associated with such enzymes. Because ATP, ADP, GTP, pyrophosphate and EDTA all antagonise the effect of KF, it would have to be assumed that these agent were capable of either activating the putative phosphatase or endowing it with substantial insensitivity to inhibition by KF. Although ATP-dependent activation of a phosphatase (protein phosphatase 1_I, see section 1.3.9.(i)) has been demonstrated in other tissues [24, 305, 375, 805] there is no precedent for phosphatase activation in response to ADP, GTP, pyrophosphate or EDTA, indeed, pyrophosphate is normally considered to be a phosphatase inhibitor. The observation that NaF, a phosphatase inhibitor often substituted for KF, is considerably less effective than KF in raising particulate fraction PDE activity also runs contrary to expected phosphatase behaviour.

In order to accommodate the alternative proposal that dephosphorylation stimulates the increase in particulate PDE activity, the protein phosphatase responsible for this effect would be required to display activation by KF and inhibition by ATP, ADP, GTP, pyrophosphate and EDTA (Table 10). However, even assuming such a phosphatase exists, in the absence of protein kinase activity (i.e. all incubations except, perhaps, those containing ATP), it is difficult to account for the ability of EDTA not only to prevent the increase in particulate PDE activity induced by KF but also to return KF-stimulated activity to near control levels (Table 7). In conclusion, then, bearing in mind the degree of anomolous behaviour required in order to satisfy the conditions imposed by experimental data, it seems extremely unlikely that phosphatase activity of any description could provide a plausible explanation for the effects of KF on particulate PDE activity.

Although experimentation designed to characterise further the effects of KF on this membrane system demonstrated that the apparent KF-induced activation of membrane associated high affinity cAMP-PDE described above was, in fact, wholly attributable to a redistribution of soluble PDE activity (present as a contaminant of the membrane system) to the particulate fraction (Figures 23, 24 and 25, Table 11) rather than activation of existing particulate enzyme, the reasoning employed to rule out participation of protein phosphorylation in this process is equally applicable to the revised model for KF-induced increases in particulate PDE activity and leads to the conclusion that the phosphorylation state of mammary high affinity cAMP-PDE is not an important factor determining whether the enzyme exists in a soluble or sedimentable (particulate) form. The observation that, unlike the

activation of hepatocyte plasma membrane insulin-sensitive PDE, under the experimental conditions chosen for this study, there are no obvious phosphorylation-mediated effects on mammary high affinity cAMP-PDE activity is perhaps not surprising since no effects of this nature have yet been described for either the 'dense-vesicle' insulin-sensitive PDE of rat hepatocytes or the insulin-sensitive PDE of adipose tissue (see section 1.2.2.(ii)).

The inhibitory effect of fluoride ion on phosphatase activity and its ability to activate adenylate cyclase are both well known phenomena with widely recognised applications but the mechanisms involved in these processes remain unclear. Recent work with bovine transducin [382] provides evidence to suggest that activation of guanine nucleotide binding protein by fluoride involves binding of F^- and Al^{3+} to the α-subunit and consequent promotion of its dissociation from $\beta\gamma$-subunits, however while proposed, previously, by Northup et al. [763], this has not yet been verified in the adenylate cyclase system. Perhaps the effect of KF on PDE activity and solubility (KF-induced precipitation of soluble PDE activity appears to be accompanied by a reduction in enzymic activity [see section 3.1.3.) will prove to be related to those involved in activation or inhibition of these other important regulatory enzymes but the complex nature of the interactions involved in the system described here makes assessment of possible mechanisms difficult, beyond suggesting that physical, rather than enzymic reactions are implicated. In this situation, however, documentation of the effects themselves may be of more value to future studies than a detailed assessment, on the molecular level, of reasons for their occurrence. In practical terms, this report of a major

modification of the properties of a soluble enzyme by widely used phosphatase inhibitors, at the concentrations commonly employed for such enzyme inhibition (50-100mM), presents a strong case for caution in interpreting the results of studies designed to measure the relative activities of phospho and dephosphoenzymes using fluoride to preserve the phosphorylated form. In this respect alone, the observations made here are obviously of significant importance to future research but, on a more positive note, KF-induced precipitation may also prove to be a useful preliminary step in purification of certain soluble enzymes and, in relation to the mammary PDE system, it would be particularly interesting to identify the properties of the PDE enzyme(s) isolated by such a procedure.

Effects of salts on high affinity cAMP-PDE activity have been observed by others [501] in adipose tissue. The membrane preparation used by these authors to demonstrate effects similar in nature to those described here was basically the same as that used in this study. Although the adipocyte membrane incubations were performed at 37°C, rather than the 30°C adopted here, and apparent activation observed in the presence of high concentrations (150mM) of KCl was not maximal until approximately 20minutes after initiation (much slower than the effects observed with KF in this study), substantial increases (approximately 2-fold) in high affinity cAMP-PDE activity associated with the crude microsomal fraction were demonstrated under 'optimal' conditions. The incubation additions described as necessary for maximum effect are 150mM KCl and 5mM $MgCl_2$ or $CaCl_2$ but little, if any, effect is observed at an incubation KCl concentration of 100mM. This latter observation is consistent with the results presented here (Table 7) and although the effect noted at higher concentrations of KCl in the

adipocyte membranes is not absolutely dependent on divalent cation, there is evidence of a certain degree of interaction. It is therefore conceivable that the effects observed following incubation of adipocyte membranes with 150mM KCl are analogous to those induced by incubation of mammary cell membranes with 100mM KF.

4.2. Protein Phosphorylation in Mammary Acini

Although various protein kinase activities have already been ascribed to mammary tissue (see section 1.3.10.), a comprehensive study of the effector-dependence of endogenous protein phosphorylation has not, until now, been attempted in this tissue. In order to determine the potential importance of effector-dependent phosphorylation and, in particular, the role of cAMP-PrK, in the regulation of mammary cell metabolism, it is first necessary to establish whether a competent combination of effector-dependent kinase and endogenous substrate is expressed in these cells. The data presented in sections 3.2. and 3.3. represent the product of work directed towards addressing this issue and have revealed a number of interesting properties of mammary cell protein phosphorylation.

The relatively slow loss of protein-bound ^{32}P observed following addition of excess unlabelled ATP to cytosolic fraction phosphorylation incubations (for example, see Figure 31) indicates a low rate of protein bound phosphate turnover and, since substantial incorporation of phosphate into protein occurred after as little as 30 seconds, indicating significant protein kinase activity, the implication is that phosphatase activity was comparatively low. Indeed, the level of protein phosphatase activity was probably sufficiently low, in relation to protein kinase activity, to preclude any significant effect on the

level of ^{32}P incorporation measured after 30 seconds. This conclusion is supported by the inability of KF, a potent inhibitor of protein phosphatase activity, to stimulate the rate of incorporation into any of the major phosphoproteins isolated (Figure 32). The reliability of this supporting evidence is, however, subject to certain reservations on the grounds that KF often substantially inhibited the accumulation of protein-bound ^{32}P (Figure 32) suggesting that the influence of KF on ^{32}P-incorporation in this system was not confined to expression of its effect on phosphatase activity. The possibility that this ill-defined inhibitory effect might be capable of masking any stimulation of ^{32}P-incorporation mediated by KF-induced phosphatase inhibition cannot, therefore, be ruled out completely. Nevertheless, the combination of intrinsically low mammary protein phosphatase activity [281] and an incubation buffer containing the protein phosphatase inhibitor β-glycerophosphate might easily give rise to sufficiently low protein phosphatase activity to be consistent with that implied by these experimental findings.

The loss of linearity of ^{32}P-incorporation into cytosolic proteins may be due to saturation of phosphorylation sites or, alternatively, exhaustion of available ATP. The former is difficult to assess but some estimate of the rate of ATP depletion not attributable to kinase activity (the amount of ^{32}P remaining unbound to protein following incubation indicates that protein kinase activity accounts for only a very small fraction of the available ATP) may be derived from ATPase activities reported by others in similar mammary tissue extracts. ATPase activity in mammary Golgi vesicles has been reported to release approximately 50-120 nmol Pi/min/mg protein [806, 828]. The protein content of each incubation in the present work was 0.05mg, so

the maximum contribution of Golgi-associated ATPase activity to the total ATPase activity might be expected to be around 2.5-6.0nmol Pi released/min. Since each incubation contained only ~ 3nmol ATP, these figures would suggest that ATP supply might rapidly become limiting for kinase activity.

This conclusion is supported by ATPase activity data obtained using mammary cell fractions identical to those employed for the protein phosphorylation studies described here [100a]. Although the data refer to activity measured at protein concentrations less than half that used here and are therefore higher than would be expected to prevail under the conditions chosen for the present study (ATPase activity is non-linear with respect to protein concentration), they indicate that substantial ATP depletion almost certainly contributed to the observed loss of linearity of ^{32}P-incorporation into mammary proteins. Indeed, under experimental conditions identical to those chosen for the present study, substantial ATP depletion (~ 50% and ~ 36%, respectively, of ATP supplied initially to the "microsomal" and "cytosolic" fraction incubations) has been demonstrated after 2 minutes of incubation [100a].

The apparent ability of vanadate markedly to stimulate incorporation of label into just two of the major phosphoproteins detected in acinar cytosols incubated with [γ-^{32}P] ATP (preliminary results, see Section 3.3.) is interesting, particularly since one of these is almost certainly acetyl-CoA carboxylase (by virtue of their abundance in mammary tissue, acetyl-CoA carboxylase and fatty acid synthase are identifiable in Coomassie blue-stained SDS polyacrylamide gels) and the other is a 47kDa protein shown to undergo cAMP-dependent phosphorylation (see section 3.3.4.). Vanadate has been shown to

stimulate the tyrosine phosphorylation of two membrane proteins from a human lymphoblastoid cell line [172], an effect thought to have three possible explanations: (i) vanadate inhibits phosphotyrosine dephosphorylation (it has been observed that vanadate inhibits phosphotyrosine phosphatase activities of human A431 cells [749], astrocytoma [469] and prostate gland [473]), (ii) vanadate directly stimulates tyrosine kinase activity, (iii) since vanadate forms complexes with protein [77], it might be capable of modifying substrate conformations to increase their susceptibility to tyrosine phosphorylation. Admittedly, these effects were observed at concentrations of vanadate several orders of magnitude lower than that used in the present study but it is not unreasonable to suppose that similar effects on tyrosine phosphorylation might be observed, even at concentrations many times higher than those required for saturation of such a system.

The definitive method for determining the identity of amino acid residues with which phosphate is associated in a phosphoprotein involves isolation of the phosphoprotein (labelled with ^{32}P) of interest, followed by partial hydrolysis and analysis of the amino acid content of the resulting hydrolysate by, for example, TLC or HPLC. This procedure is described in detail by Cooper et al. [127a] but the method can be summarised as follows. The ^{32}P-labelled phosphoprotein of interest is identified and isolated, for example, by excision from SDS gels, by affinity chromatography (for acetyl-CoA carboxylase this would be achieved using avidin-Sepharose columns) or by immune precipitation and then subjected to partial acid hydrolysis. Both alkaline hydrolysis and conditions necessary for total acid hydrolysis (for example, 5.7M HCl for 18h at 110°C) result in hydrolysis of the phosphate ester linkage of phosphotyrosine in

addition to peptide bonds, and are therefore unsuitable for determination of tyrosine-specific phosphorylation. Consequently, the period of acid treatment is reduced, for example, to 1h [127a], in order to preserve phosphotyrosine. The hydrolysate can then be analysed using one of a number of techniques for separation of amino acids, the phospho residues being identified by their associated radioactivity. Perhaps the most convenient of the techniques available currently is either strong anion exchange or reversed-phase HPLC. However, when dealing with ^{32}P-labelled samples, use of such systems may be impractical due to contamination of expensive chromatographic hardware. Less sophisticated techniques such as TLC or thin layer/paper electrophoresis may therefore be preferred. Use of this general method would establish conclusively whether, under any experimental conditions, either acetyl-CoA carboxylase or the 47kDa protein contains phosphotyrosine residues and therefore whether or not the increased phosphorylation observed in the presence of vanadate was tyrosine-specific. Essentially the same procedure could also be adopted to distinguish serine from threonine-specific phosphorylations.

The results of experiments involving treatment of phosphoprotein-containing gels, of a similar nature to those produced in this study, with 1M KOH at 55°C for 2h (according to a protocol described by Cooper et al. [127a]), suggest that neither the phosphorylations of acetyl-CoA carboxylase, nor that of the 47kDa protein is likely to be tyrosine-specific. Confirmation of these results could be obtained using the partial acid hydrolysis method described above. However, if, as now seems unlikely, the observed stimulation by vanadate of phosphate incorporation into acetyl-CoA carboxylase and the 47kDa protein were proved to be an indication that

these two cytosolic proteins are substrates for tyrosine kinase activity, several intriguing possibilities for novel potential regulatory interactions might be envisaged.

Insulin is known to stimulate acetyl-CoA carboxylase activity in liver and adipose tissue ([66, 276, 466, 848, 850], see also section 1.3.4.(ii)) and, despite a lack of direct evidence (see section 3.2.), is also thought to activate mammary acetyl-CoA carboxylase [552]. Since tyrosine kinase activity is known to be associated with the insulin receptor, it is tempting to suggest that tyrosine phosphorylation of acetyl-CoA carboxylase might provide the necessary link between insulin binding and its effect on acetyl-CoA carboxylase activity. However, rather than being associated with increased phosphorylation of acetyl-CoA carboxylase, increases in plasma insulin have been shown to be accompanied by decreased phosphorylation of the mammary enzyme [552]. Hence, whether the increase in phosphorylation of acetyl-CoA carboxylase observed here in the presence of vanadate is attributable to tyrosine or, as seems more probable, to serine phosphorylation, this effect is unlikely to have any relevance to regulation of the enzyme in response to insulin. The significance of this observation is therefore unclear and since the data are uncorroborated (single experiment), further speculation would be inappropriate.

4.3. Effector-Dependent Protein Phosphorylation

The most striking examples of effector-dependent phosphorylation revealed by in vitro incubation of mammary cell fractions with [γ-^{32}P]ATP were the Ca^{2+}-calmodulin dependent stimulation of ^{32}P- incorporation into a 100.3kDa cytosolic protein (section 3.3.1.), cAMP-dependent phosphorylation of three other

cytosolic proteins (molecular weights 38.4kDa, 47kDa and 57.3kDa) (section 3.3.4.) and polyamine-dependent stimulation of the phosphorylation of a number of proteins, particularly in the 'nuclear' and 'microsomal' fractions (section 3.3.3.). The lack of identifiable protein kinase C activity in any of the mammary cell fractions (section 3.3.2.) is consistent with recent work in mouse mammary gland [86].

4.3.1. Calcium-Dependent Phosphorylation

On SDS polyacrylamide gel electrophoresis, the 100.3kDa protein migrated close to a standard sample of the enzyme glycogen phosphorylase and its phosphorylation also displayed the same regulatory characteristics with respect to Ca^{2+}/calmodulin dependence as phosphorylation/activation of skeletal muscle glycogen phosphorylase (see section 1.3.1.). On the basis of these observations, it would therefore be reasonable to conclude that the 100.3kDa phosphoprotein observed in mammary cell cytosols is in fact glycogen phosphorylase. However, since, characteristically, phosphorylase kinase is also activated by cAMP-dependent phosphorylation (see section 1.3.1.), ^{32}P-incorporation into both phosphorylase kinase α and β-subunits and phosphorylase itself would be expected to increase in the presence of Bt_2 cAMP. cAMP-PrK mediated activation of phosphorylase kinase is achieved by a reduction in the Ca^{2+} concentration required for half maximal stimulation of the enzyme and an increase in the maximum response to Ca^{2+} (see section 1.3.1.) and therefore may not be detectable at very low Ca^{2+} concentration such as might have prevailed in incubations containing EGTA. Nevertheless, incubations performed in the presence

of Bt_2cAMP, whether in the presence or absence of Ca^{2+}, should have elicited an increase in phosphorylation of the α and β subunits of phosphorylase kinase (molecular weights 145kDa and 125kDa respectively). As a result, in the presence of Ca^{2+}, the consequent activation of phosphorylase kinase should have precipitated a marked increase in the amount of ^{32}P incorporated into phosphorylase. An alternative identity for this protein, more consistent with its observed phosphorylation characteristics, is offered by a calcium/calmodulin-dependent phosphorylation of a 100kDa cytosolic protein that has been described in several mammalian tissues including heart, liver and skeletal muscle [583] and a number of mammalian cell lines, in several of which it has also been shown to become phosphorylated during incubation of intact cells with ^{32}Pi [585]. This protein has been demonstrated to be unrelated to glycogen phosphorylase (molecular weight ~ 97kDa) on grounds of differences in migration on one and two dimensional polyacrylamide gels, distinct phosphopeptide maps obtained by four different methods, the presence of phosphothreonine (as opposed to the phosphoserine of glycogen phosphorylase), its failure to bind to glycogen and its failure to be recognised by anti-phosphorylase antibodies [583]. The finding that the 100.3kDa cytosolic protein phosphorylated by Ca^{2+}-calmodulin-dependent protein kinase during <u>in vitro</u> incubation of mammary acinar cytosols with [γ-^{32}P]ATP consistently migrated slightly behind purified phosphorylase marker protein in SDS polyacrylamide gels (e.g. Figure 38) suggests that this phosphoprotein may be analogous to the 100kDa cytosolic protein described above and therefore not in fact related to glycogen phosphorylase. The inability to demonstrate

cAMP-dependent phosphorylation of the cytosolic 100.3kDa mammary protein is also consistent with such a classification since the 100kDa substrate for Ca^{2+}-calmodulin-dependent protein kinase identified in other tissues is not phosphorylated by phosphorylase kinase [583]. A 97kDa cytosolic protein of pituitary adenoma cells, termed S97 [169], has been proposed to be identical to the 100kDa protein [583] and the reported widespread tissue distribution of this major substate for Ca^{2+}/calmodulin-dependent protein kinase [583, 585] would seem to suggest that it may fulfil an important function in mediating cytoplasmic propagation of Ca^{2+} signals. The identity of this potentially very interesting phosphoprotein has not yet been revealed but both this and the nature of the Ca^{2+}/calmodulin-dependent protein kinase responsible for its phosphorylation are currently under investigation [583]. Since, in contrast to phosphorylase, this protein is phosphorylated on threonine residues, the possibility that it may provide an identity for the 100kDa mammary protein could be investigated further using the partial acid hydrolysis technique [127a] described earlier for identification of tyrosine-specific phosphorylation (see section 4.2.).

The possibility of a further regulatory mechanism governing the phosphorylation of the 100.3kDa protein was revealed by incubations in the presence of polyamine (Figure 34). Irrespective of the subcellular fraction with which the 100.3kDa protein was associated, the magnitude of stimulation, by polyamine, of its phosphorylation was consistently high in relation to other major phosphoproteins of the same fractions (see section 3.3.3.). The stimulation observed was much more pronounced in membrane fractions, particularly the 'nuclear' and 'microsomal' fractions, than in the

'cytosolic' fraction, in contrast to the Ca^{2+} sensitivity of its phosphorylation which was obvious in the cytosolic fraction but undetectable in any of the membrane fractions. Assuming membrane bound 100.3kDa and cytosolic 100.3kDa proteins represent two forms of the same protein, a convenient explanation for these observations would be that this protein is phosphorylated by at least two protein kinases; cytosolic Ca^{2+}/calmodulin-dependent protein kinase and membrane associated polyamine-dependent protein kinase(s) since the nature of the in vitro incubations performed in this study precludes interaction between kinases exclusive to one fraction and substrates associated with another. Although the potential for such interaction would exist in vivo, cytosolic proteins might be expected to be more accessible to a cytosolic kinase than a membrane bound enzyme and, conversely, a membrane bound protein more accessible to a membrane associated kinase. Consequently, membrane bound 100.3kDa protein would be phosphorylated preferentially by polyamine-dependent protein kinase while phosphorylation of the cytosolic form would be governed, essentially, by Ca^{2+}/calmodulin-dependent protein kinase activity. This reasoning provides an attractive interpretation of the results in terms of the potential it implies for independent regulation of the phosphorylation of the 100.3kDa protein in different cellular compartments, but without knowing the identity of this protein it is difficult to assess the potential physiological significance of such an arrangement.

At least nine rat hepatocyte cytosolic proteins have been demonstrated to show increased phosphorylation in response to Ca^{2+}-mobilising hormones [238]. Of these, two are apparently substrates for protein kinase C while phosphorylation of the

remainder is stimulated equally well by treatment of hepatocytes with the Ca^{2+} ionophore A23187 and are suggested to be substrates for Ca^{2+}/calmodulin-dependent protein kinase [238]. In view of the apparent lack of endogenous protein kinase C activity in mammary cell fractions (discussed below), only the Ca^{2+}/calmodulin-dependent phosphorylations would be expected to be revealed by incubation in the presence of Ca^{2+}. Although phosphoproteins with similar molecular weights to those described for the seven putative Ca^{2+}/calmodulin-dependent protein kinase substrates of rat hepatocyte cytosols including phosphorylase (97kDa), pyruvate kinase (61kDa) and phenylalanine hydroxylase (52kDa) can be identified in mammary acinar cytosols, none, except perhaps, phosphorylase, exhibit detectable Ca^{2+}/calmodulin dependence. These observations suggest that while containing significant amounts of Ca^{2+}/calmodulin-dependent protein kinase (as evidenced by the phosphorylation characteristics of the 100.3kDa cytosolic protein), either its substrate specificity differs from the analogous hepatocyte enzyme or the mammary proteins of similar molecular weight to hepatic substrates for Ca^{2+}/calmodulin-dependent protein kinase are in fact structurally distinct and therefore mammary tissue is deficient in these substrates.

The second major type of Ca^{2+}-dependent protein kinase, Ca^{2+}/phospholipid-dependent protein kinase (protein kinase C, see section 1.3.8.(i)b), is known to be predominantly cytosolic in several non-neuronal tissues with membrane associated activity accounting for only 2% total activity in tissues such as muscle and platelets. If its distribution is similarly uneven in mammary cells, many of the kinase's established substrates such as hormone receptors [139, 334, 361, 396,

705, 757], eIF-2 [683] and ribosomal protein S6 [471] would be inaccessible, confined, as they are, to membrane fractions. It might seem incongruous for a predominantly cytosolic kinase to have exclusively particulate substrates, however, in intact cells, these substrates would in fact be accessible. Furthermore, activation of protein kinase C involves redistribution to membranes, a mechanism that would bring the kinase into closer association with these substrates and therefore augment further the effect of its activation. Nevertheless, in hepatocytes this enzyme has also been shown to phosphorylate at least two endogenous cytosolic proteins [238]. Unfortunately, as exemplified by the differences between mammary and hepatic Ca^{2+}/calmodulin-dependent protein kinase activities, there is no guarantee that cytosolic substrates for protein kinase C, corresponding to those observed in other tissues, will be found in mammary cytosols. Notwithstanding this, if, as suggested earlier (section 4.2.), fragments of insulin receptor bearing serine/threonine phosphorylation sites were present in the acinar cytosols, protein kinase C would be expected to participate in their phosphorylation ([139], see sections 1.3.8.(i)b and 1.3.8.(ii)). Since there is no evidence for increased phosphorylation (in response to Ca^{2+} or phorbol ester) of the 47kDa cytosolic protein suggested to be an insulin receptor fragment, it must be assumed either that such fragments do not, after all, populate cytosolic fractions or that protein kinase C activity is negligible in these rat mammary cytosols. Evidence in support of the latter is provided by work with protein kinase C in mouse mammary glands at various stages of the reproductive cycle [86]. The activity of mouse mammary protein kinase C falls throughout pregnancy, reaching a minimum value by the fourth day of lactation.

At this point, protein kinase C activity is very low and remains so throughout lactation. Assuming rat mammary tissue behaves in a similar manner, protein kinase C activity would indeed be expected to be negligible in the mammary extracts used in this study since the subcellular fractions were prepared from mammary glands of rats in mid-lactation (10-12 days post-partum) and therefore likely to contain minimal levels of such activity. It would therefore seem reasonable to conclude that protein kinase C activity of lactating mammary tissue is too low to be credited with a significant role in the acute regulation of cellular metabolism. There is evidence to suggest, however, that protein kinase C inhibits mammary cell differentiation [86] and reduction in its activity may therefore represent an important part of the longer-term mechanisms governing cell function during mammary gland development.

4.3.2. cAMP-Dependent Protein Phosphorylation

Of the two cAMP-dependent phosphorylations observed consistently, phosphorylation of the 57.3kDa protein showed the greater response to incubation with Bt_2cAMP (Figure 35) or cAMP itself (Table 15). The only major cytosolic phosphoproteins with molecular weights in the region of 57.3kDa described by Garrison et al. [238] in rat hepatocytes are fructose 2, 6-bisphosphatase (55kDa) and an unidentified 56kDa protein. Phosphorylation of the 56kDa protein is unaffected by incubation with glucagon but the same treatment provokes a large (approximately 15-fold) increase in fructose 2,6-bisphosphatase phosphorylation indicating the involvement of cAMP-dependent protein kinase (cAMP-PrK). The fructose 2,6-bisphosphatase/6-phosphofructo2-kinase (F2,6-P_2ase/PFK2) dimer is known to be an excellent

substrate for cAMP-PrK [617, 622] and is phosphorylated on one serine residue per 55kDa subunit [180, 183]. Whether phosphorylase kinase is also capable of phosphorylating the F2,6-P_2ase/PFK2 dimer [233] or not [662] is controversial but even assuming it is, there is no guarantee that such an effect would be observed in mammary tissue. While F2,6-P_2ase/PFK2 therefore displays phosphorylation characteristics broadly consistent with those observed for the 57.3kDa phosphoprotein of mammary acinar cytosols, it does not represent the only known protein displaying properties indicative of a possible identity for the unknown protein. Another such candidate is the R-subunit of type II cAMP-PrK itself (see section 1.3.8.(i)a) which is autophosphorylated on one serine residue per subunit [82, 196 317, 629, 639], has a molecular weight of 52-55kDa [556] and is predominantly cytosolic in most tissues, including liver and muscle [318]. Although not normally regarded as a cytosolic enzyme, HMG-CoA reductase may also be envisaged to provide a possible identity for the unknown 57.3kDa protein since a 50-56kDa soluble peptide is released from the membrane bound enzyme by limited proteolysis [398] and microsomal HMG-CoA reductase has been shown to be phosphorylated by cAMP-PrK <u>in vitro</u> [245, 398]. The soluble proteolytic fragment contains the enzyme active site [398] and its presence in mammary acinar cytosols could therefore be verified by assaying for HMG-CoA reductase activity. Although such assays were not performed on fresh samples, a frozen sample of cytosol used in a previous experiment and known to contain the 57.3kDa substrate for cAMP-PrK was screened for HMG-CoA reductase activity. Despite the fact that little, if any, HMG-CoA reductase activity is normally expected to survive freezing, measurable activities were detectable in these

samples [715], suggesting that sufficient quantities of the soluble HMG-CoA reductase fragment might be expected to be found in the cytosols used for in vitro phosphorylations in this study. While not representing evidence in support of a positive identification for the 57.3kDa protein, the indications are that soluble HMG-CoA reductase may provide an equally plausible alternative to F2,6-P_2ase/PFK2 complex and type II cAMP-PrK R-subunit in any speculation regarding the identity of this mammary phosphoprotein. The apparent enhancement of response to Bt_2cAMP observed in mammary cytosols incubated in the presence of Ca^{2+} (Figure 36) might be regarded as evidence in favour of F2,6-P_2ase/PFK2 which, as mentioned above, has been suggested to be a substrate for the Ca^{2+}-dependent enzyme phosphorylase kinase [233]. However, the same effect of Ca^{2+} was observed for cAMP-dependent phosphorylation of the 47kDa protein and a more likely explanation for this phenomenon (as discussed in section 3.3.4.) is afforded by increased sensitivity of the technique employed to detect cAMP-dependent phosphorylation, resulting from divalent cation-dependent suppression of background phosphorylation. In addition, if these divalent cation-dependent effects were indeed mediated by phosphorylase kinase, increased phosphorylation of the two substrates for cAMP-dependent phosphorylation would be expected to occur in the presence of Ca^{2+} alone and not, as observed (see section 3.3.4.), solely in concert with Bt_2cAMP.

In the absence of further relevent information, it is difficult to assess properly the likely identity of the 57.3kDa protein. However, on the basis of the (optimistic) assumption that the true identity of this protein will be ultimately selected from among the three enzymes cited above, then whichever is finally

adopted will command an important role in the regulation of mammary cell metabolism since all three enzymes have well documented regulatory functions in other tissues (see sections 1.3.2., 1.3.5. and 1.3.8.(i)a).

Although cAMP-dependent phosphorylation of the 47kDa protein of mammary acinar cytosols is not as responsive to Bt_2cAMP as that of the 57.3kDa protein, it is equally consistent and appears to share a similar cAMP sensitivity (see section 3.3.4.). Once again, the identity of this protein is uncertain with possibilities including the E_1 component of branched chain 2-oxoacid dehydrogenase (46kDa, see section 1.3.3.(ii)) and a proteolytic fragment of the insulin receptor (45-50kDa, see section 1.3.8.(ii)a). The latter is attractive in a number of respects but is subject to uncertainties associated with the assumption that sufficient quantities of the receptor might be solubilised, during the homogenisation procedure, to account for the observed effects. Since the β_1 fragment of the receptor might be expected to remain membrane-associated, whereas the 47kDa phosphoprotein was identified in mammary acinar cytosols, there would seem to be little justification for suggesting that they may represent the same peptide. If however, substantial internalisation and processing of insulin receptors proceeds during preparation of acini, homogenisation might be expected to liberate, from lysosomes, a sufficient quantity of receptors at various stages of degradation to establish a population of "cytosolic" β_1-fragments, perhaps still bound to their counterpart α-subunits or subunit fragments (reducing conditions encountered later during preparation for SDS-polyacrylamide gel electrophoresis would lead to dissociation of these α-β subunit pairs, allowing identification of the 45-50kDa β_1 receptor fragments).

Alternatively, the homogenisation procedure itself may be sufficiently vigorous to solubilise membrane bound receptors which would then be susceptible to attack by endogenous proteases released from ruptured lysosomes.

Similar misgivings might be expressed with respect to the branched chain dehydrogenase (BCDH) complex but since BCDH is an enzyme of the mitochondrial matrix and no steps were taken to ensure preservation of the structural integrity of mammary cell mitochondria during preparation of the subcellular fractions, it is not inconceivable that sufficient numbers of mitochondria might have been disrupted to create a detectable 'cytosolic' population of the enzyme. Although the E_1 component is known to be a substrate for cAMP-PrK *in vitro*, phosphorylation of the enzyme, catalysed by this kinase, appears to have no effect on BCDH activity ([604, 637], see section 1.3.3.(ii)) and is unlikely to represent a physiologically relevant event since BCDH would normally be confined to the interior of mitochondria and therefore inaccessible to endogenous cAMP-PrK. Consequently, if the 47kDa protein were to be identified positively as the E_1 component of BCDH, the potential regulatory significance of its phosphorylation in mammary acinar 'cytosols' would be, at best, obscure and unlikely to have any importance *in vivo*.

Three unidentified cytosolic proteins whose phosphorylation is enhanced by incubation of hepatocytes with glucagon and are, therefore, presumably substrates for cAMP-PrK, share molecular weights similar to the 47kDa protein described here. Two of these share the molecular weight of 45kDa, while the third is a 49kDa protein and, unlike the two 45kDa proteins, is apparently, also phosphorylated by Ca^{2+}/ calmodulin-dependent protein kinase [238]. The latter characteristic is

not shared by the 47kDa mammary protein and the probability of homology between the 49kDa hepatocyte protein and the mammary protein is therefore diminished as a result, leaving only the two 45kDa hepatocyte proteins as possible candidates for homology between the two cell types. One of these may also correspond to a 46kDa hepatocyte protein reported by LeCam [464] to be phosphorylated, at the same sites, in response to both insulin and glucagon. This property is particularly interesting in that the 45-50kDa fragment of insulin receptor β-subunit (see section 1.3.8.(ii)a), would also be expected to become phosphorylated in response to both insulin and glucagon. However, both effects on phosphorylation of the 46kDa hepatic protein are predominantly attributable to increases in phosphoserine with no indication of insulin-stimulated tyrosine phosphorylation [464]. While serine/threonine phosphorylation of the insulin receptor might also be expected to occur at the same sites following insulin or glucagon treatment (see section 1.3.8.(ii)a), tyrosine phosphorylations in response to insulin should be equally obvious. It might, therefore, be suggested that the 46kDa hepatocyte protein is unlikely to correspond to the 46kDa insulin receptor fragment, on grounds that the hepatocyte protein shows no evidence of tyrosine phosphorylation. However, the evidence supporting tyrosine phosphorylation of the 47kDa mammary protein is too tenuous to draw any conclusions as to which of these two hepatocyte proteins is more likely to have phosphorylation characteristics akin to its own.

Although not observed consistently, the cAMP-dependent phosphorylation of a 38.4kDa protein (Figure 37), identified in the same mammary cytosols that also gave rise to the two prominent substrates for mammary cAMP-PrK described above, perhaps deserves

comment. However, there are few immediately obvious candidates for the identity of this protein and of the cytoplasmic hepatocyte proteins whose phosphorylation is stimulated by pre-treatment of the cells with glucagon [238], only fructose 1,6-bisphosphatase (42kDa) exhibits a molecular weight comparable to that of the mammary phosphoprotein (38.4kDa). While interesting, in itself, such a classification would offer little opportunity for speculation regarding the role of its phosphorylation in metabolic regulation since phosphorylation of fructose 1,6-bisphosphatase is known to be without effect on this enzymes's activity [622]. The possibility that the 38.4kDa protein does not in fact represent any recognisable native protein but is, instead, a proteolytic fragment of one of the other two endogenous substrates for cAMP-dependent phosphorylation should not be ignored and the intermittent nature of its detection might be presented as evidence in favour of this alternative.

Indeed, since there is no evidence to the contrary, it remains possible that all three substrates for endogenous mammary cAMP-Prk are in fact proteolytic fragments of larger native proteins. Inclusion of protease inhibitors in the homogenisation and incubation buffers should provide some indication of the general importance of proteolytic effects in determining the molecular weights of substrates for cAMP-dependent phosphorylation. However, even under such conditions, proteolytic peptides such as intermediates of insulin receptor degradation might be expected to be in evidence, since lysosomes (the ultimate site of receptor processing) might, as suggested earlier (section 4.2.) be imagined to release such peptides when ruptured during homogenisation. Possibilities of this kind could be probed using antibodies to the insulin receptor. While there is no guarantee

that these antibodies would necessarily recognise any proteolytic fragment of insulin receptor, a positive result would provide strong evidence in favour of such an identity.

4.3.3. Polyamine-Dependent Protein Phosphorylation

Polyamines appear to be potent stimulators of the phosphorylation of most major mammary phosphoproteins, although the effect on cytosolic proteins is more variable (Figure 34). Spermine and spermidine were the only two agents shown to affect noticably the extent of ^{32}P-incorporation into proteins contained in the "microsomal", "mitochondrial" and "nuclear" fractions. Furthermore, unlike cAMP and Ca^{2+}, polyamine appeared to influence incorporation into all proteins studied. Indeed, in all the particulate fractions, treatment with spermine provoked a consistent and often marked increase in protein phosphorylation. Such generalised effects may suggest that polyamine was affecting the availability of ATP, rather than the activities of any kinase(s) or phosphatase(s). As indicated earlier (see section 4.2.), the major limiting factor in the phosphorylation incubations was likely to be ATP-depletion due to ATPase activity. It might therefore be suggested that polyamine-dependent inhibition of ATPase activity could account for the observed effects of this agent on protein phosphorylation in particulate fractions. Various effects of polyamine on ATPase activities have been reported in a number of different tissues, including rabbit muscle [150], rabbit kidney [762], rat brain [633] and human erythrocytes [23]. However, no significant effect of spermine was observed on ATPase activity in any of the mammary cell fractions under the conditions prevailing in the phosphorylation experiments (results not shown). It is therefore

unlikely that the observed polyamine-dependent increases in ^{32}P-incorporations were merely manifestations of an effect on ATP-availability.

Since polyamine is thought to interact with the substrate protein, rather than the kinase itself, to achieve activation (see section 1.3.8.(i)c), it might be speculated that in the mammary system, polyamine simply modifies the structure of all proteins, generally rendering them more susceptible to phosphorylation by their own particular endogenous kinases. Such an argument would obviate the need to infer the existence, in mammary tissue, of a specific polyamine-dependent protein kinase (poly A-PrK) or group of protein kinases. However, the striking subcellular fraction-specific nature of the phosphorylation enhancement profile for the two individual polyamines is difficult to explain in terms of this model without resorting to the proposal that each membrane type provides an environment capable of imposing a characteristic influence on the interaction of its consituent proteins with polyamine. Since, in addition, there are no previous reports of polyamine-dependent enhancement of phosphorylation catalysed by anything other than specific, otherwise modulator-independent protein kinases, a more probable explanation of the observed effects of polyamine could be constructed on the basis of a population of two or more distinct types of poly A-PrK. If it is assumed that phosphoproteins associated with various subcellular fractions but sharing the same molecular weight, are in fact identical proteins, more direct evidence for the existence of at least one poly A-PrK may be provided by the observation that membrane-associated forms of both the 47kDa protein and the 100.3kDa protein show marked enhancement of phosphorylation in the

presence of polyamine but no evidence of cAMP dependent or Ca^{2+} calmodulin-dependent phosphorylation, respectively (see sections 3.3.4 and 3.3.1). Since such effects are prominent in mammary cytosols (see section 3.3.1 and Figures 36 and 37), their absence from membrane fractions may imply a lack of significant membrane-associated cAMP-PrK or Ca^{2+} calmodulin-dependent protein kinase activities. It would therefore appear that the effects of polyamine on ^{32}P-incorporation into the 47 and 100.3kDa proteins are not mediated by stimulation of these two kinases, leading to the obvious conclusion that at least one other endogenous kinase is capable of phosphorylating these two mammary proteins and that phosphorylation mediated by this enzyme is polyamine-dependent.

Having adopted the assumption that the observed effects are indeed mediated by the activity of poly A-PrK, the fraction-specific patterns of stimulation of particulate protein phosphorylation by spermine and spermidine evident, particularly in the 'nuclear' and 'microsomal' fractions (Figure 34), may be explained by the existence of two forms of particulate poly A-PrK with distinct regulatory characteristics. At least one polyamine-dependent protein kinase, nuclear casein kinase NII, has been identified in nuclei [104, 435, 660, 661, 775] and, although not yet screened for polyamine-dependence, a casein kinase associated with the Golgi apparatus of bovine mammary gland [48, 49] may prove to have regulatory properties similar to those of the polyamine-dependent cytosolic enzyme, casein kinase G (casein kinase II, see section 1.3.8.(i)c), recently identified in mouse mammary tissue [468]. The evidence presented here strongly suggests the existence of microsomal poly A-PrK in rat mammary tissue and since Golgi vesicles would be expected to sediment in the 'microsomal'

fraction, an enzyme analogous to the bovine Golgi-associated casein kinase represents an attractive candidate for the identity of this kinase. To be consistent with the observed polyamine response, the putative Golgi-associated poly A-PrK must display a marked preference for spermine over spermidine as an activator, while, in contrast, the nuclear poly A-PrK must either show little polyamine specificity (with respect to spermine and spermidine) or a preference for spermidine. In the latter situation, the pronounced spermine induced increases in phosphorylation observed in the 'nuclear' fraction (Figure 34) would be proposed to arise from contamination with Golgi-associated poly A-PrK. The pattern of polyamine-dependent stimulation displayed by 'mitochondrial' fraction protein phosphorylations is more reminiscent of that observed in the 'nuclear' fraction than that of the 'microsomal' fraction (Figure 34) suggesting either contamination with nuclear-type poly A-PrK or the existence of a distinct mitochondrial poly A-PrK, similar to the nuclear form. The reduced responses of mitochondrial protein phosphorylations to polyamine, in comparison with those observed in the 'nuclear' fraction argues in favour of the latter but the existence of a distinct mitochondrial form cannot be dismissed.

The most extensively characterised polyamine-dependent protein kinase, casein kinase G (CKG, see section 1.3.8.(i)c) has been purified from cytosols of at least two different tissues (bovine lung [105], bovine adrenal cortex [108]) and has also been identified in mouse mammary gland [468]. It is surprising, therefore that the polyamine-dependent phosphorylation reported here was much less obvious in mammary acinar cytosols than in either 'nuclear' or 'microsomal' fractions. This does not necesarily imply that rat mammary cytosols are substantially deficient in polyamine-dependent protein kinase, it

may simply indicate that the major substrates for the cytosolic kinase are membrane-associated and therefore not accessible under the experimental conditions employed in this study. Considering the aliphatic character of polyamines, it is not difficult to imagine that many of the substrates for polyamine-dependent protein kinase might be membrane-associated where interactions between polyamine and the hydrophobic environment of biological membranes might be envisaged to participate in the activation mechanism. Non-membrane proteins may then be poor substrates for polyamine-stimulated phosphorylation, thus accounting for the limited response, to polyamine, of protein phosphorylation in the mammary cytosols.

Incubation of isolated hepatocytes with 7.5-10mM spermine has been shown to promote dephosphorylation of three cytosolic proteins with molecular weights of 46kDa, 34kDa (ribosomal protein S6) and 22kDa [17]. Of these, only the 34kDa phosphoprotein appears not to have a representative in the mammary complement of major cytosolic phosphoproteins. Neither of the mammary proteins with similar molecular weights (21.5kDa and 47kDa) to these hepatocyte proteins showed any spermine-dependent reduction in ^{32}P-incorporation (Figure 34). However, whether this discrepancy indicates some kind of tissue-specific effect or is more related to differences in the behaviour of intact cells and broken cell systems to treatment with polyamine cannot be deduced from the available information. The discrepant responses of the two systems to this polyamine might also reflect a dose-dependent effect since it is unknown whether the concentration of polyamine achieved in the cytoplasm of hepatocytes exposed to 7.5-10mM spermine is comparable with the 1mM spermine added to mammary cytosol incubations in this study.

The whole field of polyamine-mediated effects on enzyme activities is fraught with apparent paradox. The complex nature of the protein-polyamine interactions responsible for this confusion predisposes towards a situation in which the reliability of interpretations put upon the results generated by the present investigation into polyamine-dependent phosphorylation is questionable. Notwithstanding this, it is clear that polyamine-mediated modulation of protein phosphorylation represent a potentially major regulatory mechanism in lactating mammary tissue.

4.4. General Discussion

The most significant discoveries arising from work reported here are that mammary high affinity cAMP-PDE is susceptible to regulatory influences very similar to those found in other tissues and that despite an apparent lack of effect of increased intracellular cAMP concentrations on either steady-state protein phosphorylation or cellular metabolism, mammary tissue does in fact possess functional cAMP-PrK whose activity, *in vitro*, is susceptible to modulation by cAMP concentrations within the physiological range. Perhaps equally important is the observation that while enzymes such as cAMP-PrK and Ca^{2+} calmodulin-dependent protein kinase are represented in mammary tissue, the number of substrates attributable to both these kinases together forms only a very small fraction of the number attributable to poly A-PrK alone. As discussed earlier, this picture may be distorted, to a certain extent, by the constraints of the experimental design since interaction between kinases confined to one fraction and substrates residing in another is precluded by the very nature of the study. Further work is therefore necessary to establish the potential

importance of such interactions before reliable assessment of the relative significance of both cAMP-PrK and Ca^{2+} calmodulin-dependent protein kinase can be attempted. It seems unlikely, however, that future experimentation of this kind will reveal a sufficient number of additional substrates for these two kinases to rival the comprehensive range of proteins apparently subject to polyamine-dependent phosphorylation.

The observation that neither exposure to insulin nor elevation of intracellular cAMP levels are capable of affecting the steady state phosphorylation of any protein detectable in one-dimensional SDS polyacrylamide gel electrophoretic separations of unfractionated homogenates derived from mammary acini incubated under appropriate conditions (see section 3.2.) is surprising. In other tissues, increased intracellular cAMP concentrations are known to raise markedly the level of phosphorylation of a number of proteins including glycogen phosphorylase, phosphorylase kinase, glycogen synthase, phosphofructokinase, pyruvate kinase, fructose 2,6-bisphosphatase and acetyl-CoA carboxylase [116, 238, 323, 325, 868]. Treatment of adipocytes with insulin has been shown to stimulate the incorporation of ^{32}P into a peptide of molecular weight 123kDa [18] and stimulation of the phosphorylation of both peripheral plasma membrane insulin sensitive PDE [511, 512] and a 46kDa cytosolic protein [590] has also been demonstrated in response to insulin treatment of hepatocytes. In addition, it has been suggested, recently, that insulin is capable of regulating the phosphorylation state and therefore activity of acetyl-CoA carboxylase in rat mammary gland in vivo [552] but there is at present no evidence to suggest that such an effect can be reproduced in mammary acini [102]. Equally puzzling is the inability

of raised intracellular cAMP concentrations to inactivate mammary acinar acetyl-CoA carboxylase, an observation supported by the inability to identify any cAMP-dependent changes in the phosphorylation pattern of tryptic peptides derived from acetyl- CoA carboxylase purified from mammary acini incubated in the presence of ^{32}Pi [102]. In view of the apparent prominence of poly A-PrK activity in lactating mammary tissue ([468], see section 3.3.3.), one possible explanation for the inability to observe cAMP-dependent acetyl-CoA carboxylase phosphorylation is that the level of polyamine-dependent phosphorylation of this enzyme is sufficiently high to block or mask any minor changes in phosphorylation attributable to the activity of other kinases, such as cAMP-PrK. If the mechanism for the proposed blocking of cAMP-PrK mediated phosphorylation involved competition with a more active kinase, such as poly A-PrK, for the same phosphorylation sites, acetyl-CoA carboxylase would be expected to be permanently inactive. However, since acetyl-CoA carboxylase activity is known to be high in lactating mammary acini [102, 656], polyamine-dependent phosphorylation at sites distinct from the cAMP-dependent phosphorylation sites might, instead, be imagined to induce conformational changes capable of preventing cAMP dependent phosphorylation of the sites responsible for enzyme inactivation. In the latter situation, acetyl-CoA carboxylase would constantly be fully active, perhaps explaining the inability of insulin treatment of mammary acini to elevate acetyl-CoA carboxylase activity further [656]. The ability of acetyl-CoA carboxylase to influence the rate of de novo fatty acid synthesis would therefore be lost and acute regulation of this pathway would then rely entirely on control exerted at other points in the synthetic sequence. This contention is

supported by the observation that insulin treatment stimulates the rate of lipogenesis in rat mammary acini without influencing the activity of acetyl-CoA carboxylase [656]. In the absence of acetyl- CoA carboxylase-governed control of fatty acid synthesis, perhaps the most obvious alternative enzyme activities at which regulation of this pathway may be exerted are the glucose transporter, PFK1 and PDH. Glucose uptake is known to be stimulated by insulin in other tissues and there is mounting evidence [72a, 73, 370a, 582a] to suggest that this is also the case for the mammary gland. Furthermore, there is evidence to suggest that monosaccharide transport is a rate-limiting factor in the utilisation of carbohydrate by mammary gland [776] and recent work with mammary tissue of starved/re-fed rats [73, 582a] appears to show a good correlation between rate of monosaccharide transport and the contribution of glucose to de novo fatty acid synthesis.

The rate of lactose synthesis appears to be governed by intracellular glucose concentration [73], yet no increase in synthetic rates has been recorded in response to insulin (see section 1.4.2.). This apparent paradox may be explained by increased glucose utilisation for lipogenesis, if not due to elevated acetyl-CoA carboxylase activity, perhaps as a result of activation of PFK1 and/or PDH. The increase in glucose uptake, in response to insulin, would not, therefore, necessarily be translated into raised intracellular glucose concentrations. Consequently, in the absence of a direct effect of insulin on the lactose synthetic pathway, increased glucose uptake would, effectively, be "funnelled" into lipogenesis, at the expense of lactose synthesis.

Whatever the reasons for defective regulation of acetyl-CoA carboxylase activity by reversible phosphorylation in mammary acini, this enzyme undoubtedly represents a major phosphoprotein of mammary acinar cytosols, as demonstrated by its steady-state level of ^{32}P-incorporation following equilibration of mammary acini with ^{32}Pi ([102], see section 3.2.). Curiously, however, incubation of mammary acinar cytosols with [γ-^{32}P]ATP *in vitro* reveals remarkably little acetyl-CoA carboxylase phosphorylation, suggesting that it is a comparatively poor substrate for the endogenous cytosolic kinases identified in this study, including cAMP-PrK and poly A-PrK. One obvious possible solution to this apparent paradox is that in the intact cell, acetyl-CoA carboxylase is phosphorylated by a membrane-bound kinase such as the particulate polyamine-dependent protein kinase(s) since such a phosphorylation would not be detected in the *in vitro* incubation system. Inconsistency of this nature is not confined to the phosphorylation of acetyl-CoA carboxylase; cAMP and Ca^{2+} both show marked effects on the initial rates of protein phosphorylation measured *in vitro* but modulation of the intracellular concentrations of these agents in intact mammary acini has no obvious effects on the observed level of steady-state phosphorylation of any endogenous proteins ([100, 103], see sections 3.2, 3.3.1. and 3.3.4.). Unlike the previous example (acetyl-CoA carboxylase phosphorylation), separation of kinase and substrate as a result of *in vitro* experimental protocol cannot be invoked to explain these effects unless phosphorylation of the cytosolic substrates for cAMP-PrK and Ca^{2+} calmodulin-dependent PrK is supposed to be masked, *in vivo*, by overwhelming phosphorylation mediated by membrane-bound kinase activity. However, it is equally probable that these substrates are

quantitatively minor components of mammary cytosols, in comparison with proteins such as acetyl-CoA carboxylase or ATP-citrate lyase. In this situation, under conditions where the level of phosphorylation of the more abundant proteins becomes appreciable, the amount of ^{32}P associated with any particular protein is likely to be more dependent on the actual number of protein molecules present than on the rate of their phosphorylation. Under these circumstances, the amount of ^{32}P incorporated into quantitatively minor proteins might easily be imagined to be insignificant in comparison with phosphoproteins such as acetyl CoA carboxylase and ATP-citrate lyase and, perhaps even indistinguishable from background phosphorylation. Consequently, the less abundant the protein, the less likelihood there is of observing any changes in its steady-state level of phosphorylation, even if measurements of its initial rate of phosphorylation indicate that it is an excellent substrate for one of the effector-dependent protein kinases. The only workable alternative solution would seem to be that in the intact cell, stimulation of phosphorylation is accompanied by a parallel increase in dephosphorylation capable of exactly balancing the effect of increased kinase activity and therefore preventing any expression of increased net phosphate-incorporation. In the in vitro system, control of this nature might be expected to be at least partially disabled by the inclusion of the phosphatase inhibitor β-glycerophosphate in all incubation buffers, thus allowing any increase in kinase activity to be reflected by increases in net phosphate-incorporation. While the existence of such a finely balanced homeostatic mechanism might seem improbable and difficult to justify in terms of functional significance, an analogous situation is known to exist in the control of mammary intracellular cAMP concentrations.

Mammary adenylate cyclase activity can be stimulated markedly by β-adrenergic agonists such as isoproterenol [449] but incubation of mammary acini with these agents does not result in raised intracellular cAMP concentrations unless a PDE inhibitor such as IBMX or Ro 7-2956 is also present in the incubation medium [101]. The implication is therefore that β-adrenergic stimulation of adenylate cyclase also provokes a concomitant increase in cAMP-PDE activity sufficient to preserve resting levels of net cAMP production. The increases in high affinity cAMP-PDE activity in response to agents capable of stimulating cAMP synthesis, demonstrated in both adipose tissue [597, 721, 878] and liver [312, 490], provide support for this assertion but no corresponding effects on mammary high affinity cAMP-PDE have yet been identified. While confirmation of the mechanism(s) involved in antagonism of stimulated intracellular cAMP accumulation in mammary tissue therefore remains elusive, there is no doubt that mammary cell cAMP levels are extremely resistant to regulation by external stimuli. The discovery, made here, that insulin treatment of mammary acini stimulates high affinity cAMP-PDE activity was, consequently, of great interest since it provided a potential mechanism whereby intracellular cAMP levels might be depressed in mammary tissue. However, while local cAMP concentration gradients may be modified by changes in membrane-bound high affinity cAMP-PDE activity [212], it is now known that insulin treatment of mammary acini does not in fact provoke any changes in overall intracellular cAMP concentration [275].

The picture emerging from all this apparently paradoxical information is that elaborate mechanisms have evolved in lactating mammary tissue to prevent cAMP-dependent regulation of metabolic

processes, in the short term, despite the existence of both a competent system for coupling external stimuli to the activity of enzymes involved in cAMP metabolism and a functional effector system comprising at least one form of cAMP-PrK. The same would also appear to be true for acute regulation of mammary metabolism by Ca^{2+}-dependent phosphorylation, although the characteristics of mechanisms involved in modulation of intracellular Ca^{2+} concentrations have not yet been established in this tissue. Both polyamine-dependent protein kinase activity [468] and polyamine levels [843] are high in lactating mammary tissue but the potential for acute regulation of mammary protein phosphorylation represented by such a situation may not in fact be exploited by mammary cells. Various agents including Bt_2cAMP, insulin and prolactin have been demonstrated to influence mammary ornithine decarboxylase activity [5, 57] and since the activity of this enzyme generally determines the rate of polyamine synthesis (see section 1.3.8.(i)c), these agents would be expected to modulate mammary cell polyamine concentrations and consequently the activity of poly A-PrK, leading to changes in the level of phosphorylation of a number of endogenous proteins (see section 3.3.3.). That no such changes are observed following exposure of mammary acini to either insulin or agents capable of elevating intracellular cAMP concentrations (see section 3.2.), would seem, once again, to indicate that mammary protein phosphorylation is invulnerable to acute regulation despite the presence of systems capable of translating external stimuli into changes in kinase activity.

From a simplistic viewpoint, based on the generalisation that phosphorylation stimulates catabolism and dephosphorylation stimulates synthetic activity, the inability of increases in kinase activity to

effect increases in mammary protein phosphorylation might be imagined to have evolved in order to maintain maximum lactogenic rates independent of 'trivial' (with respect to mammary cell function) acute regulatory stimuli. The secretory cells of mammary tissue (mammary epithelial cells) have no efferent autonomic nerve supply and any regulatory stimuli must therefore be humoral. The systems discussed above would then serve to insulate further mammary lactogenic activity from 'irrelevent' stimuli and allow metabolic regulation to be governed solely by substrate availability. The only important factors influencing mammary cell metabolism in the short term would, therefore, be serum concentration of lactogenic precursors and their rate of uptake across the plasma membrane. Restriction of synthetic rates during food deprivation would, according to this scheme, be achieved by reduced glucose uptake in response to the low levels of circulating insulin and, as discussed earlier, the insulin-stimulated increase in lipogenesis in mammary acini, known not to involve increases in acetyl-CoA carboxylase activity [656], would be explained by increased glucose uptake. It should, however, perhaps be re-emphasised that observations in mammary acini may not be representative of the response of mammary cells to insulin in intact animals where dietary manipulation of circulating insulin levels have been shown to influence acetyl-CoA carboxylase activity [552]. The most obvious objection to an explanation of this kind, beside the dangers of over-simplification, is that it provides no indication of why mammary cells should retain elaborate regulatory systems, as exemplified by the adenylate cyclase/PDE/cAMP-PrK system, when, in evolutionary terms, such systems would appear to be superfluous and, therefore, obsolete. Perhaps an answer to this criticism might be afforded by a proposal asserting that

protein phosphorylation is essential for maintenance of long term, rather than acute regulatory mechanisms. There is already good evidence that both protein kinase C [86, 758] and poly A-PrK [468] are involved in the control of mammary gland development. While the activities of Ca^{2+} calmodulin-dependent protein kinase and cAMP-PrK have not yet been demonstrated to correlate with cellular growth or differentiation (but see below), both may prove to have important regulatory effects on protein synthesis. In other tissues, both Ca^{2+} calmodulin-dependent protein kinase [262] and cAMP-PrK [170, 837, 839] are known to phosphorylate ribosomal protein S6 and cAMP-PrK also phosphorylates histone H 1[454, 505]. Interestingly, the onset of lactation in rats and mice is accompanied by a reduction in the level of cAMP in mammary cells [651, 681, 682] and treatment of a variety of mammary culture preparations with Bt_2 cAMP or PDE inhibitors for extended periods has been shown to suppress both lactose and fatty acid synthesis [75, 487, 488, 682, 722]. The apparent ability of cAMP to limit lactogenesis might therefore justify the development of systems capable of nullifying the effect of stimuli normally expected to raise intracellular cAMP concentrations and therefore activate cAMP-PrK. However, the potential to express such effects may be important in relation to initiation of involution at the end of lactation, thus rationalising the existence of systems which, during lactation, are apparently antagonistic to the major function of the tissue. Although there is no guarantee that these effects of cAMP are mediated by cAMP-PrK (as discussed earlier [section 4.2.], raised intracellular cAMP concentrations appear to be without effect on the steady state level of protein phosphorylation in mammary acini), there is, at present, no known alternative mechanism for effecting a cellular

response to cAMP. These observations therefore imply that, despite evidence to the contrary, under the right conditions, cAMP-dependent protein phosphorylation can be induced in mammary cells. Evidence in favour of some, as yet, undetermined role for cAMP-PrK is provided by the observation that prolactin stimulates induction of cAMP-PrK synthesis in mouse mammary stem cells [500]. However, it is difficult to understand why cAMP-PrK synthesis should be induced at a time when, as described above, cAMP-dependent effects are known to antagonise lactose and fatty acid synthesis. Perhaps the best explanation would be that a certain level of cAMP-dependent phosphorylation is required for normal cell function and that at the lower intracellular cAMP concentrations prevailing during lactation, such levels can only be maintained by increasing the cellular complement of cAMP-PrK.

In conclusion then, it might easily be imagined that a certain level of activity of these kinases is required in order to maintain lactogenesis but that acute regulation might interfere with the balance of protein synthesis necessary for normal milk production, perhaps resulting in modification of milk composition. The value of developing a system capable of preventing such undesirable interference during lactation, while preserving potential flexibility of response would then be obvious. The elaborate nature of the two tier 'fail-safe' mechanism represented by the resistance of intracellular cAMP levels to modulation and the inability of increased cAMP-PrK activity to be translated into raised levels of steady-state protein phosphorylation, might therefore be regarded as a reflection of the importance of maintaining correct milk composition regardless of acute external stimuli normally irrelevent to mammary tissue during lactation.

References

1. Adelstein, R.S., Conti, M.A., Hathaway, D.R. and Klee, C.B. (1978) J. Biol. Chem. 253, 8347-8350.

1a. Agius, L., Robinson, A.M., Girard, J.R. and Williamson, D.H. (1979) Biochem. J. 180, 689-692.

2. Ahmad, F., Ahmad, P.M., Pieretti, L. and Watters, G.T. (1978) J. Biol. Chem. 253, 1733-1737.

3. Ahmad, Z., De Paoli-Roach, A.A. and Roach, P.J. (1982) J. Biol. Chem. 256, 8348-8355.

4. Ahmad, Z., Lee, F-T., De Paoli-Roach, A.A. and Roach, P.J. (1984) J. Biol. Chem. 259, 8743-8747.

5. Aisbitt, R.P.G. and Barry, J.M. (1973) Biochim. Biophys. Acta 320, 610-616.

6. Aitken, A., Bilham, T. and Cohen, P. (1982) Eur. J. Biochem. 126, 235-246.

7. Ajiro, K., Borum, T.W., Shulman, S.D., McFadden, G.M. and Cohen, L.H. (1981) Biochemistry 20, 1454-1464.

8. Albert, K.A., Helmer-Matyjek, E., Nairn, A.C., Muller, T.H., Haycock, J.W., Greene, L.A., Goldstein, M. and Greengard, P. (1984) Proc. Natl. Acad. Sci. USA 81, 7713-7717.

9. Allen, E.H. and Sneyd, J.G.T. (1975) Biochem. Biophys. Res. Commun. 62, 594-601.

9a. Allred, J.B., Roman-Lopez, C.R., Pope, T.S. and Goodson, J. (1985) Biochem. Biophys. Res. Commun. 129, 453-460.

10. Aloyo, V.J., Zwiers, H. and Gispen, W.H. (1983) J. Neurochem. 44, 649-653.

11. Antoniw, J.F. and Cohen, P. (1975) Eur. J. Biochem. 68, 45-54.

12. Antonio, J.F., Nimmo, H.G., Yeaman, S.J. and Cohen, P. (1977) Biochem. J. 162, 423-433.

13. Appleman, M.M. and Terasaki, W.L. (1975) Adv. Cycl. Nucl. Res. 5, 152-162.

14. Arch, J.R.S. and Newsholme, E.A. (1976) Biochem. J. 158, 603-622.

15. Assimacopoulos-Jeannet, F., McCormack, J.G., Prentki, M., Jeanrenaud, B. and Denton, R.M. (1982) Biochim. Biophys. Acta 717, 86-90.

16. Atmar, V.J., Kuehn, G.D. and Casillas, E.R. (1981) J. Biol. Chem. 256, 8275-8278.

17. Auberger, P., Samson, M., Le Cam, G. and Le Cam, A. (1984) Biochim. Biophys. Acta 801, 461-469.

18. Avruch, J., Leone, G.R. and Martin, D.B. (1976) J. Biol. Chem. 251, 1511-1515.

19. Avruch, J., Nemenoff, R.A., Blackshear, P.J., Pierce, M.W. and Osathanondh, R. (1982) J. Biol. Chem. 257, 15162-15166.

20. Bachrach, U. (1980) In Polyamines in Biomedical Res. pp. 81-107 (Gaugas, J.M. ed.) J. Wiley and Sons, New York.

21. Balduzzi, P.C., Notter, M.F.D., Morgan, H.R. and Shibuya, M. (1981) J. Virol. 40, 268-275.

21a. Baldwin, R.L. and Martin, R.J. (1968) Endocrinology 86, 506-510.

21b. Baldwin, R.L. and Yang, Y.T. (1974) in Lactation, (Larson, B.L. and Smith, V.R. eds.) vol. 1 pp. 349-411, Academic Press, London and New York.

22. Ball, E.H., Seth, P.K. and Sanwal, B.D. (1980) J. Biol. Chem. 255, 2962-2968.

22a. Ballard, F.J., Hanson, R.W. and Kronfeld, D.S. (1969) Fed. Proc. 28, 218-231.

23. Ballas, S.K., Clark, M.R., Mohandas, N. and Shohet, S.B. (1983) Clinica. Chemica. Acta 129, 287-293.

24. Ballou, L.M., Brautigan, D.L. and Fischer, E.H. (1983) Biochemistry 22, 3393-3399.

25. Barrit, G.J., Parker, J.C. and Wandsworth, J.C. (1981) J. Physiol. 312, 29-55.

26. Baxter, M.A. and Coore, H. (1979) Biochem. Biophys. Res. Commun. 87, 433-440.

27. Baxter, M.A., Goheer, M.A. and Coore, H.G. (1979) FEBS Lett. 97, 27-31.

28. Beavo, J.A., Bechtel, P.J. and Krebs, E.G. (1974) Proc. Natl. Acad. Sci. USA 71, 3580-3583.

29. Beavo, J.A., Hardman, J.G. and Sutherland, E.W. (1971) J. Biol. Chem. 246, 3841-3846.

30. Beg, Z.H., Allman, D.W. and Gibson, D.W. (1973) Biochem. Biophys. Res. Commun. 54, 1362-1369.

31. Beg, Z.H. and Brewer, H.B.Jr. (1981) Current Topics in Cellular Regulation vol. 20 pp. 139-184 (Horecker, B.L. and Stadtman, E.R. eds.) Academic Press, New York.

32. Beg, Z.H., Stonik, J.A. and Brewer, H.B. Jr. (1978) Proc. Natl. Acad. Sci. USA 75, 3678-3682.

33. Beg, Z.H., Stonik, J.A. and Brewer, H.B.Jr. (1979) Proc. Natl. Sci. USA 76, 4375-4379.

34. Beg, Z.H., Stonik, J.A. and Brewer, H.B.Jr. (1980) J. Biol. Chem. 255, 8541-8545.

35. Beg, Z.H., Stonik, J.A. and Brewer, H.B.Jr. (1984) Proc. Natl. Acad. Sci. USA 81, 7293-7297.

36. Beg, Z.H., Stonik, J.A. and Brewer, H.B.Jr. (1985) J. Biol. Chem. 260, 1682-1687.

37. Belfrage, P., Fredrikson, G., Nilson, N.O. and Stralfors, P. (1979) INSERM Colloq. 87, 161-178.

38. Belfrage, P., Fredrikson, G., Nilson, N.O. and Stralfors, P. (1981) Int. J. Obesity 5, 635-641.

39. Benjamin, W.B. and Singer, I. (1975) Biochemistry 14, 3301-3309.

40. Bennett, M.K., Erondu, N.E. and Kennedy, M.B. (1983) J. Biol. Chem. 258, 12735-12744.

41. Benovic, J.L., Pikes, L.J., Cerione, R.A., Staniszewski, C., Yoshimasa, T., Codina, J., Caron, M.G. and Lefkowitz, R.J. (1985) J. Biol. Chem. 260, 7094-7101.

42. Bergmeyer, H.U. and Bernt, E. (1974) In Methods of Enzymatic Analysis, 2nd edition (Bergmeyer, H.U. ed.) vol. 2 pp. 574-579, Verlag Chemie, Weinheim.

43. Bergmeyer, H.U., Bernt, E., Schmidt, F. and Stork, H. (1974) In Methods of Enzymatic Analysis, 2nd edition (Bergmeyer, H.U. ed.) vol. 2 pp. 1196-1201, Verlag, Chemie, Weinheim.

44. Bergstrand, H., and Lundquist, B. (1978) Mol. Cell. Biochem. 21, 9-19.

45. Berridge, M.J. (1981) Mol. Cell. Endocrinol. 24, 115-140.

46. Berridge, M.J. (1984) Biochem. J. 220, 345-360.

46a. Bershtein, C.M. and Aleksandrov, V.A. (1977) Bull. Exp. Biol. Med. (USSR) (Eng. Transl.) 84, 1177-1180.

47. Billah, M.M. and Michell, R.H. (1979) Biochem. J. 182, 661-668.

48. Bingham, E.W. and Farrell, H.M. (1977) J. Dairy Sci. 60, 1199-1207.

49. Bingham, E.W. and Groves, M.L. (1979) J. Biol. Chem. 254, 4510-4515.

50. Blackshear, P.J., Nemenoff, R.A. and Avruch, J. (1984) Endocrinology 114, 141-152.

52. Bortz, W.M. and Lynen, F. (1963) Biochem. Z. 337, 505-509.

53. Bove, J. and Hegardt, F.G. (1978) FEBS Lett. 90, 198-202.

54. Bradbury, E.M., Inglis, R.J. and Matthews, H.R. (1974) Nature 247, 257-261.

55. Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.

56. Brooks, C.L. and Landt, M. (1985) Arch. Biochem. Biophys. 240, 663-673.

57. Brosnan, M.E., Ilic, V. and Williamson, D.H. (1982) Biochem. J. 202, 693-698.

58. Brostrom, C.O. and Wolff, D.J. (1976) Arch. Biochem. Biophys. 172, 301-311.

59. Brown, A.E., Lok, M.P. and Elovson, J. (1976) Biochim. Biophys. Acta 426, 418-432.

60. Brown, M.S., Goldstein, J.L. and Dietschy, J.M. (1979) J. Biol. Chem. 254, 5144-5149.

61. Brown, D.A. and Simoni, R.D. (1984) Proc. Natl. Acad. Sci. USA 81, 1674-1678.

62. Brown, M.S., Brunschede, G.Y. and Goldstein, J.L. (1975) J. Biol. Chem. 250, 2502-2509.

63. Brown, W.E. and Rodwell, V.W. (1983) Biochim. Biophys. Acta 751, 218-229.

64. Browning, E.T., Brostrom, C.O. and Groppi, V.E. (1976) Mol. Pharmacol. 12, 32-40.

65. Brownsey, R.W. (1981) Biochem. Soc. Trans. 9, 515-518.

66. Brownsey, R.W. and Denton, R.M. (1982) Biochem. J. 202, 77-86.

67. Brownsey, R.W. and Hardie, D.G. (1980) FEBS Lett. 120, 67-70.

68. Brownsey, R.W., Hughes, W.A., Denton, R.M. and Mayer, R.J. (1977) Biochem. J. 168, 441-445.

69. Brownsey, R.W., Hughes, W.A. and Denton, R.M. (1979) Biochem. J. 184, 23-32.

70. Builder, S.E., Beavo, J.A. and Krebs, E.G. (1980) J. Biol. Chem. 255, 2350-2354.

71. Builder, S.E., Beavo, J.A. and Krebs, E.G. (1980) J. Biol. Chem. 255, 3514-3519.

72. Burgess, G.M., Godfrey, P.P., McKinney, J.S., Berridge, M.J., Irvine, R.F. and Putney, J.W. (1984) Nature 309, 63-66.

73. Bussmann, L.E., Ward, S. Kuhn, N.J. (1984) Biochem. J. 219, 173-180.

74. Butcher, R.W., Ho, R.J., Meng, H.C. and Sutherland, E.W. (1965) J. Biol. Chem. 240, 4515-4523.

75. Cameron, C.M. and Rillema, J.A. (1983) Proc. Soc. Exp. Biol. Med. 173, 306-311.

76. Canellakis, E.S., Viceps-Madore, D., Kyriakidis, D.A. and Heller, J.S. (1979) In Current Topics in Cellular Regulation pp. 156-202 (Horecker, B.C. and Stadtman, E.R. eds.) Academic Press, New York.

77. Cantley, L.C.Jr. and Aisen, P. (1979) J. Biol. Chem. 254, 1781-1784.

78. Carlson, C.A. and Kim, K.H. (1973) J. Biol. Chem. 248, 378-380.

79. Carlson, C.A. and Kim, K.H. (1974) Arch. Biochem. Biophys. 164, 478-489.

80. Carlson, C.A. and Kim, K.H. (1974) Arch. Biochem. Biophys. 164, 490-501.

81. Carmichael, D.F. (1980) Fed. Proc. 40, 1609.

82. Carmichael, D.F., Geahlen, R.L., Allen, S.M. and Krebs, E.G. (1982) J. Biol. Chem. 257, 10440-10445.

82a. Carrick, D.T. and Kuhn, N.J. (1978) Biochem. J. 174, 319-325.

83. Casnellie, J.E. and Greengard, P. (1974) Proc. Natl. Acad. Sci. USA 71, 1891-1895.

84. Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. (1982) J. Biol. Chem. 257, 7847-7851.

85. Castano, J.G., Nieto, A. and Feliu, J.L. (1979) J. Biol. Chem. 254, 5576-5579.

86. Caufield, J.J. and Bolander, F.F.Jr. (1986) J. Endocrinology (in press).

87. Cercek, B., Wilson, S.R. and Houslay, M.D. (1983) Biochem. J. 213, 89-97.

88. Chambard, J.-C., Franchi, A., Le Cam, A. and Pouyssegur, J. (1983) J. Biol. Chem. 258, 1706-1713.

89. Chang, T.-Y., Limanek, J.S. and Chang, C.C.Y. (1981) J. Biol. Chem. 256, 6174-6180.

89a. Chatwin, A.L., Linzell, J.L. and Setchell, B.P. (1969) J. Endocrinol. 44, 247-254.

90. Cheung, W.Y. (1969) Biochim. Biophys. Acta 191, 303-315.

91. Cheung, W.Y. (1970) J. Biol. Chem. 246, 2859-2869.

92. Chin, D.J., Gil, G., Russell, D.W., Liscum, L., Luskey, K.L., Basu, S.K., Okayama, H., Berg, P., Goldstein, J.L. and Brown, M.S. (1984) Nature 308, 613-617.

93. Chow, J.C., Higgins, M.J.P. and Rudney, H. (1975) Biochem. Biophys. Res. Commun. 63, 1077-1084.

93a. Christie, W.W., Hunter, M.L. and Clegg, R.A. (1981) Biochim. Biophys. Acta 666, 284-290.

94. Chuang, D.T., Nui, W.L. and Cox, R.P. (1981) Biochem. J. 200, 59-67.

95. Clarenburg, R. and Chaikoff, J.L. (1966) J. Lipid Res. 7, 27-37.

96. Clarke, C.F., Edwards, P.A., Lau, S.-F., Tanaka, R.D. and Fogleman, A.M. (1983) Proc. Natl. Acad. Sci. USA 80, 3305-3308.

97. Claus, T.H., Schlumpf, J.R., El-Maghrabi, M.R., Pilkis, J. and Pilkis, S.J. (1980) Proc. Natl. Acad. Sci. USA 77, 6501-6505.

98. Claus, T.H., Schlumpf, J.R., El-Maghrabi, M.R. and Pilkis, S.J. (1982) J. Biol. Chem. 257, 7541-7548.

99. Clegg, R.A. (1981b) Biochim. Biophys. Acta 664, 397-408.

100. Clegg, R.A. (1987) Curr. Topics Cell. Regul. 28 (in press).

100a. Clegg, R.A. (personal communication).

101. Clegg, R.A. and Mullaney, I. (1985) Biochem. J. 230, 239-246.

102. Clegg, R.A., Mullaney, I., Robson, N.A., Zammit, V.A. (1986) Biochem. J. 240, 13-18.

103. Clegg, R.A., West, D.W. and Aitchison, R.E.D. (1987) Biochem. J. 241, 447-454.

104. Cochet, C. and Chambaz, E.M. (1983) Mol. Cell. Endocrinol. 30, 247-266.

105. Cochet, C., Feige, J.-J. and Chambaz, E.M. (1983) Biochim. Biophys. Acta 744, 147-154.

106. Cochet, C., Gill, G.N., Meisenhelder, J., Cooper, J.A. and Hunter, T. (1984) J. Biol. Chem. 259, 2553-2558.

107. Cochet, C., Job, D., Pirollet, F. and Chambaz, E.M. (1980) Endocrinology 106, 750-757.

108. Cochet, C., Job, D., Pirollet, F. and Chambaz, E.M. (1981) Biochim. Biophys. Acta 658, 191-201.

109. Codina, J., Hildebrandt, J., Sunyer, T., Sekura, R.D., Manclark, C.R., Iyengar, R. and Birnbaumer, L. (1984) Adv. Cyclic. Nucl. and Protein Phos. Res. vol. 17 pp. 153-159 (Greengard, P. et al. eds.) Raven Press, New York.

110. Cohen, P. (1973) Eur. J. Biochem. 34, 1-14.

111. Cohen, P. (1978) Curr. Top. Cell. Regul. 14, 117-196.

112. Cohen, P. (1980) Eur. J. Biochem. 111, 564-574.

113. Cohen, P. (1980) FEBS Lett. 119, 301-306.

114. Cohen, P. in Molecular Aspects of Cellular Regulation vol. 1 (Cohen, P. ed.) pp. 255-268 (Elsevier, Amsterdam, 1980).

115. Cohen, P. (1982) Nature, 296, 613-620.

116. Cohen, P. (1983) Phil. Trans. R. Soc. Lond. B302, 13-25.

117. Cohen, P. (1985) Abstract. 8th International Conference on Metabolic Interconversion of Enzymes. Pitlochry, Scotland.

118. Cohen, P. (1985) Abstract. 614th Biochem. Soc. Meeting, Oxford.

119. Cohen, P. and Antoniw, J.F. (1973) FEBS Lett. 34, 43-47.

120. Cohen, P., Nimmo, H.G. and Antoniw, J.F. (1977) Biochem. J. 162, 435-444.

121. Cohen, P., Rylatt, D.B. and Nimmo, G.A. (1977) FEBS Lett. 76, 182-186.

122. Cohen, P., Yellowlees, D., Aitken, A., Donella-Deana, A., Hemmings, B.A. and Parker, P.J. (1982) Eur. J. Biochem. 124, 21-35.

123. Cohen, S., Carpenter, G. and King, L.E.Jr. (1980) J. Biol. Chem. 255, 4834-4842.

123a. Cook, K.G., Bradford, A.P., Yeaman, S.J., Aitken, A., Fearnley, I.M. and Walker, J.E. (1984) Eur. J. Biochem. 145, 587-591.

124. Cook, K.G., Colbran, R.J., Snee, J. and Yeaman, S.J. (1983) Biochim. Biophys. Acta 752, 46-53.

124a. Cook, K.G., Lawson, R. and Yeaman, S.J. (1983) FEBS Lett. 157, 59-62.

124b. Cook, K.G., Lawson, R. and Yeaman, S.J. (1983) FEBS Lett. 164, 85-88.

125. Cook, K.G., Lee, F.-T. and Yeaman, S.J. (1981) FEBS Lett. 145, 271-276.

126. Cook, K.G., Yeaman, S.J., Stralfors, P., Fredrikson, G. and Belfrage, P. (1982) Eur. J. Biochem. 125, 245-249.

127. Cooper, D.M. (1983) Current Topics in Membrane Transport vol. 18, pp. 67-84.

127a. Cooper, J.A., Sefton, B.M. and Hunter, T. (1983) Meth. Enzymol. 99, 387-402.

128. Corbin, J.D. and Krebs, E.G. (1969) Biochem. Biophys. Res. Commun. 36, 328-335.

129. Corbin, J.D., Rannels, S.R., Flockhart, D.A., Robinson-Steiner, A.M., Tigani, M.C., Doskeland, S.O., Suva, R.M., Suva, R. and Miller, J.P. (1982) Eur. J. Biochem. 125, 1259-1266.

130. Corbin, J.D., Reinmann, E.M., Walsh, D.A. and Krebs, E.G. (1970) J. Biol. Chem. 245, 4849-4851.

131. Corbin, J.D., Sugden, P.H., West, L., Flockhart, D.A., Lincoln, T.M. and McCarthy, D. (1978) J. Biol. Chem. 253, 3997-4003.

132. Costa, M. (1977) Biochem. Biophys. Res. Commun. 78, 1311-1318.

133. Couchie, D., Erneux, C. and Dumont, J.E. (1981) Biochem. J. 199, 441-446.

134. Cox, A.J., Malnoe, A. and Stein, E.A. (1981) J. Biol. Chem. 256, 3218-3222.

134a. Cowie, A.T., Forsyth, I.A. and Hart, I.C. (1980) Hormonal Control of Lactation, Springer-Verlag, Berlin.

135. Cowie, A.T., Duncombe, W.G., Folley, S.J., French, T.H., Glascock, R.F., Massart, L., Peeters, G.J. and Popjak, G. (1951) Biochem. J. 49, 610-615.

135a. Craft, I.L. (1970) Clin. Sci. 38, 287-295.

136. Creba, J.A., Downes, C.P., Hawkins, P.T., Brewster, G., Michell, R.H. and Kirk, C.J. (1983) Biochem. J. 212, 733-747.

136a. Cripps, A.W. and Williams, V.J. (1975) Br. J. Nutr. 33, 17-32.

137. Crompton, M., Kessar, P. and Al-Nasser, I. (1983) Biochem. J. 216, 333-342.

138. Cushman, S.W., Wardzala, L.J., Simpson, I.A., Karnieli, E., Hissin, H.J., Wheeler, T.J., Hinkle, P. and Salans, L.B. (1983) Horm. Cell. Regul. 7, 73-84.

139. Czech, M.P. (1985) Ann. Rev. Physiol. 47, 357-381.

139a. Dabrowska, R. and Hartshorne, D.J. (1978) Biochem. Biophys. Res. Commun. 85, 1352-1359.

140. Dabrowska, R., Sherry, J.M.F., Aromatorio, D.K. and Hartshorne, D.J. (1978) Biochemistry 17, 253-258.

141. Dahmus, M.E. (1981) J. Biol. Chem. 256, 3319-3325.

142. Dahmus, M.E. (1981) J. Biol. Chem. 256, 3332-3339.

142a. Damuni, Z., Humphreys, J.S. and Reed, L.J. (1986) Proc. Natl. Acad. Sci. USA, 83, 285-289.

143. Damuni, Z., Merryfield, M.L., Humphreys, J.S. and Reed, L.J. (1984) Proc. Natl. Acad. Sci. USA 81, 4335-4338.

144. Danner, D.J., Lemmon, S.K. and Elsas, L.J. (1978) Biochem. Med. 19, 27-38.

145. Darfler, F.J., Mahan, L.C., Koachman, A.M. and Insel, P.A. (1982) J. Biol. Chem. 257, 11901-11907.

146. Davis, C.W. (1982) Biochim. Biophys. Acta 705, 1-7.

147. De Jonge, H.R. (1976) Nature 262, 590-592.

148. De Jonge, H.R. and Rosen, O.M. (1977) J. Biol. Chem. 252, 2780-2783.

149. Demaille, J.G., Peters, K.A. and Fischer, E.H. (1977) Biochemistry 16, 3080-3086.

150. De Meis, L. and De Paula, H.J. (1967) Arch. Biochem. Biophys. 119, 16-21.

151. De Meyts, P., Bianco, A.R. and Roth, J. (1976) J. Biol. Chem. 251, 1877-1888.

152. De Meyts, P., Roth, J., Neville, D.M.Jr., Gavin, J.R. III and Lesniak, M.A. (1973) Biochem. Biophys. Res. Commun. 55, 154-161.

153. Denton, R.M. (1985) Abstracts of 13th International Congress of Biochemistry, Amsterdam, Code No. MO-C39.

154. Denton, R.M., Coore, M.G., Martin, B.R. and Randle, P.J. (1971) Nature NB 231, 115-116.

155. Denton, R.M., Hughes, W.A., Bridges, B., Brownsey, R.W., McCormack, J.G. and Stansbie, D. (1978) Hormones and Cellular Regulation Vol. 2. (Dumont, J. and Nunez, J. eds.) pp. 191-208. (Elsevier/North-Holland Biomedical Press).

156. Denton, R.M., McCormack, J.G. and Marshall, S.E. (1984) Biochem. J. 217, 441-452.

157. Denton, R.M., Randle, P.J. and Martin, B.R. (1972) Biochem. J. 128, 161-163.

158. De Paoli-Roach, A.A. and Roach, P.J. (1982) Arch. Biochem.

158a. De Paoli-Roach, A.A., Roach, P.J. and Larner, J. (1979) J. Biol. Chem. 254, 4212-4219.

159. De Paoli-Roach, A.A., Roach, P.J., Pham,K., Kramer, G. and Hardesty, B. (1981) J. Biol. Chem. 256, 8871-8874.

160. De Reimer, S.A., Kaczmarek, L.K., Lai, Y., McGuinness, T.L. and Greengard, P. (1984) J. Neurosci. 4, 1618-1625.

161. Desai, D. and Hollenberg, C.H. (1975) J. Med. Sci. 11, 540-550.

162. Dietschy, J.M. and Brown, M.S. (1974) J. Lipid Res. 15, 508-516.

163. Dils, R.R. (1983) in Biochemistry of Lactation. 141-157 (Mepham, T.B. ed.) Elsevier, Amsterdam.

164. Donner, D.B. and Corin, R.E. (1980) J. Biol. Chem. 255, 9005-9008.

165. Dotan, I. and Shechter, I. (1982) Biochim. Biophys. Acta 713, 427-434.

166. Downward, J., Waterfield, M.D. and Parker, P.J. (1985) J. Biol. Chem. 260, 14538-14546.

167. Duceman, B.W., Rose, K.M. and Jacob, S.T. (1981) J. Biol. Chem. 256, 10755-10758.

168. Dugan, R.E. and Porter, J.W. (1977) in Biochemical Actions of Hormones (Litwack, G.P. ed.) Vol. IV pp. 197-247. Academic Press, New York.

169. Durst, D.S. and Martin, T.F.J. (1982) J. Biol. Chem. 257, 7566-7573.

170. DuVernay, V.H.Jr. and Traugh, J.A. (1978) Biochemistry 17, 2045-2049.

171. Earp, H.S., Austin, K.S., Buessow, S.C., Dy, R. and Gillespie, G.Y. (1984) Proc. Natl. Acad. Sci. USA 81, 2347-2351.

172. Earp, H.S., Rubin, R.A., Austin, K.S. and Dy, R.C. (1983) FEBS Lett. 161, 180-184.

173. Easom, R.A. and Zammit, V.A. (1984) Biochem. J. 220, 739-745.

174. Edwards, P.A., Fogleman, A.M. and Tanaka, R.D. (1983) in 3-Hydroxy 3-methylglutaryl Co Enzyme A reductase (Sabine, J.R. ed.) 93-105 CRC press, Florida.

175. Edwards, P.A., Lan, S.-F., Tanaka, R.D. and Fogleman, A.M. (1983) J. Biol. Chem. 258, 7272-7275.

176. Edwards, P.A., Lemongello, D. and Fogleman, A.M. (1979) J. Lipid Res. 20, 2-7.

177. Ek, B. and Heldin, C.H. (1982) J. Biol. Chem. 257, 10486-10492.

178. Ekerdt, R. and Papahadjopoulous, D. (1982) Proc. Natl. Acad. Sci. USA 79, 2273-2277.

179. Ekman, P., Dahlqvist, U., Humble, E. and Engstrom, L. (1976) Biochim. Biophys. Acta. 358, 289-298.

180. El-Maghrabi, M.R., Claus, T.H., Pilkis, J., Fox, E. and Pilkis, S.J. (1982) J. Biol. Chem. 257, 7603-7607.

181. El-Maghrabi, M.R., Claus, T.H., Pilkis, J. and Pilkis, S.J. (1981) Biochem. Biophys. Res. Commun. 101, 1071-1077.

182. El-Maghrabi, M.R., Claus, T.H., Pilkis, J. and Pilkis, S.J. (1981) Proc. Natl. Acad. Sci. USA 79, 315-319.

183. El-Maghrabi, M.R., Fox, E., Pilkis, J. and Pilkis, S.J. (1982) Biochem. Biophys. Res. Commun. 106, 794-802.

184. El-Maghrabi, M.R., Pate, T.M., Pilkis, J. and Pilkis, S.J. (1984) J. Biol. Chem. 259, 13104-13110.

185. Elsas, L., Danner, D., Lubitz, D., Fermhoff, P. and Dembure, P. (1981) in Metabolic and Clinical Implications of Branched Chain Amino and Ketoacids (Walser, M. and Williamson, J.R. eds.) 369-382. Elsevier, New York.

185a. Embi, N., Rylatt, D.B. and Cohen, P. (1979) Eur. J. Biochem. 100, 339-347.

186. Embi, N., Rylatt, D.B. and Cohen, P. (1979) Eur. J. Biochem. 107, 519-527.

186a. Embi, N., Rylatt, D.B. and Cohen, P. (1979) in Modulation of Protein Function (Atkinson, D.E. and Fox, C.F., eds.) pp. 257-271.

187. Emerman, J.T., Bartley, J.C. and Bissell, M.J. (1980) Biochem. J. 192, 695-702.

188. Endo, T., Naka, M. and Hidaka, H. (1982) Biochem. Biophys. Res. Commun. 105, 942-948.

189. Engfeldt, P., Arner, P., Bolinder, J. and Ostman, J. (1982) J. Clin. Endocr. and Metab. 55, 983-988.

190. Engfeldt, P., Arner, P. and Ostman, J. (1982) Metabolism 31, 910-916.

191. Engstrom, L. (1980) in Molecular Aspects of Cellular Regulation vol. 1 (Cohen, P. ed.) 11-31 (Elsevier, Amsterdam).

192. Epstein, P.M., Hersch, E.M. and Thompson, W.J. (1976) Fed. Proc. Am. Soc. Exp. Biol. 35, 511.

193. Epstein, P.M., Pledger, W.J., Gardner, E.A., Stancel, G.M., Thompson, W.J. and Strada, S.J. (1978) Biochim. Biophys. Acta. 527, 442-455.

194. Epstein, P.M., Strada, S.J., Sarada, K. and Thompson, W.J. (1982) Arch. Biochem. Biophys. 218, 119-133.

195. Erikson, S.K., Shrewsbury, M.A., Gould, R.G. and Cooper, A.D. (1980) Biochim. Biophys. Acta 620, 70-79.

196. Erlichman, J., Rosenfield, R. and Rosen, O.M. (1974) J. Biol. Chem. 249, 5000-5003.

197. Erlichman, J., Sakar, D., Fleischer, N. and Rubin, C.S. (1980) J. Biol. Chem. 255, 8179-8184.

198. Exton, J.H. (1979) Biochem. Pharmacol. 28, 2237-2240.

199. Exton, J.H. (1979) J. Cyclic Nucleotide Res. 5, 277-287.

200. Exton, J.H. (1980) Am. J. Physiol. 238, E3-E12.

201. Exton, J.H. and Park, C.R. (1968) J. Biol. Chem. 243, 4189-4196.

202. Exton, J.H. and Park, C.R. (1969) J. Biol. Chem. 244, 1424-1433.

203. Fain, J.N. (1980) in Biochemical Action of Hormones (Litwack, G.P. ed.) vol. VII pp. 119-204, Academic Press, New York.

204. Fain, J.N. (1984) Metabolism 33, 672-679.

205. Farese, R.V. (1984) Mol. Cell. Endocrinol. 35, 1-14.

206. Farese, R.V., Larson, R.E., Sabir, M.A. and Gomez-Sanchez, C.E. (1983) Endocrinology 113, 1377-1386.

207. Fatania, H.R., Lau, K.S. and Randle, P.J. (1981) FEBS Lett. 132, 285-288.

208. Fatania, H.R., Lau, K.S. and Randle, P.J. (1982) FEBS Lett. 147, 35-38.

209. Faust, J.R., Luskey, K.L., Chin, D.J., Goldstein, J.L. and Brown, M.S. (1982) Proc. Natl. Acad. Sci. USA 79, 5205-5209.

210. Feige, J.J., Pirollet, F., Cochet, C. and Chambaz, E.M. (1980) FEBS Lett 121, 139-142.

211. Feldman, R.A., Wang, L.-H., Hanafusa, H. and Balduzzi, P.C. (1982) J. Virol. 42, 228-236.

211a. Fell, B.F., Smith, K.A. and Campbell, R.M. (1963) J. Pathol. Bacteriol. 85, 179-188.

212. Fell, D.A. (1980) J. Theor. Biol. 84, 361-385.

213. Ferrer, A. and Hegardt, F.G. (1984) Arch. Biochem. Biophys. 230, 227-237.

213a. Field, B. and Coore, H.G. (1976) Biochem. J. 156, 333-337.

214. Finkel, R.S. and Volpe, J.J. (1979) Biochim. Biophys. Acta 572, 461-471.

215. Fleischer, N., Rosen, O.M. and Reichlin, M. (1976) Proc. Natl. Acad. Sci. USA 73, 54-58.

216. Flint, D.J., Sinnett-Smith, P.A., Clegg, R.A. and Vernon, R.G. (1979) Biochem. J. 182, 421-427.

217. Flockerzi, V., Speichermann, N. and Hofmann, F. (1978) J. Biol. Chem. 253, 3395-3399.

218. Flockhart, D.A. and Corbin, J.D. (1982) CRC Crit. Rev. Biochem. 12, 133-186.

219. Flory, W., Peczon, B.D., Koeppe, R.E. and Spivey, H.O. (1974) Biochem. J. 141, 127-131.

220. Font, E., Stiges, M. and Hegardt, F.G. (1981) Arch. Biochem. Biophys. 210, 224-229.

221. Forn, J. and Greengard, P. (1976) Arch. Biochem. Biophys. 176, 721-733.

222. Foster, J.L., Guttman, J. and Rosen, O.M. (1981) J. Biol. Chem. 256, 5029-5036.

223. Foulkes, J.G. and Cohen, P. (1979) Eur. J. Biochem. 97, 251-256.

224. Foulkes, J.G. and Cohen, P. (1980) Eur. J. Biochem. 105, 195-203.

225. Foulkes, J.G., Cohen, P., Strada, S.J., Everson, W.V. and Jefferson, L.S. (1982) J. Biol. Chem. 257, 12493-12496.

226. Foulkes, J.G., Jefferson, L.S. and Cohen, P. (1980) FEBS Lett. 112, 21-24.

227. Francis, S.H. and Kono, T. (1982) Mol. Cell. Biochem. 42, 109-116.

228. Fredrikson, G., Stralfors, P., Nilsson, N.O. and Belfrage, P. (1981) J. Biol. Chem. 256, 6311-6320.

229. Friedman, D.J. and Larner, J. (1963) Biochemistry 2, 669-675.

230. Fukunaga, K., Yamamoto, H., Matsui, K., Higashi, K. and Miyamoto, E. (1982) J. Neurochem. 39, 1607-1617.

231. Fukunaga, K., Yamamoto, H., Tanaka, E., Iwasa, T. and Miyamoto, E. (1984) Life Sci. 35, 493-500.

232. Furuya, E. and Uyeda, K. (1981) J. Biol. Chem. 256, 7109-7112.

233. Furuya, E., Yokoyama, M. and Uyeda, K. (1982) Biochem. Biophys. Res. Commun. 105, 264-270.

234. Gabrielli, B., Wettenhall, R.E.H., Kemp, B.E., Quinn, M. and Bizonova, L. (1984) FEBS Lett. 175, 219-225.

235. Gain, K.R. and Appleman, M.M. (1978) Adv. Cycl. Nucl. Res. 9, 221-231.

236. Garland, P.B. (1964) Biochem. J. 92, 10C-12C.

237. Garland, P.B. and Randle, P.J. (1964) Biochem. J. 91, 6C-7C.

238. Garrison, J.C., Johnsen, D.E. and Campanile, C.P. (1984) J. Biol. Chem. 259, 3238-3292.

239. Gavin, J.R. III, Roth, J., Neville, D.M.Jr., De Meyts, P. and Buell, D.N. (1974) Proc. Natl. Acad. Sci. USA 71, 84-88.

240. Geahlen, R.L., Carmichael, D.F., Hashimoto, E. and Krebs, E.G. (1982) Adv. Enzyme Regul. 20, 195-209.

241. Geahlen, R.L., Allen, S.M. and Krebs, E.G. (1981) J. Biol. Chem. 256, 4536-4540.

242. Geahlen, R.L. and Krebs, E.G. (1980) J. Biol. Chem. 255, 1164-1169.

243. Geahlen, R.L. and Krebs, E.G. (1980) J. Biol. Chem. 255, 9375-9379.

244. Gibbons, G.F., Pullinger, C.R., Munday, M.R. and Williamson, D.H. (1983) Biochem. J. 212, 843-848.

245. Gibson, D.M. and Ingebritsen, T.S. (1978) Life Sci. 23, 2649-2664.

246. Gibson, D.M., Parker, R.A., Stewart, C.S. and Evensen, K.J. (1982) Adv. Enzyme Regul. 20, 263-283.

247. Gietzen, K., Sardof, I. and Bader, H. (1982) Biochem. J. 207, 541-548.

248. Gietzen, K., Xu, Y.-H., Galla, H.-J. and Bader, H. (1982) Biochem. J. 207, 637-640.

249. Gil, G., Stiges, M., Bove, J. and Hegardt, F.G. (1980) FEBS Lett. 110, 195-199.

250. Gil, G., Stiges, M. and Hegardt, F.G. (1981) Biochim. Biophys. Acta 663, 211-221.

251. Gill, G.N., and McCune, R.W. (1981) Curr. Topics Cell. Regul. 15, 1-45.

252. Gill, G.N., Walton, G.M. and Sperry, P.J. (1977) J. Biol. Chem. 252, 4269-4275.

253. Gilman, A.G. (1984) J. Clin. Invest. 73, 1-4.

254. Ginsberg, B.H. (1977) Biochemical Actions of Hormones Vol. IV pp. 313-349 (Litwack, G. ed.) Academic press, New York.

255. Glass, D.B. and Krebs, E.G. (1979) J. Biol. Chem. 254, 9728-9738.

256. Glass, D.B. and Krebs, E.G. (1980) Ann. Rev. Pharmacol. Toxicol. 20, 363-388.

257. Glass, D.B. and Krebs, E.G. (1982) J. Biol. Chem. 257, 1196-1200.

258. Goldenring, J.R., Gonzalez, B., McGuire, J.S.Jr. and De lorenzo, R.J. (1983) J. Biol. Chem. 258, 12632-12640.

259. Goldfarb, S. (1972) FEBS Lett. 24, 153-155.

260. Goldfine, I.D. (1981) Biochemical Actions of Hormones Vol. VIII pp. 273-305 (Litwack, G. ed.) Academic Press, New York.

261. Goodridge, A.G. (1972) J. Biol. Chem. 247, 6946-6952.

262. Gorelick, F.S., Cohn, J.A., Freedman, S.D., De la Haunt, N.G., Gershoni, J.M. and Jamieson, J.D. (1983) J. Cell. Biol. 97, 1294-1298.

263. Goren, E.N. and Rosen, O.M. (1971) Arch. Biochem. Biophys. 142, 720-723.

264. Goris, J., Defreyn, G. and Merlevede, W. (1979) FEBS Lett. 99, 279-282.

265. Goren, E.N. and Rosen, O.M. (1972) Arch. Biochem. Biophys. 153, 384-397.

266. Granot, J., Mildvan, A.S. and Kaiser, E.T. (1980) Arch. Biochem. Biophys. 205, 1-17.

267. Green, A. (1983) FEBS Lett 152, 261-264.

268. Gregolin, C. Ryder, E. and Lane, M.D. (1968) J. Biol. Chem. 243, 4227-4235.

269. Gregolin, C., Ryder, E., Warner, R.C., Kleinschmidt, A.K., Chang, H.C. and Lane, M.D. (1968) J. Biol. Chem. 243, 4236-4245.

270. Gregolin, C., Ryder, E., Warner, R.C., Kleinschmidt, A.K. and Lane, M.D. (1966) Proc. Natl. Acad. Sci. USA 56, 1751-1758.

270a. Grigor, M.R., Geursen, A., Sneyd, M.J. and Warren, S.M. (1982) Biochem. J. 208, 611-618.

271. Grigorescu, F., White, M.F. and Kahn, C.R. (1983) J. Biol. Chem. 258, 13708-13716.

272. Grinstead, G.F., Trzaskos, J.M., Billheimer, J.T. and Gaylor, J.L. (1983) Biochim. Biophys. Acta. 751, 41-45.

273. Grunberger, G. and Gordon, P. (1982) Am. J. Physiol. 6, E319-E324.

274. Guan, W.R. and Cheung, W.Y. (1980) Arch. Biochem. Biophys. 204, 191-198.

275. Haddox, M.K., Newton, N.E., Hartle, O.K. and Goldberg, N.D. (1972) Biochem. Biophys. Res. Commun. 47, 653-661.

275a. Haiech, J. and Demaille, J.G. (1983) Phil. Trans. R. Soc. Lond. B. 302, 91-98.

276. Halestrap, A.P. and Denton, R.M. (1974) Biochem. J. 142, 365-377.

276a. Hallowes, R.C., Wang, D.Y., Lewis, D.J., Strong, C.R. and Dils, R. (1973) J. Endocr. 57, 265-275.

277. Hammond, J.M., Jarret, L., Mariz, I.K. and Doughaday, W.H. (1972) Biochem. Biophys. Res. Commun. 49, 1122-1128.

277a. Hamosh, M., Clary, T.R., Chernick, S.S. and Scow, R. (1970) Biochim. Biophys. Acta 210, 473-482.

278. Hanson, R.S. and Beavo, J.A. (1982) Proc. Natl. Acad. Sci. USA 79, 2788-2792.

279. Hardeman, E.C., Jenke, H.-S., and Simoni, R.D. (1983) Proc. Natl. Acad. Sci. USA 80, 1516-1520.

280. Hardie, D.G. (1980) in Molecular Aspects of Hormone Regulation on vol. I, pp. 33-62 (Cohen, P. ed.) Elsevier, Amsterdam.

281. Hardie, D.G. and Cohen, P. (1978) Eur. J. Biochem. 92, 25-34.

282. Hardie, D. G. and Cohen, P. (1978) FEBS Lett. 91, 1-7.

283. Hardie, D.G. and Guy, P.S. (1980) Eur. J. Biochem. 110, 167-177.

284. Hardie, D.G., Holland, R., Haystead, T. and Munday, M.R. (1985) Abstract 8th International conference on metabolic interconversion of enzymes, Pitlochry, Scotland.

285. Hardie, D.G., Matthews, H.R. and Bradbury, E.M. (1976) Eur. J. Biochem. 66, 37-42.

286. Hardie, D.G. and Munday, M.R. (personal commun.).

287. Hardman, J.G. and Sutherland, E.W. (1968) J. Biol. Chem. 244, 6363-6370.

288. Haring, H.U., Kasuga, M. and Kahn, C.R. (1982) Biochem. Biophys. Res. Commun. 108, 1538-1545.

289. Harris, R.A., Paxton, R. and Parker, R.A. (1982) Biochem. Biophys. Res. Commun. 107, 1497-1503.

290. Hartl, F.T. and Roskoski, R.Jr. (1982) Biochemistry, 21, 5175-5183.

291. Hartl, F.T. and Roskoski, R.Jr. (1983) J. Biol. Chem. 258, 3950-3955.

292. Harwood, H.J.Jr., Brandt, K.G. and Rodwell, V.W. (1984) J. Biol. Chem. 259, 2810-2815.

293. Haselbacher, G.K., Humbel, R.E. and Thomas, G. (1979) FEBS Lett. 100, 185-190.

294. Hashimoto, S. (1981) Biomed. Res. 2(A), 472-482.

295. Hashimoto, E., Takio, K. and Krebs, E.G. (1981) J. Biol. Chem. 256, 5604-5607.

295a. Hathaway, D.R. and Adelstein, R.S. (1979) Proc. Natl. Acad. Sci. USA 76, 1653-1657.

296. Hathaway, D.R., Adelstein, R.S. and Klee, C.B. (1981) J. Biol. Chem. 256, 8183-8189.

297. Hathaway, G.M. and Traugh, J.A. (1979) J. Biol. Chem. 254, 762-768.

298. Hathaway, G.M. and Traugh, J.A. (1981) in Current Topics in Cellular Regulation vol. 21 pp. 101-127. (Horecker, B. and Stadtman, E. eds.) Academic Press, New York.

299. Hayakawa, T., Perkins, J.P. and Krebs, E.G. (1973) Biochemistry 12, 574-580.

300. Hazeki, O. and Ui, M. (1981) J. Biol. Chem. 256, 2856-2862.

301. Heby, O. (1981) Differentiation 19, 1-20.

302. Helfman, D.M., Shoji, M. and Kuo, J.F. (1981) J. Biol. Chem. 256, 6327-6334.

303. Hemmings, B.A., Aitken, A., Cohen, P., Rymond, M. and Hofmann, F. (1982) Eur. J. Biochem. 127, 473-481.

305. Hemmings, B.A., Resink, T.J. and Cohen, P. (1982) FEBS Lett. 150, 319-324.

306. Hemmings, B.A., Yellowlees, D., Kernohan, J.C. and Cohen, P. (1981) Eur. J. Biochem. 119, 443-451.

307. Henneberg, R. and Rodwell, V.W. (1981) Fed. Proc. 40, 1604.

308. Hepp, K.D. and Renner, R. (1972) FEBS Lett. 20, 191-194.

309. Hers, H.-G. and Van Schaftingen, E. (1982) Biochem. J. 206, 1-12.

310. Heyworth, C.M., Rawal, S. and Houslay, M.D. (1983) FEBS Lett. 154, 87-91.

311. Heyworth, C.M. and Houslay, M.D. (1983) Biochem. J. 214, 547-552.

312. Heyworth, C.M., Wallace, A.V. and Houslay, M.D. (1983) Biochem. J. 214, 99-110.

313. Heyworth, C.M., Wallace, A.V., Wilson, S.R. and Houslay, M.D. (1984) Biochem. J. 222, 183-187.

314. Hiraga, A., Kikuchi, K., Tamura, S. and Tsuiki, S. (1981) Eur. J. Biochem. 119, 503-510.

315. Ho, H.C., Wirch, E., Stevens, F.C. and Wang, J.H. (1977) J. Biol. Chem. 252, 43-50.

316. Hochman, J., Katz, A. and Bachrach, U. (1978) Life Sci. 22, 1481-1484.

317. Hofmann, F., Beavo, J.A., Bechtel, P.J. and Krebs, E.G. (1975) J. Biol. Chem. 250, 7795-7801.

318. Hofmann, F., Bechtel, P.J. and Krebs, E.G. (1977) J. Biol. Chem. 252, 1441-1447.

319. Hofmann, F. and Flockerzi, V. (1983) Eur. J. Biochem. 130, 599-603.

320. Hofmann, F., Sensheimer, H.-P. and Gobel, G. (1983) FEBS Lett. 164, 350-354.

321. Hofmann, P., Tobey, R.A. and Gurley, L.R. (1975) Biochem. Biophys. Res. Commun. 63, 126-131.

322. Holland, R., Hardie, D.G., Clegg, R.A. and Zammit, V.A. (1984) Biochem. J. 217, 743-749.

323. Holland, R., Witters, L.A. and Hardie, D.G. (1984) Eur. J. Biochem. 140, 325-333.

324. Holub, B.J., Kuksis, A. and Thompson, W. (1970) J. Lipid Res. 11, 558-564.

325. Honeyman, T.W., Strohsnitter, W., Scheid, C.R. and Schimmel, R.J. (1983) Biochem. J. 212, 489-498.

326. Houslay, M.D. and Marchmont, R.J. (1981) Biochem. J. 198, 703-706.

327. Houslay, M.D., Wallace, A.V., Marchmont, R.J., Martin, B.R. and Heyworth, C.M. (1983) Adv. Cyclic Nucleotide Res. 16, 159-176.

328. Houslay, M.D., Wallace, A.V., Wilson, S.R., Marchmont, R.J. and Heyworth, C.M. (1983) Hormones and Cellular Regulation 7, 105-120.

329. Hrapchak, R.J. and Ramussen, H. (1972) Biochemistry 11, 4458-4465.

330. Huang, F.L. and Glinsmann, W.H. (1976) Eur. J. Biochem. 70, 419-426.

331. Huang, K.P., Itarte, E., Singh, T.J. and Akatsuka, A. (1982) J. Biol. Chem. 257, 3236-3242.

332. Hue, L. (1982) Biochem. J. 206, 359-365.

333. Hue, L., Blackmore, P.F. and Exton, J.H. (1981) J. Biol. Chem. 256, 8900-8903.

334. Huganir, R.L., Albert, K.A. and Greengard, P. (1983) Soc. Neurosci. Abstr. 9, 578.

335. Hughes, W.A., Brownsey, R.W. and Denton, R.M. (1980) Biochem. J. 192, 469-481.

336. Hughes, W.A. & Halestrap, A.P. (1981) Biochem. J. 196, 459-469.

337. Hunter, C.F. and Rodwell, V.W. (1980) J. Lipid Res. 21, 399-405.

338. Hunter, T. and Cooper, J.A. (1985) Ann. Rev. Biochem. 54, 897-930.

339. Hunzicker-Dunn, M. (1982) Biochim. Biophys. Acta 714, 395-406.

340. Huttunen, J.K., Ellingboe, J., Pittman, R.C. and Steinberg, D. (1970) Biochim. Biophys. Acta 218, 333-346.

341. Huttunen, J.K. and Steinberg, D. (1971) Biochim. Biophys. Acta 239, 411-427.

342. Huttunen, J.K., Steinberg, D. and Mayer, S.E. (1970) Proc. Natl. Acad. Sci. USA 67, 290-295.

343. Huttunen, J.K., Steinberg, D. and Mayer, S.E. (1970) Biochem. Biophys. Res. Commun. 41, 1350-1356.

344. Illiano, G. and Cuatrecasas, P. (1972) Science 175, 906-908.

345. Ingebritsen, T.S. (1983) in 3-hydroxy3-methylglutaryl Co-enzyme A Reductase, pp. 93-105 (Sabine, J.R. ed.) CRC press, Florida.

346. Ingebritsen, T.S., Blair, J., Guy, P., Witters, L. and Hardie, D.G. (1983) Eur. J. Biochem. 132, 275-281.

347. Ingebritsen, T.S. and Cohen, P. (1983) Eur. J. Biochem. 132, 255-261.

348. Ingebritsen, T.S., Foulkes, J.G. and Cohen, P. (1983) Eur. J. Biochem. 132, 263-274.

349. Ingebritsen, T.S., Geelen, M.J.H., Parker, R.A., Evenson, K.J. and Gibson, D.M. (1979) J. Biol. Chem. 254, 9986-9989.

350. Ingebritsen, T.S., Lee, H.-S., Parker, R.A. and Gibson, D.M. (1978) Biochem. Biophys. Res. Commun. 81, 1268-1277.

351. Ingebritsen, T.S., Parker, R.A. and Gibson, D.M. (1981) J. Biol. Chem. 256, 1138-1144.

352. Ingebritsen, T.S., Stewart, A.A. and Cohen, P. (1983) Eur. J. Biochem. 132, 297-307.

353. Inoue, M., Kishimoto, A., Takai, Y. and Nishizuka, Y. (1977) J. Biol. Chem. 252, 7610-7616.

354. Inoue, H. and Lowenstein, J.M. (1973) J. Biol. Chem. 247, 4825-4832.

355. Jacob, S.T., Rose, K.M. and Canellakis, Z.N. (1983) Adv. Polyamine Res. Vol. 4, pp. 631-646 (Bachrach, U., Kaye, A. & Chayen, R. eds.) Raven press, New York.

356. Jacobs, B.O. and Krahl, M.E. (1973) Biochim. Biophys. Acta 319, 410-415.

357. Jacobs, S. and Cuatrecasas, P. (1983) Ann. Rev. Pharmacol. Toxicol. 23, 461-479.

358. Jacobs, S., Hazum, E., Schecter, Y. and Cuatrecasas, P. (1979) Proc. Natl. Acad. Sci. USA 76, 4918-4921.

359. Jacobs, S., Hazum, E. and Cuatrecasas, P. (1980) J. Biol. Chem. 255, 6937-6940.

360. Jacobs, S., Kull, F.C.Jr., Earp, H.S., Svoboda, M.E., Van Wyk, J.J. and Cuatrecasas, P. (1983) J. Biol. Chem. 258, 9581-9584.

361. Jacobs, S., Sahyoun, N.E., Saltiel, A.R. and Cuatrecasas, P. (1983) Proc. Natl. Acad. Sci. USA 80, 6211-6213.

362. Jakobs, K.H. and Aktories, K. (1983) Biochim. Biophys. Acta 732, 352-358.

363. Jakobs, K.H., Aktories, K. and Schultz, G. (1984) Adv. Cyclic. Nucl. and Protein Phos. Res. vol. 17, pp. 135-143 (Greengard, P. et al. eds.) Raven Press, New York.

364. Jakobs, K.H., Bauer, S. and Watanabe, Y. (1985) Eur. J. Biochem. 151, 425-430.

365. Jakobs, K.H., Gehring, U., Gaugler, B., Pfeuffer, T. and Schultz, G. (1983) Eur. J. Biochem. 130, 605-611.

366. Jakoi, L. and Quarfordt, S.H. (1974) J. Biol. Chem. 249, 5840-5844.

367. Jastorff, B., Hoppe, J. and Morr, M. (1979) Eur. J. Biochem. 101, 555-561.

368. Jenness, R. (1977) in Lactation: A Comprehensive Treatise (Larson, B.L. and Smith, V.R. eds.) vol. 3 p. 21 Academic Press, New York.

369. Job, D., Cochet, C., Pirollet, F. and Chambaz, E.M. (1979) FEBS Lett. 98, 303-308.

370. Job, D., Pirollet, F., Cochet, C. and Chambaz, E.M. (1979) FEBS Lett. 108, 508-512.

371. Joost, H.-G., and Steinfelder, H.-J. (1983) Mol. Cell. Biochem. 57, 177-183.

372. Joseph, S.K., Thomas, A.P., Williams, R.J., Irvine, R.F. and Williamson, J.R. (1984) J. Biol. Chem. 259, 3077-3081.

373. Jungas, R.L. (1966) Proc. Natl. Acad. Sci. USA 56, 757-763.

374. Jungas, R.L. (1970) Endocrinology 86, 1368-1375.

375. Jurgensen, S., Shacter, E., Huang, C.Y., Chock, P.B., Yang, S.D., Vandenheede, J.R. and Merlevede, W. (1984) J. Biol. Chem. 259, 5864-5870.

376. Kagimoto, T. and Uyeda, K. (1979) J. Biol. Chem. 254, 5584-5587.

377. Kaibuchi, K., Takai, Y. and Nishizuka, Y. (1981) J. Biol. Chem. 256, 7146-7149.

378. Kakiuchi, S. and Yamazaki, R. (1970) Biochem. Biophys. Res. Commun. 41, 1104-1110.

379. Kakiuchi, S., Yamazaki, R., Teshima, Y. and Uenishi, K. (1973) Proc. Natl. Acad. Sci. USA 70, 3526-3530.

380. Kakiuchi, S., Yamazaki, R., Teshima, Y., Uenishi, K. and Miyamato, E. (1975) Biochem. J. 146, 109-120.

381. Kalderon, A.E. and Ravanshenas, S.F. (1974) Histochem. 39, 229-242.

382. Kanako, Y., Moss, J. and Vaughan, M. (1985) J. Biol. Chem. 260, 11493-11497.

383. Kaneko, I., Hazuma-Shimada, Y., Kuroda, M. and Endo, A. (1977) Biochem. Biophys. Res. Commun. 76, 1207-1213.

384. Kasuga, M., Fujita-Yamaguchi, Y., Blithe, D.L., White, M.F. and Kahn, C.R. (1983) J. Biol. Chem. 258, 10973-10980.

385. Kasuga, M., Fujita-Yamaguchi, Y., Blithe, D.L. and Kahn, C.R. (1983) Proc. Natl. Acad. Sci. USA 80, 2137-2141.

386. Kasuga, M., Karlsson, F.A. and Kahn, C.R. (1982) Science 215, 185-187.

387. Kasuga, M., Zick, Y., Blithe, D.L., Crettaz, M. and Kahn, C.R. (1982) Nature 298, 667-669.

388. Kasuga, M., Zick, Y., Blithe, D.L., Karlsson, F.A., Haring, H.U. and Kahn, C.R. (1982) J. Biol. Chem. 257, 9891-9894.

389. Katada, T., Gilman, A.G., Watanabe, Y., Bauer, S. and Jakobs, K.H. (1985) Eur. J. Biochem. 151, 431-437.

390. Katada, T. and Ui, M. (1979) J. Biol. Chem. 254, 469-479.

391. Katoh, N. and Kuo, J.F. (1982) Biochem. Biophys. Res. Commun. 106, 590-595.

392. Katoh, N., Wise, B.C. and Kuo, J.F. (1983) Biochem. J. 209, 189-195.

393. Katoh, N., Wrenn, R.W., Wise, B.C., Shoji, M. and Kuo, J.F. (1981) Proc. Natl. Acad. Sci. USA 78, 4813-4817.

394. Keith, M.L., Kennelly, P.J. and Rodwell, V.W. (1983) J. Protein Chem. 2, 209-220.

395. Keith, M.L., Rodwell, V.W., Rogers, D.H. and Rudney, H. (1979) Biochem. Biophys. Res. Commun. 90, 969-975.

396. Kelleher, D.J., Pessin, J.E., Ruoho, A.E. and Johnson, G.L. (1984) Proc. Natl. Acad. Sci. USA 81, 4316-4320.

397. Kennedy, M.B. and Greengard, P. (1981) Proc. Natl. Acad. Sci. USA 78, 1293-1297.

398. Kennelly, P.J. and Rodwell, V.W. (1985) J. Lipid Res. 26, 903-914.

399. Kerbey, A.L., Randle, P.J. and Kearns, A. (1981) Biochem. J. 195, 51-59.

400. Kerlavage, A.R. and Taylor, S.S. (1982) J. Biol. Chem. 257, 1749-1754.

401. Khan, C.R., Freychet, P., Roth, J. and Neville, D.M.Jr. (1974) J. Biol. Chem. 249, 2249-2257.

402. Khan, M.N., Posner, B.I., Khan, R.J. and Bergeron, J.J.M. (1982) J. Biol. Chem. 257, 5969-5976.

403. Khan, M.N., Posner, B.I., Verma, A.K., Khan, R.J. and Bergeron, J.J.M. (1981) Proc. Natl. Acad. Sci. USA 78, 4980-4984.

404. Khatra, B.S., Chiasson, J.L., Shikama, H., Exton, J.H. and Soderling, T.R. (1980) FEBS Lett. 114, 253-256.

405. Khoo, J.C., Jarrett, L., Mayer, S.E. and Steinberg, D. (1972) J. Biol. Chem. 247, 4812-4818.

406. Khoo, J.C., Steinberg, D. and Lee, E.Y.C. (1978) Biochem. Biophys. Res. Commun. 80, 418-423.

407. Khoo, J.C., Steinberg, D., Huang, J.J. and Vagelos, P.R. (1976) J. Biol. Chem. 251, 2882-2890.

408. Khoo, J.C., Steinberg, D., Thompson, B. and Mayer, S.E. (1972) J. Biol. Chem. 248, 3823-3830.

409. Khoo, J.C., Steinberg, D., Thompson, B. and Mayer, S.E. (1973) J. Biol. Chem. 248, 3823-3830.

410. Kiechle, F.L. and Jarett, L. (1981) FEBS Lett. 133, 279-282.

411. Kiechle, F.L., Jarett, L., Popp, D.A. and Kotagal, N. (1981) J. Biol. Chem. 256, 2945-2951.

412. Kiechle, F.L., Strauss III, J.F., Tanaka, T. and Jarett, L. (1982) Fed. Proc. 41, 1082.

413. Kikkawa, U., Takai, Y., Minakuchi, R., Inohara, S. and Nishizuka, Y. (1982) J. Biol. chem. 257, 13341-13348.

414. Kikkawa, U., Takai, Y., Tanaka, Y., Miyake, R. and Nishizuka, Y. (1983) J. Biol. Chem. 258, 11442-11445.

415. Kim, K-.H. (1979) Mol. Cell. Biochem. 28, 27-43.

416. Kirk, C.J. (1982) Cell. Calcium 3, 399-411.

417. Kirk, C.J., Creba, J.A., Downes, C.P. and Michell, R.H. (1981) Biochem. Soc. Trans. 9, 377-379.

418. Kirk, C.J., Michell, R.H. and Hems, D.A. (1981) Biochem. J. 194, 155-165.

419. Kirsten, E.S. and Watson, J.A. (1974) J. Biol. Chem. 249, 6104-6109.

420. Kishimota, A., Takai, Y., Mori, T., Kikkawa, U. and Nishizuka, Y. (1980) J. Biol. Chem. 255, 2273-2278.

421. Klee, C.B., Crouch, T.H. and Krinks, M.H. (1979) Biochemistry 18, 722-729.

422. Klee, C.B., Crouch, T.H. and Krinks, M.H. (1979) Proc. Natl. Acad. Sci. USA 76, 6260-6273.

423. Klee, C.B., Crouch, T.H. and Richman, P.G. (1980) Ann. Rev. Biochem. 49, 489-515.

424. Klee, C.B. and Krinks, M.H. (1978) Biochemistry 17, 120-126.

425. Klee, W.A., Koshi, G., Tocque, B. and Simonds, W.F. (1984) Adv. Cyclic. Nucl. and Protein Phos. Res. vol. 17 pp. 153-159 (Greengard, P. et al. eds.) Raven Press, New York.

425a. Knopp, R.H., Herrera, E. and Freinkel, N. (1970) J. Clin. Invest. 49, 1438-1445.

426. Knutson, V.P., Ronnett, G.V. and Lane, M.D. (1985) J. Biol. Chem. 260, 14180-14188.

427. Koepfer-Hobelsberger, B. and Wieland, O.H. (1984) Mol. Cell. Endocrinol. 36, 123-129.

428. Koizumi, J., Mabuchi, H. and Takeda, R. (1982) Biochem. Biophys. Res. Commun. 108, 240-246.

429. Kono, T., Robinson, F.W. and Sarver, J.A. (1975) J. Biol. Chem. 250, 7826-7835.

430. Kono, T., Robinson, F.W., Sarver, J.A., Vega, F.V. and Pointer, R.H. (1977) J. Biol. Chem. 252, 2226-2233.

431. Kosmakos, F.C. and Roth, J. (1980) J. Biol. Chem. 255, 9860-9869.

432. Kountz, P., El-Maghrabi, M.R. and Pilkis, S.J. (1983) Fed. Proc. 42, 2080.

433. Kraft, A.S. and Anderson, W.B. (1983) Nature 30, 621-623.

434. Kraft, A.S., Anderson, W.B., Cooper, H.C. and Sando, J.J. (1982) J. Biol. Chem. 257, 13193-13196.

435. Kranias, E.G. and Jungmann, R.A. (1978) Biochim. Biophys. Acta 517, 439-446.

436. Krebs, E.G. (1983) Phil. Trans. R. Soc. Lond. B302, 3-11.

437. Krebs, E.G. and Beavo, J.A. (1979) Ann. Rev. Biochem. 48, 923-959.

438. Krebs, E.G. and Fischer, E.H. (1956) Biochim. Biophys. Acta 20, 150-157.

439. Krebs, E.G., Graves, D.J. and Fischer, E.H. (1959) J. Biol. Chem. 234, 2867-2873.

440. Krebs, H.A. and Henseleit, K. (1932) Hoppe-Seyler's Z. Physiol. Chem. 210, 33-66.

441. Kuehn, G.D. (1984) in Enzyme Regulation by Reversible Phosphorylation vol. 3 pp. 185-207 (Cohen, P. ed.) Elsevier, Amsterdam.

442. Kuehn, G.D. and Atmar, V.J. (1983) Advances in Polyamine Research vol. 4 pp. 615-629 (Bachrach, U., Kaye, A. and Chayen, R. eds.) Raven Press, New York.

442a. Kuhn, N.J. (1977) in Comparative Aspects of Lactation (Peaker, M. ed.), pp. 165-192, Academic Press, London.

442b. Kuhn, N.J. (1978) Biochem. Soc. Trans. 6, 539-543.

443. Kuhn, N.J. (1983) in Biochemistry of Lactation pp. 351-379 (Mepham, T.B. ed.) Elsevier, Amsterdam.

443a. Kumaresan, P. and Turner, C.W. (1968) Proc. Soc. Exp. Biol. Med. 129, 957.

444. Kuo, J.F., Andersson, R.G.G., Wise, B.C., Mackerlova, L., Salomonsson, I., Brackett, N.L., Katoh, N. Shoji, M. and Wrenn, R.W. (1980) Proc. Natl. Acad. Sci. USA 77, 7039-7043.

445. Kuo, J.F. (1975) Biochem. Biophys. Res. Commun. 65, 1214-1220.

446. Kuo, J.F. (1975) J. Cyclic Nucleotide. Res. 1, 151-157.

447. Kuo, J.F. and Greengard, P. (1969) Proc. Natl. Acad. Sci. USA 64, 1349-1355.

448. Kuo, J.F. and Greengard, P. (1970) J. Biol. Chem. 245, 2493-3498.

449. Ladha, S., West, D.W. and Clegg, R.A. (1985) Biochem. Soc. Trans. 13, 866-867.

450. Laemmli, U.K. (1970) Nature 227, 680-685.

451. Lake, R.S. and Salzman, N.P. (1972) Biochemistry 11, 4817-4826.

452. Lambert, B. and Jacquemin, C. (1979) FEBS Lett. 105, 19-22.

453. Lane, M.D., Moss, J. and Polakis, S.E. (1974) Curr. Top. Cell. Regul. 8, 139-195.

454. Langan, T.A. (1969) Proc. Natl. Acad. Sci. USA 64, 1276-1283.

455. Langan, T.A. (1971) Ann. N.Y. Acad. Sci. 185, 166-185.

456. Langan, T.A. (1973) Adv. Cyclic Nucleotide Res. 3, 99-153.

457. LaPorte, D.C., Toscano, W.A. Jr. and Storm, D.R. (1979) Biochemistry 18, 2820-2825.

458. LaPorte, D.C., Wierman, B.H. and Storm, D.R. (1980) Biochemistry 19, 3814-3819.

459. Laskey, R.A. (1983) Phil. Trans. R. Soc. Lond. B302, 143-150.

460. Laskey, R.A., Harland, R.M., Earnshaw, W.C. and Dingwall, C. (1981) in International Cell Biology (Schweiger, H. ed.) 163-167 (Springer-Verlag, Berlin).

461. Lastick, S.M. and McConkey, E.H. (1981) J. Biol. Chem. 256, 583-585.

462. Lau, K.S., Fatania, H.R. and Randle, P.J. (1981) FEBS Lett. 126, 66-70.

463. Lau, K.S., Fatania, H.R. and Randle, P.J. (1982) FEBS Lett. 144, 57-62.

464. LeCam, A. (1982) J. Biol. Chem. 257, 8376-8385.

465. Lee, K.-H. and Kim, K.H. (1977) J. Biol. Chem. 252, 1748-1751.

466. Lee, K.-H., Thrall, T. and Kim, K.-H. (1973) Biochem. Biophys. Res. Commun. 54, 1133-1140.

467. Lehrer, G., Panini, S.R., Rogers, D.H. and Rudney, H. (1981) J. Biol. Chem. 256, 5612-5619.

468. Leiderman, L.J., Criss, W.E. and Oka, T. (1985) Biochim. Biophys. Acta 844, 95-104.

469. Leis, J.F. and Kaplan, N.O. (1982) Proc. Natl. Acad. Sci. USA 79, 6509-6511.

470. Lent, B.A., Lee, K.-H. and Kim, K.-H. (1978) J. Biol. Chem. 253, 8149-8156.

471. Le Peuch, C.J., Ballester, R. and Rosen, O.M. (1983) Proc. Natl. Acad. Sci. USA 80, 6858-6862.

472. Levy, J., Teuerstein, I., Marbach, M., Radian, S. and Sharoni, Y. (1984) Biochem. Biophys. Res. Commun. 123, 1227-1233.

473. Li, H.-C., Chernoff, J., Chen, L.B. and Kirschonbaum, A. (1984) Eur. J. Biochem. 138, 45-51.

474. Lincoln, T.M., Dills, W.L. and Corbin, J.D. (1977) J. Biol. Chem. 252, 4269-4275.

475. Lincoln, T.M., Flockhart, D.A. and Corbin, J.D. (1978) J. Biol. Chem. 253, 6002-6009.

476. Lincoln, T.M., Hall, C.L., Park, C.R. and Corbin, J.D. (1976) Proc. Natl. Acad. Sci. USA 73, 2559-2563.

477. Linn, T.C., Pettit, F.H., Hucho, F. and Reed, L.J. (1969) Proc. Natl. Acad. Sci. USA 64, 227-234.

478. Linn, T.C., Pettit, F.H. and Reed, L.J. (1969) Proc. Natl. Acad. Sci. USA 62, 234-241.

479. Liscum, L., Cummings, R.D., Anderson, R.G.W., De Martino, G.N., Goldstein, J.L. and Brown, M.S. (1983) Proc. Natl. Acad. Sci. USA 80, 7165-7169.

480. Liscum, L., Luskey, K.L., Chin, D.J., Ho, Y.K., Goldstein, J.L. and Brown, M.S. (1983) J. Biol. Chem. 258, 8450-8455.

481. Liu, A. Y.-C. and Greengard, P. (1976) Proc. Natl. Acad. Sci. USA, 73, 568-572.

482. Liu, A. Y.-C., Walter, U. and Greengard, P. (1981) Eur. J. Biochem. 114, 539-548.

483. Ljungstrom, O., Berglund, L. and Engstrom, L. (1976) Eur. J. Biochem. 68, 497-506.

484. Ljungstrom, O., Hjelmquist, G. and Engstrom, L. (1974) Biochim. Biophys. Acta 429, 374-382.

485. Lockwood, D.H. and East, L.E. (1974) J. Biol. Chem. 249, 7717-7722.

486. Lohmann, S.M., Walter, U. and Greengard, P. (1980) J. Biol. Chem. 255, 9985-9992.

487. Loizzi, R.F. (1978) Horm. Metab. Res. 10, 415-419.

488. Loizzi, R.F., de Pont, J.J.H.M.M. and Bonting, S.L. (1975) Biochim. Biophys. Acta 392, 20-25.

489. Loten, E.G., Assimacopoulos-Jeannet, F.D., Exton, J.H. and Park, C.R. (1978) J. Biol. Chem. 253, 746-757.

490. Loten, E.G., Francis, S.H. and Corbin, J.D. (1980) J. Biol. Chem. 255, 7838-7844.

491. Loten, E.G. and Sneyd, J.G.T. (1970) Biochem. J. 120, 187-193.

492. Loten, E.G. and Sneyd, J.G.T. (1973) Endocrinology 93, 1315-1322.

493. Lovell-Smith, C., Manganiello, V.C. and Vaughan, M. (1977) Biochim. Biophys. Acta 497, 447-458.

494. Macaulay, S.L., Kiechle, F.L. and Jarett, L. (1982) Fed. Proc. 41, 1082.

495. Macaulay, S.L., Kiechle, F.L. and Jarett, L. (1983) Arch. Biochem. Biophys. 225, 130-136.

496. Macara, I.G., Marinetti, G.V. and Balduzzi, P.C. (1984) Proc. Natl. Acad. Sci. USA 81, 2728-2732.

497. Mackall, J. and Lane, M.D. (1977) Biochem. J. 162, 635-642.

498. MacKenzie III, Ch. W. (1982) J. Biol. Chem. 257, 5581-5593.

499. Maeno, H., Johnson, E.M. and Greengard, P. (1977) J. Biol. Chem. 252, 5164-5174.

500. Majunder, G.C. and Turkington, R.W. (1972) J. Biol. Chem. 247, 7202-7217.

501. Makino, H., de Bushiazzo, P.M., Pointer, R.H., Jordan, J.E. and Kono, T. (1980) J. Biol. Chem. 255, 7845-7849.

502. Makino, H., Kanatsuka, A., Osegawa, M. and Kamugai, A. (1982) Biochim. Biophys. Acta 704, 31-36.

503. Makino, H. and Kono, T. (1980) J. Biol. Chem. 255, 7850-7854.

504. Malchow, D., Fuchilla, J. and Nanjundiah, V. (1975) Biochim. Biophys. Acta 385, 421-428.

505. Mallette, L.E., Neblett, M., Exton, J.H. and Langan, T.A. (1973) J. Biol. Chem. 248, 6289-6291.

506. Manganiello, V.C. and Vaughan, M. (1973) J. Biol. Chem. 248, 7164-7170.

507. Manning, R., Dils, R. and Mayer, R.J. (1976) Biochem. J. 153, 463-468.

508. Manning, D.R. and Gilman, A.G. (1983) J. Biol. Chem. 258, 7059-7063.

509. Marchmont, R.J., Ayad, S.R. and Houslay, M.D. (1981) Biochem. J. 195, 645-652.

510. Marchmont, R.J. and Houslay, M.D. (1980) Biochem. J. 187, 381-392.

511. Marchmont, R.J. and Houslay, M.D. (1980) Nature 286, 904-906.

512. Marchmont, R.J. and Houslay, M.D. (1981) Biochem. J. 195, 653-660.

513. Marsh, J.W., Westley, J. and Steiner, D.F. (1984) J. Biol. Chem. 259, 6641-6649.

514. Marshall, S., Green, A. and Olefsky, J.M. (1981) J. Biol. Chem. 256, 11464-11470.

515. Marshall, S.E., McCormack, J.G. and Denton, R.M. (1984) Biochem. J. 218, 249-260.

516. Massague, J., Pilch, P.F. and Czech, M.P. (1980) Proc. Natl. Acad. Sci. USA 77, 7137-7141.

517. Massague, J., Pilch, P.F. and Czech, M.P. (1981) J. Biol. Chem. 256, 3182-3190.

518. Matsuhashi, M., Matsuhashi, S. and Lynen, F. (1963) Biochem. Z. 340, 263-289.

519. Matsumoto, Y., Yasuda, H., Mita, S., Marunouchi, T. and Yamada, M. (1980) Nature 284, 181-183.

520. Matthews, H.R. in Molecular Aspects of Cellular Regulation Vol. 1 (Cohen, P. ed.) 235-251 (Elsevier, Amsterdam, 1980).

521. McCafferey, P.D., Friedman, B. and Rosner, M.R. (1984) J. Biol. Chem. 259, 12502-12507.

522. McCormack, J.G. and Denton, R.M. (1977) Biochem. J. 166, 627-630.

523. McCormack, J.G. and Denton, R.M. (1980) Biochem. J. 180, 533-544.

524. McCormack, J.G. and Denton, R.M. (1984) Biochem. J. 218, 235-247.

525. McGuinness, T.L., Lai, Y. and Greengard, P. (1984) Soc. Neurosci. Abstr. 10, 919.

526. McGuinness, T.L., Lai, Y. and Greengard, P. (1985) J. Biol. Chem. 260, 1696-1704.

527. McGuinness, T.L., Lai, Y., Greengard, P., Woodgett, J.R. and Cohen, P. (1983) FEBS Lett. 163, 329-334.

528. McNeillie, E.M., Clegg, R.A. and Zammit, V.A. (1981) Biochem. J. 200, 639-644.

529. McNeillie, E.M. and Zammit, V.A. (1982) Biochem. J. 204, 273-280.

530. Meggio, F., Donella-Deana, A. and Pinna, L.A. (1981) J. Biol. Chem. 256, 11958-11961.

531. Meggio, F., Donella-Deana, A., Pinna, L.A. and Moret, V. (1977) FEBS Lett. 75, 192-196.

532. Menon, A.S., Devi, S.U. and Ramasarma, T. (1982) Biochem. Biophys. Res. Commun. 109, 619-625.

532a. Mercer, S.W. and Williamson, D.H. (1986) Biochem. J. 239, 489-492.

533. Meredith, M.J. and Lane, M.D. (1978) J. Biol. Chem. 253, 3381-3383.

534. Michell, R.H. (1975) Biochim. Biophys. Acta 415, 81-147.

535. Michell, R.H., Kirk, C.J., Jones, L.M., Downes, C. and Creba, J.A. (1981) Phil. Trans. R. Soc. Lond. B296, 123-137.

536. Middleton, B., Hatton, J. and White, D.A. (1981) J. Biol. Chem. 256, 4827-4831.

537. Miller, S.G. and Kennedy, M.B. (1984) Soc. Neurosci. Abstr. 10, 544.

538. Mills, J.S., Epstein, P.M., Strada, S.J. and Thompson, W.J. (1978) Adv. Cycl. Nucl. Res. 9, 758.

539. Minakuchi, R., Takai, Y., Yu, B. and Nishizuka, Y. (1981) J. Biochem. 89, 1651-1654.

540. Mitropoulos, K.A., Venkatesan, S., Reeves, B.E.A. and Balasubramanium, S. (1981) Biochem. J. 194, 265-271.

541. Mohindru, A. and Roads, A.R. (1982) Biochem. J. 205, 427-435.

542. Moore, J.B. Jr., and Schroedter, D.E. (1982) Arch. Biochem. Biophys. 213, 276-287.

543. Mori, T., Takai, Y., Minakuchi, R., Yu, B. and Nishizuka, Y. (1980) J. Biol. Chem. 255, 8378-8380.

544. Mori, T., Takai, Y., Yu, B., Takahashi, Y., Nishizuka, Y. and Fujikura, T. (1982) J. Biochem. 91, 427-431.

545. Morrill, M.E., Thompson, S.T. and Stellwagen, E. (1979) J. Biol. Chem. 254, 4371-4373.

546. Morris, D.R. and Fillingame, R.H. (1974) Ann. Rev. Biochem. 43, 303-325.

547. Moss, J., Manganiello, V.C. and Vaughan, M. (1977) J. Biol. Chem. 252, 5211-5215.

548. Moss, J., Yamagishi, M., Kleinschmidt, A.K. and Lane, M.D. (1972) Biochemistry 11, 3779-3786.

549. Mullaney, I. and Clegg, R.A. (1984) Biochem. J. 219, 801-809.

550. Munday, K.A., Giles, I.G. and Poat, P.C. (1980) Comp. Biochem. Physiol. 67B, 403-411.

551. Munday, M.R. and Hardie, D.G. (1984) Eur. J. Biochem. 141, 617-627.

552. Munday, M.R. and Hardie, D.G. (1986) Biochem. J. 237, 85-91.

553. Munday, M.R. and Williamson, D.H. (1981) Biochem. J. 196, 831-837.

553a. Munday, M.R. and Williamson, D.H. (1983) Biochem. J. 214, 183-187.

554. Murray, A.W., Froscio, M. and Rogers, A. (1976) Biochem. Biophys. Res. Commun. 71, 1175-1181.

554a. Nagato, T., Yoshida, H., Yoshida, A. and Uehara, Y. (1980) Cell Tissue Res. 209, 1-10.

555. Nairn, A.C. and Greengard, P. (1983) Soc. Neurosci. Abstr. 9, 1029.

556. Nairn, A.C., Hemmings, H.C. Jr. and Greengard, P. (1985) Ann. Rev. Biochem. 54, 931-976.

556a. Nakanishi, S. and Numa, S. (1970) Eur. J. Biochem. 16, 163-173.

557. Nambi, P., Peters, J.R., Sibley, D.R. and Lefkowitz, R.J. (1985) J. Biol. Chem. 260, 2165-2171.

558. Narindrasorasak, S., Tan, L.U., Seth, P.K. and Sanwal, B.D. (1982) J. Biol. Chem. 257, 4618 4626.

559. Nestler, E.J. and Greengard, P. (1982) Nature 296, 452-454.

560. Nilsen-Hamilton, M., Hamilton, R.T., Allen, W.R. and Potter-Perigo, S. (1982) Cell 31, 237-242.

561. Nilsson, N.O., Stralfors, P., Fredrikson, G. and Belfrage, P. (1980) FEBS Lett. 111, 125-130.

562. Nimmo, G.A. and Cohen, P. (1978) Eur. J. Biochem. 87, 353-365.

563. Nimmo, H.G. and Cohen, P. (1977) Adv. Cyclic. Nucleotide Res. 8, 145-266.

564. Nishikawa, M., Hidaka, H. and Adelstein, R.S. (1983) J. Biol. Chem. 258, 14069-14072.

565. Nishimura, J. and Deuel, T.F. (1983) FEBS Lett. 156, 130-134.

566. Nishimura, J., Huang, J.S. and Deuel, T.F. (1982) Proc. Natl. Acad. Sci. USA 79, 4203-4207.

567. Nishizuka, Y. (1984) Nature 308, 693-698.

568. Nishizuka, Y. (1984) Science 225, 1365-1370.

569. Nordstrom, J.L., Rodwell, V.W. and Mitschelen, J.J. (1977) J. Biol. Chem. 252, 8924-8934.

570. Northup, J.K., Smigel, M.D., Sternweis, P.C. and Gilman, A.G. (1983) J. Biol. Chem. 258, 11369-11376.

571. Northup, J.K., Sternweis, P.C., Smigel, M.D., Schleifer, L.S., Ross, E.M. and Gilman, A.G. (1980) Proc. Natl. Acad. Sci. USA 77, 6516-6520.

572. Novak-Hofer, I. and Thomas, G. (1985) J. Biol. Chem. 260, 10314-10319.

573. Numa, S., Ringlemann, E. and Lynen, F. (1965) Biochem. Z. 343, 243-257.

574. Odyssey, R. (1980) FEBS Lett. 121, 306-308.

575. Odyssey, R. (1982) Biochem. J. 204, 353-356.

576. Ogiwara, H., Tanabe, T., Nikawa, J. and Numa, S. (1978) Eur. J. Biochem. 89, 33-41.

577. Øgreid, D., Døskeland, S.O. and Miller, J. (1983) J. Biol. Chem. 258, 1041-1049.

577a. O'Hea, E.K. and Leveille, G.A. (1969) J. Nutr. 99, 338-344.

578. Oka, T., Perry, J.W., Takemoto, T., Sakai, T., Terada, N. and Inoue, H. (1981) Adv. in Polyamine Res. 3, 309-320.

579. Oku, H., Ide, T. and Sugano, M. (1984) J. Lipid Res. 25, 254-261.

580. Olefsky, J.M. and Kobayashi, M. (1978) Metabolism 27, 1917-1929.

581. Olefsky, J.M., Kobayashi, M. and Chang, H. (1979) Diabetes 28, 460-471.

581a. Ota, K. and Yokoyama, A. (1967) J. Endocrinol. 38, 251-261.

581b. Otway, S. and Robinson, D.S. (1968) Biochem. J. 106, 677-682.

582. Ozawa, E. (1973) J. Neurochem. 20, 1487-1488.

582a. Page, T. and Kuhn, N.J. (1986) Biochem. J. 239, 269-274.

583. Palfrey, H.C. (1983) FEBS Lett. 157, 183-190.

584. Palfrey, H.C. (1984) Fed. Proc. 43, 1466.

585. Palfrey, H.C. (1984) Fed. Proc. 43, 1467.

586. Palfrey, H.C., Lai, Y. and Greengard, P. (1984) in Proc. 6th Int. Conf. Red Cell Structure and Metabolism. pp 291-308 (Brewer, G. Ed) Liss, New York,

587. Palfrey, H.C., Rothlein, J.F. and Greengard, P. (1983) J. Biol. Chem. 258, 9496-9503.

588. Pall, M.L. (1981) Microbiol. Rev. 45, 462-480.

589. Papahadjopoulous, D., Poste, G. and Vail, W.J. (1979) Methods Membr. Biol. 10, 1-21.

590. Parker, P.J., Caudwell, F.B. and Cohen, P. (1983) Eur. J. Biochem. 130, 227-234.

591. Parker, P.J., Embi, N., Caudwell, F.B. and Cohen, P. (1982) Eur. J. Biochem. 124, 47-55.

592. Parker, R.A., Ingebritsen, T.S., Gibson, D.M. and Geelen, M.J.H. (1981) in Protein Phosphorylation, Vol 8, p. 607 (Rosen, O.M. and Krebs, E.G. Eds) Cold Spring Harbour Laboratory, New York.

593. Parker, P.J. and Randle, P.J. (1978) Biochem. J. 171, 751-757.

594. Parker, P.J. and Randle, P.J. (1978) FEBS Lett. 95, 153-156.

595. Parker, P.J., Stabel, S. and Waterfield, M.D. (1984) EMBO J. 3, 953-959.

596. Pato, M.D., Adelstein, R.S., Crouch, D., Safer, B., Ingebritsen, T.S. and Cohen, P. (1983) Eur. J. Biochem. 132, 283-287.

597. Pawlson, L.G., Lovell-Smith, C.J., Manganiello, V.C. and Vaughan, M. (1974) Proc. Natl. Acad. Sci. USA 71, 1639-1642.

598. Payne, M.E., Schworer, C.M. and Soderling, T.R. (1983) J. Biol. Chem. 258, 2376-2382.

599. Perisic, O. and Traugh, J.A. (1983) J. Biol. Chem. 258, 9589-9592.

600. Perisic, O., Trevillyan, J.M., Byus, C.V. and Traugh, J.A. (1984) Fed. Proc. 43, 1977.

601. Pessin, J.E., Gitomer, W., Oka, Y., Oppenheimer, C.L. and Czech, M.P. (1983) J. Biol. Chem. 258, 7386-7394.

602. Peterson, B., Beckner, S. and Blecher, M. (1978) Biochim. Biophys. Acta 542, 470-485.

603. Petruzzelli, L.M., Ganguly, S., Smith, C.J., Cobb, M.H., Rubin, C.S. and Rosen, O.M. (1982) Proc. Natl. Acad. Sci. USA 79, 6792-6796.

604. Pettit, F.H., Yeaman, S.J. and Reed, L.J. (1978) Proc. Natl. Acad. Sci. USA 75, 4881-4885.

605. Peuch, J.C., Ballester, R. and Rosen, O.M. (1983) Proc. Natl. Acad. Sci. USA 80, 6858-6862.

606. Pfeuffer, T., Gaugler, B. and Metzger, H. (1983) FEBS Lett. 164, 154-160.

607. Pfeuffer, T. and Metzger, H. (1982) FEBS Lett. 146, 369-375.

608. Phillips, C.E. and Ness, G.C. (1984) Biochem. Biophys. Res. Commun. 119, 772-778.

609. Pichard, A.L. and Cheung, W.Y. (1976) J. Biol. Chem. 251, 5726-5737.

610. Picq, M., prigent, A.F., Nemoz, G., Andre, A.C. and Pacheco, H. (1982) J. Med. Chem. 25, 1193-1198.

611. Picton, C., Woodgett, J., Hemmings, B.A. and Cohen, P. (1982) FEBS Lett. 150, 191-196.

612. Pires, E.M.V. and Perry, S.V. (1977) Biochem. J. 167, 137-146.

613. Pirollet, F., Feige, J.J., Cochet, C., Job, D. and Chambaz, E.M. (1981) Biochem. Biophys. Res. Commun. 100, 613-620.

614. Pittman, R.C., Khoo, J.C. and Steinberg, D. (1975) J. Biol. Chem. 250, 4505-4511.

615. Pizarro, M., Puente, J. and Sapag-Hagar, M. (1981) FEBS Lett. 136, 127-130.

616. Pilkis, S.J., Chrisman, T., Burgess, B., McGrane, M., Colosia, A., Pilkis, J., Claus, T. and El-Maghrabi, M.R. (1983) Adv. Enzyme Regul. 21, 147-173.

617. Pilkis, S.J., Chrisman, T., El-Maghrabi, M.R., Colosia, A., Fox, E., Pilkis, J. and Claus, T.H. (1983) J. Biol. Chem. 258, 1495-1503.

618. Pilkis, S.J., El-Maghrabi, M.R., McGrane, M., Pilkis, J. and Claus, T.H. (1981) J. Biol. Chem. 256, 11489-11495.

619. Pilkis, S.J., El-Maghrabi, M.R., Pilkis, J. and Claus, T.H. (1981) J. Biol. Chem. 256, 3619-3622.

620. Pilkis, S.J., El-Maghrabi, M.R., Pilkis, J., Claus, T.H. and Cummings, D.A. (1981) J. Biol. Chem. 256, 3171-3174.

621. Pilkis, S.J., Pilkis, J., El-Maghrabi, M.R. and Claus, T.H. (1985) J. Biol. Chem. 200, 7551-7556.

622. Pilkis, S.J., Regen, B.H., Stewart, T., Chrisman, T., Pilkis, J., Kountz, P., Pate, T., McGrane, M., El-Maghrabi, M.R. and Claus, T.H. (1984) in Molecular Aspects of Cellular Regulation, Vol 3 (Cohen, P. ed.) 95-155 (Elsevier, Amsterdam).

623. Pilkis, S.J., Regen, D.M., Stewart, H.B., Pilkis, J., Pate, T.M. and El-Maghrabi, M.R. (1984) J. Biol. Chem. 259, 949-958.

624. Pilkis, S.J., Schworer, C., Soderling, T.R. and El-Maghrabi, M.R. (1985) Abstr. 8th International Conference on Metabolic Interconversions of Enzymes, Pitlochry, Scotland

624a. Pires, E.M.V. and Perry, S.V. (1977) Biochem. J. 167, 137-146.

625. Pledger, W.J., Thompson, W.J. and Strada, S.J. (1975) Biochim. Biophys. Acta 391, 334-340.

626. Pledger, W.J., Thompson, W.J. and Strada, S.J. (1976) Biochem. Biophys. Res. Commun. 70, 58-65.

627. Popjak, G. and Beeckmans, M.-L. (1950) Biochem. J. 46, 547-558.

628. Potter, R.L. and Taylor, S.S. (1979) J. Biol. Chem. 254, 2413-2418.

629. Potter, R.L. and Taylor, S.S. (1979) J. Biol. Chem. 254, 9000-9005.

630. Potter, R.L. and Taylor, S.S. (1980) J. Biol. Chem. 255, 9706-9712.

631. Pratt, M.L., Maher, J.F. and Roche, T.E. (1982) Eur. J. Biochem. 125, 349-355.

632. Pratt, M.L. and Roche, T.E. (1979) J. Biol. Chem. 254, 7191-7196.

633. Quarfoth, G., Ahmed, K. and Foster, D. (1978) Biochim. Biophys. Acta 526, 580-590.

634. Ramachandran, C.K., Gray, S.L. and Melnykovych, G. (1978) Arch. Biochem. Biophys. 189, 205-211.

635. Randle, P.J. (1983) Phil. Trans. R. Soc. Lond. B302, 47-57.

636. Randle, P.J., Garland, P.B., Hales, C.N., Newsholme, E.A., Denton, R.M. and Pogson, C.I. (1966) Recent Prog. Horm. Res. 22, 1-48.

637. Randle, P.J., Fatania, H.R. and Lau, K.S. (1984) in Molecular Aspects of Cellular Regulation, Vol 3 (Cohen, P. ed.) pp. 1-26 (Elsevier, Amsterdam).

638. Rangel-Aldo, R., Kupiec, J.W. and Rosen, O.M. (1979) J. Biol. Chem. 254, 2499-2508.

639. Rangel-Aldo, R. and Rosen, O.M. (1971) J. Biol. Chem. 252, 7140-7145.

640. Rannels, S.R. and Corbin, J.D. (1980) J. Biol. Chem. 255, 7085-7088.

641. Rannels, S.R. and Corbin, J.D. (1981) J. Biol. Chem. 256, 7871-7876.

642. Raphael, B.C., Patton, S. and McCarthy, R.D. (1975) FEBS Lett. 58, 47-49.

643. Reed, B.C., Ronnett, G.V. and Lane, M.D. (1981) Proc. Natl. Acad. Sci. USA 78, 2908-2912.

644. Reed, L.J. and Damuni, Z. (1985) Abstr. 8th International Conference on Metabolic Interconversions of Enzymes, Pitlochry, Scotland.

645. Reed, L.J., Pettit, F.H., Bleile, D.H. and Wu, T.L. (1981) in Metabolic Interconversions of Enzymes, 1980 (Holzer, H. Ed.) pp. 124-133, Springer-Verlag, Heidelberg.

646. Reynolds, C.H. (1982) Biochem. J. 202, 125-132.

647. Ricard, J., Meunier, J.-C. and Buc, J. (1974) Eur. J. Biochem. 49, 195-208.

648. Richards, C.S., Furuya, E. and Uyeda, K. (1981) Biochem. Biophys. Res. Commun. 100, 1673-1679.

649. Richards, C.S., Yokoyama, M., Furuya, E. and Uyeda, K. (1982) Biochem. Biophys. Res. Commun. 104, 1073-1079.

650. Richert, L., Castagna, M., Beck, J.-P., Rong, S., Luu, B. and Ourisson, G. (1984) Biochem. Biophys. Res. Commun. 120, 192-198.

651. Rillema, J.A. (1976) Proc. Soc. Expt. Biol. Med. 151, 748-751.

652. Rion, J.-P., Claus, T.H., Flockhart, D.A., Corbin, J.D. and Pilkis, S.J. (1977) Proc. Natl. Acad. Sci. USA 74, 4615-4619.

653. Rittschof, D. and Traugh, J.A. (1982) Eur. J. Biochem. 123, 333-336.

653a. Roach, P.J., De Paoli-Roach, A.A. and Larner, J. (1978) J. Cyclic Nucl. Res. 4, 245-257.

654. Roach, P.J. and Goldman, M. (1983) Proc. Natl. Acad. Sci. USA 80, 7170-7173.

654a. Robinson, A.M., Girard, J.R. and Williamson, D.H. (1978) Biochem. J. 176, 343-346.

654b. Robinson, A.M. and Williamson, D.H. (1977) Biochem. J. 164, 153-159.

655. Robinson-Steiner, A.M. and Corbin, J.D. (1983) J. Biol. Chem. 258, 1032-1040.

656. Robson, N.A., Clegg, R.A. and Zammit, V.A. (1984) Biochem. J. 217, 743-749.

657. Rodwell, V.W., Nordstrom, J.L. and Mitschelen, J.J. (1976) Adv. Lipid Res. 14, 1-74 (Paoletti, R. and Kritchevsky, D. Eds) Academic Press, New York.

658. Roitelman, J. and Shechter, I. (1984) J. Biol. Chem. 259, 870-877.

659. Romsos, D.R. and Leveille, G.A. (1974) Adv. Lipid Res. 12, 97-146.

660. Rose, K.M., Bell, L.E., Siefken, D.A. and Jacob, S.T. (1981) J. Biol. Chem. 256, 7468-7477.

661. Rose, K.M., Stetler, D.A. and Jacob, S.T. (1981) Proc. Natl. Acad. Sci. USA 78, 2833-2837.

662. Rosen, O.M., Herrera, R., Olowe, Y., Petruzzelli, L.M. and Cobb, M. (1983) Proc. Natl. Acad. Sci. USA 80, 3237-3240.

663. Rosen, O.M., Rangel-Aldo, R. and Erlichman, J. (1977) Curr. Top. Cell. Regul. 12, 39-74.

664. Ross, A.C. and Rowe, J.F. (1984) Proc. Soc. Exp. Biol. Med. 176, 42-47.

665. Ross, E.M. and Gilman, A.G. (1980) Ann. Rev. Biochem. 49, 533-564.

666. Roth, R.A., Mesirow, M.L. and Cassell, D.J. (1983) J. Biol. Chem. 258, 14456-14460.

667. Rubin, C.S., Fleischer, N., Sarkar, D. and Erlichman, J. (1981) Cold Spring Harbor Conf. Cell Prolif. 8, 1333-1346.

668. Rubin, C.S., Rangel-Aldo, R., Sarkar, D., Erlichman, J. and Fleischer, N. (1979) J. Biol. Chem. 254, 3797-3805.

669. Russel, T.R., Terasaki, W.C. and Appleman, M.M. (1973) J. Biol. Chem. 248, 1334-1340.

670. Russel, T.R., Thompson, W.J., Schneider, F.W. and Appleman, M.M. (1972) Proc. Natl. Acad. Sci. USA 69, 1791-1795.

671. Rymond, M. and Hofmann, F. (1982) Eur. J. Biochem. 125, 395-400.

672. Saitoh, T. and Schwartz, J.H. (1985) J. Cell. Biol. 100, 835-842.

673. Sakai, T., Makino, H. and Tanaka, R. (1978) Biochim. Biophys. Acta 527, 442-455.

674. Sakai, T., Thompson, W.J., Lavis, V.R. and Williams, R.H. (1974) Arch. Biochem. Biophys. 162, 331-339.

675. Sakai, T., Yamanaka, H., Tanaka, R., Makino, H. and Kasai, H. (1977) Biochim. Biophys. Acta 483, 121-123.

676. Sakakibara, R., Kitajima, S. and Uyeda, K. (1984) J. Biol. Chem. 259, 41-46.

677. Sale, G.J. and Randle, P.J. (1980) Biochem. J. 188, 409-421.

678. Sale, G.J. and Randle, P.J. (1981) Eur. J. Biochem. 120, 535-540.

679. Sale, G.J. and Randle, P.J. (1982) Biochem. J. 203, 99-108.

680. Sale, G.J. and Randle, P.J. (1982) Biochem. J. 206, 221-229.

681. Sapag-Hagar, M. and Greenbaum, A.L. (1974) Eur. J. Biochem. 47, 303-312.

682. Sapag-Hagar, M., Greenbaum, A.L., Lewis, D.J. and Hallowes, R.C. (1974) Biochem. Biophys. Res. Commun. 59, 261-268.

683. Schatzman, R.C., Grifo, J.A., Merrick, W.C. and Kuo, J.F. (1983) FEBS Lett. 159, 167-170.

684. Schulman, H. (1984) J. Cell Biol. 99, 11-19.

685. Schulman, H. and Greengard, P. (1978) Nature 271, 478-479.

686. Schulman, H. and Greengard, P. (1978) Proc. Natl. Acad. Sci. USA 75, 5432-5436.

687. Schwoch, G., Hamann, A. and Hilz, H. (1980) Biochem. J. 192, 223-230.

688. Scott, C.W. and Mumby, M.C. (1985) J. Biol. Chem. 260, 2274-2280.

689. Seals, J.R. and Czech, M.P. (1980) J. Biol. Chem. 255, 6529-6531.

690. Seals, J.R. and Czech, M.P. (1981) J. Biol. Chem. 256, 2894-2899.

691. Seamon, K.B. and Daly, J.W. (1981) J. Biol. Chem. 256, 9799-9801.

692. Sefton, B.M. and Hunter, T. (1984) Adv. Cyclic Nucleotide and Protein Phyosphorylation Res. Vol. 18, pp. 195-226 (Greengard, P. and Robinson, G.A. Eds) Raven Press, New York.

693. Severson, D.L., Fletcher, T., Groves, G., Hurley, B. and Sloan, S. (1981) Can. J. Biochem. 59, 418-429.

694. Severson, D.L., Khoo, J.C. and Steinberg, D. (1977) J. Biol. Chem. 252, 1484-1489.

695. Severson, D.L. and Sloan, S.K. (1977) Biochem. Biophys. Res. Commun. 79, 1045-1050.

696. Shah, S.S. (1981) Arch. Biochem. Biophys. 211, 439-446.

697. Sharma, R.K., Wang, T.H., Wirch, E. and Wang, J.H. (1980) J. Biol. Chem. 255, 5916-5923.

698. Sharoni, Y., Feldman, B., Teuerstein, I. and Levy, J. (1984) Endocrinology 115, 1918-1924.

699. Sharoni, Y., Graziani, Y., Karny, N., Feldman, B. and Levy, J. (1984) Eur. J. Cancer Clin. Oncol. 20, 277-281.

700. Sharoni, Y., Radian, S. and Levy, J. (1985) FEBS Lett. 189, 133-136.

701. Sharoni, Y., Teuerstein, I., Shirman, A., Feldman, B. and Levy, J. (1984) Endocrinology 115, 2297-2302.

702. Shia, M.A., Rubin, J.B. and Pilch, P.F. (1983) J. Biol. Chem. 258, 14450-14455.

703. Shields, S.M., Vernon, P.J. and Kelly, P.T. (1984) J. Neurochem. 43, 1599-1609.

704. Sibley, D.R., Nambi, P., Peters, J.R. and Lefkowitz, R.J. (1984) Biochem. Biophys. Res. Commun. 121, 973-979.

705. Sibley, D.R., Peters, J.R., Nambi, P., Caron, M.G. and Lefkowitz, R.J. (1984) J. Biol. Chem. 259, 9742-9749.

706. Siess, E.A., Brocks, D.G. and Wieland, O.H. (1978) Hoppe-Seyler's Z. Physiol. Chem. 359, 785-789.

707. Simpson, I.A. and Hedo, J.A. (1984) Science 223, 1301-1304.

708. Sinensky, M. and Logel, J. (1983) J. Biol. Chem. 258, 8547-8549.

708a. Sinnett-Smith, P.A., Vernon, R.G. and Mayer, R.J. (1980) Biochem. J. 186, 937-944.

709. Sipat, A.B. and Sabine, J.R. (1981) Biochem. J. 194, 889-893.

710. Skoglund, G., Hansson, A. and Ingelman-Sundberg, M. (1985) Eur. J. Biochem. 148, 407-412.

711. Skuster, J.R., Jesse Chan, K.F. and Graves, D.J. (1980) J. Biol. Chem. 255, 2203-2210.

712. Snigel, M., Katada, T., Northup, J.K., Bokoch, G.M., Ui, M. and Gilman, A.G. (1984) Adv. Cyclic Nucleotide and Protein Phos. Res. Vol. 17, pp. 1-18 (Greengard, P. et al. Eds) Raven Press, New York.

713. Smith, C.J., Wejksnora, P.J., Warner, J.R., Rubin, C.S. and Rosen, O.M. (1979) Proc. Natl. Acad. Sci. USA 76, 2725-2729.

714. Smith, D.L., Chen, C.-C., Bruegger, B.B., Holtz, S.L., Halpern, R.M. and Smith, R.A. (1974) Biochemistry 13, 3780-3785.

715. Smith, R.A.W. (1985) (personal communication).

716. Smith, R.A.W., Middleton, B. and West, D.W. (1986) Biochem. Soc. Trans. 14, 123-124.

716a. Smith, R.W. (1973) J. Dairy Res. 40, 353-360.

716b. Smith, R.W. (1975) Biochim. Biophys. Acta 411, 22-29.

716c. Smith, R.W. and Walsh, A. (1976) Lipids 11, 418-420.

717. Smoake, J.A., McMahon, K.L. and Wright, R.K. (1981) J. Biol. Chem. 256, 8531-8535.

718. Smoake, J.A. and Solomon, S.S. (1980) Biochem. Biophys. Res. Commun. 94, 424-430.

719. Smoake, J.A., Song, S.Y. and Cheung, W.Y. (1974) Biochim. Biophys. Acta 341-402.

720. Sneft, G., Schultz, G., Munske, K. and Hoffman, M. (1968) Diabetologia 4, 330-335.

721. Solomon, S.S. (1975) Endocrinology 96, 1366-1373.

722. Speake, B., Dils, R. and Mayer, R.J. (1976) Biochem. J. 154, 369-370.

723. Stadel, J.M., Nambi, P., Shorr, R.G.L., Sawyer, D.R., Caron, M.G. and Lefkowitz, R.J. (1983) Proc. Natl. Acad. Sci. USA 80, 3173-3177.

724. Stadtmauer, L.A. and Rosen, O.M. (1983) J. Biol. Chem. 258, 6682-6685.

725. Standaert, M.L. and Pollet, R.J. (1984) J. Biol. Chem. 259, 2346-2354.

726. Stansbie, D., Brownsey, R.W., Crettaz, M. and Denton, R.M. (1976) Biochem. J. 160, 413-416.

727. Stein, J.C., Sarkar, D. and Rubin, C.S. (1984) J. Neurochem. 42, 547-553.

728. Steinberg, R.A. (1983) J. Cell. Biol. 97, 1072-1080.

729. Steinberg, R.A. (1984) in Biochemical Actions of Hormones. Vol. XI, pp. 25-65 (Litwack, G. Ed.) Academic Press, New York.

730. Steinberg, R.A., O'Farrell, P.H., Friedrich, U. and Coffino, P. (1977) Cell 10, 381-391.

730a. Steingrimsdottir, L., Greenwood, M.R.C. and Brasel, J.A. (1980) J. Nutr. 110, 600-609.

731. Sternweis ,P.C., Northup, J.K., Smigel, M.D. and Gilman, A.G. (1981) J. Biol. Chem. 256, 11517-11526.

732. Stetler, D.A. and Rose, K.M. (1982) Biochemistry 21, 3721-3728.

733. Stevens, L. and Stevens, E. (1980) Polyamines in Biomedical Research, pp. 167-183 (Gaugas, J.M. ed.) J. Wiley & Sons, New York.

734. Stewart, A.A., Hemmings, B.A., Cohen, P., Goris, J. and Merlevede, W. (1981) Eur. J. Biochem. 115, 197-205.

735. Stewart, A.A., Ingebritsen, T.S. and Cohen, P. (1983) Eur. J. Biochem. 132, 289-295.

736. Stiges, M., Gil, G. and Hegardt, F.G. (1984) J. Lipid Res. 25, 497-506.

737. Strada, S.J., Goris, J., Sarada, K., Chang, L. and Thompson, W.J. (1981) Fed. Proc. 40, 1726.

738. Strada, S.J. and Thompson, W.J. (1978) Adv. Cycl. Nucl. Res. 9, 265-283.

739. Stralfors, P. and Belfrage, P. (1983) J. Biol. Chem. 258, 15146-15152.

740. Stralfors, P. and Belfrage, P. (1984) in Molecular Aspects of Cellular Regulation, Vol. 3 (Cohen, P. ed.) 27-62.

741. Streb, H., Irvine, R.F., Berridge, M.J. and Schulz, I. (1983) Nature 306, 67-69.

742. Strewler, G.J. and Manganiello, V.C. (1979) J. Biol. Chem. 254, 11891-11898.

743. Strewler, G.J., Manganiello, V.C. and Vaughan, M. (1978) J. Biol. Chem. 253, 390-394.

744. Strulovici, B., Cerione, R.A., Kilpatrick, B.F., Caron, M.G. and Lefkowitz, R.J. (1984) Science 225, 837-840.

745. Sugden, P.H., Hutson, N.J., Kerbey, A.L. and Randle, P.J. (1978) Biochem. J. 169, 433-435.

746. Sugden, P.H., Kerbey, A.L., Randle, P.J., Waller, C.A. and Reid, K.B.M. (1979) Biochem. J. 181, 419-426.

747. Sugden, P.H. and Randle, P.J. (1978) Biochem. J. 173, 659-668.

748. Suzuki, K. and Kono, T. (1979) J. Biol. Chem. 254, 9786-9794.

749. Swarup, G., Cohen, S. and Garbers, D.L. (1982) Biochem. Biophys. Res. Commun. 107, 1104-1109.

750. Swarup, G., Dasgupta, J.D. and Garbers, D.L. (1983) J. Biol. Chem. 258, 10341-10347.

751. Swenson, T.L. and Porter, J.W. (1985) J. Biol. Chem. 260, 3791-3797.

752. Tabor, C.W. and Tabor, H. (1976) Ann. Rev. Biochem. 45, 285-306.

753. Taira, T., Kii, R., Sakai, K., Tabuchi, H., Takimoto, S., Nakamura, S., Takahashi, J., Hashimoto, E., Yamamura, H. and Nishizuka, Y. (1982) J. Biochem. 91, 883-888.

754. Takai, Y., Kishimoto, A., Inoue, M. and Nishizuka, Y. (1977) J. Biol. Chem. 252, 7603-7609.

755. Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T. and Nishizuka, Y. (1979) J. Biol. Chem. 254, 3692-3695.

756. Takai, Y., Kishimoto, A., Kikkawa, U., Mori, T. and Nishizuka, Y. (1979) Biochem. Biophys. Res. Commun. 91, 1218-1224.

757. Takayama, S., White, M.F., Lauris, V. and Kahn, C.R. (1984) Proc. Natl. Acad. Sci. USA 81, 7797-7801.

758. Taketani, Y. and Oka, T. (1983) Proc. Natl. Acad. Sci. USA 80, 1646-1649.

759. Takio, K., Smith, S.B., Krebs, E.G., Walsh, K.A. and Titani, K. (1982) Proc. Natl. Acad. Sci. USA 79, 2546-2548.

760. Tanabe, T., Wade, K., Okazaki, T. and Numa, S. (1975) Eur. J. Biochem. 57, 15-24.

761. Tanaka, T. and Hidaka, H. (1980) J. Biol. Chem. 255, 11078-11080.

762. Tashima, Y., Hasegawa, M., Lane, L.K. and Schwartz, A. (1981) J. Biochem. 89, 249-255.

763. Teague, W.M., Pettit, F.H., Yeaman, S.J. and Reed, L.J. (1979) Biochem. Biophys. Res. Commun. 87, 244-252.

764. Teo, T.S. and Wang, J.H. (1973) J. Biol. Chem. 248, 588-595.

765. Terao, K. and Ogata, K. (1979) J. Biochem. (Tokyo) 86, 597-603.

766. Teshima, Y. and Kakiuchi, S. (1978) J. Cyclic Nucleotide Res. 4, 219-231.

767. Thomas, A.P., Marks, J.S., Coll, K.E. and Williamson, J.R. (1983) J. Biol. Chem. 258, 5716-5725.

768. Thomas, G., Martin-Perez, J., Siegmann, M. and Otto, A.M. (1982) Cell 30, 235-242.

769. Thompson, W.J. and Appleman, M.M. (1971) Biochemistry 10, 311-316.

770. Thompson, W.J. and Appleman, M.M. (1971) J. Biol. Chem. 246, 3145-3150.

771. Thompson, W.J., Brooker, G. and Appleman, M.M. (1974) Methods Enzymol. 38, 205-212.

772. Thompson, W.J., Epstein, P.M. and Strada, S.J. (1979) Biochemistry 18, 5228-5236.

773. Thompson, W.J., Little, S.A. and Williams, R.H. (1973) Biochemistry 12, 1889-1894.

774. Thompson, W.J. and Strada, S.J. (1978) Receptors and Hormone Action, Vol. III, pp. 553-577 (O'Malley, B.W. and Birnbaumer, L. Eds.) Academic Press, New York.

775. Thornburg, W. and Lindell, T.J. (1977) J. Biol. Chem. 252, 6660-6665.

776. Threadgold, L.C. and Kuhn, N.J. (1984) Biochem. J. 218, 213-219.

777. Tipper, J.P. and Witters, L.A. (1982) Biochim. Biophys. Acta 715, 162-169.

778. Tolbert, M.E.M., White, A.C., Aspry, K., Cutts, J. and Fain, J.N. (1980) J. Biol. Chem. 255, 1938-1944.

778a. Topper, Y.J. and Freeman, C.S. (1980) Physiol. Rev. 60, 1049-1106.

778b. Topper, Y.J. and Oka, T. (1974) in Lactation, vol. 1 (Larson, B.L. and Smith, V.R. eds.) pp. 327-348, Academic Press, New York.

779. Topping, D.L., Goheer, A., Coore, H.G. and Mayes, P.A. (1977) Biochem. Soc. Trans. 5, 1000-1001.

780. Trevillyan, J.M., Perisic, O., Traugh, J.A. and Byus, C.V. (1985) J. Biol. Chem. 260, 3041-3044.

781. Tsai, S.C., Belfrage, P. and Vaughan, M. (1970) J. Lipid Res. 11, 466-472.

782. Tse-Dinh, Y.C., Wong, T.W. and Goldberg, A.R. (1984) Nature 312, 785-786.

783. Tsou, K. and Greengard, P. (1982) Proc. Natl. Acad. Sci. USA 79, 6075-6079.

784. Tucker, M.M., Robinson, J.B. and Stellwagen, E. (1981) J. Biol. Chem. 256, 9051-9058.

785. Tung, H.Y.L., Pelech, S., Fisher, M.J., Pogson, C.I. and Cohen, P. (1985) Eur. J. Biochem. 149, 305-313.

786. Turley, S.D., Anderson, J.M. and Dietschy, J.M. (1981) J. Lipid Res. 22, 551-569.

787. Turner, R.S., Chou, C.-H.J., Kibler, R. and Kuo, J.F. (1982) J. Neurochem. 39, 1397-1404.

787a. Uno, I., Ueda, T. and Greengard, P. (1977) J. Biol. Chem. 252, 5164-5174.

788. Uyeda, K., Furuya, E. and Luby, L. (1981) J. Biol. Chem. 256, 8394-8399.

789. Uzunov, P., Shein, H.H. and Weiss, B. (1973) Science 180, 304-306.

790. Van Berkel, T.J.C., Koster, J.F., Kruyt, J.K. and Hulsmann, W.C. (1974) Biochim. Biophys. Acta 370, 450-458.

791. Vance, D.E. and Pelech, S.L. (1984) TIBS 9, 17-20.

792. Vandenheede, J.R., Yang, S.-D., Goris, J. and Merlevede, W. (1980) J. Biol. Chem. 255, 11768-11774.

792. Van Inwegen, R.G., Pledger, W.J., Strada, S.J. and Thompson, W.J. (1976) Arch. Biochem. Biophys. 175, 700-709.

793. Van Inwegen, R.G., Robinson, G.A., Thompson, W.J., Armstrong, K.J. and Stouffer, J.E. (1975) J. Biol. Chem. 250, 2452-2456.

794. Van Inwegen, R.G., Swafford, R.L., Strada, S.J. and Thompson, W.J. (1977) Arch. Biochem. Biophys. 178, 58-68.

795. Van Obberghen, E. and Kowalski, A. (1982) FEBS Lett. 143, 179-182.

796. Van Schaftingen, E., Davies, D.R. and Hers, H.-G. (1981) Biochem. Biophys. Res. Commun. 103, 362-368.

797. Van Schaftingen, E., Davies, D.R. and Hers, H.-G. (1982) Eur. J. Biochem. 124, 143-149.

798. Van Schaftingen, E. and Hers, H.-G. (1981) Biochem. Biophys. Res. Commun. 101, 1078-1084.

799. Van Schaftingen, E. and Hers, H.-G. (1981) Proc. Natl. Acad. Sci. USA 78, 2861-2863.

800. Van Schaftingen, E., Hue, L. and Hers, H.-G. (1980) Biochem. J. 192, 887-895.

801. Van Schaftingen, E., Hue, L. and Hers, H.-G. (1980) Biochem. J. 192, 897-901.

802. Vaughan, M. (1960) J. Biol. Chem. 235, 3049-3053.

803. Vega, F.V., Key, R.J., Jordan, J.E. and Kono, T. (1980) Arch. Biochem. Biophys. 203, 167-173.

804. Vernon, R.G. (1977) Biol. Neonate 32, 15-23.

805. Villa-Moruzzi, E., Ballou, L.M. and Fischer, E.H. (1984) J. Biol. Chem. 259, 5857-5863.

806. Virk, S.S., Kirk, C.J. and Shears, S.B. (1985) Biochem. J. 226, 741-748.

807. Volpe, J.J. (1979) J. Biol. Chem. 254, 2568-2571.

808. Volpe, J.J. and Obert, K.A. (1981) J. Biol. Chem. 256, 2016-2021.

809. Vulliet, P.R., Woodgett, J.R. and Cohen, P. (1984) J. Biol. Chem. 259, 13680-13683.

810. Waisman, D.M., Singh, T.J. and Wang, J.H. (1978) J. Biol. Chem. 253, 3387-3390.

811. Walaas, S.I., Nairn, A.C. and Greengard, P. (1983) J. Neurosci. 3, 291-301.

812. Walaas, S.I., Nairn, A.C. and Greengard, P. (1983) J. Neurosci. 3, 302-311.

813. Wallace, A.V., Heyworth, C.M. and Houslay, M.D. (1984) Biochem. J. 222, 177-182.

814. Wallace, R.W., Tallant, E.A., Dockter, M.E. and Cheung, W.Y. (1982) J. Biol. Chem. 257, 1845-1854.

815. Walsh, D.A. and Ashby, C.D. (1973) Recent Prog. Hormone Res. 29, 329-359.

816. Walsh, D.A., Ashby, C.D., Gonzalez, C., Calkins, D., Fischer, E.H. and Krebs, E.G. (1971) J. Biol. Chem. 246, 1977-1985.

817. Walsh, D.A., Perkins, J.P. and Krebs, E.G. (1968) J. Biol. Chem. 243, 3763-3765.

818. Walsh, M.P., Valet, B., Autric, F. and Demaille, J.G. (1979) J. Biol. Chem. 254, 12136-12144.

819. Walter, U. (1981) Eur. J. Biochem. 118, 339-346.

820. Walter, U., De Camilli, P., Lohmann, S.M., Miller, P. and Greengard, P. (1981) Cold Spring Harbor Conf. Cell. Prolif. 8, 141-157.

821. Walter, U. and Greengard, P. (1981) Curr. Top. Cell. Regul. 19, 219-256.

822. Walter, U., Kanof, P., Schulman, H. and Greengard, P. (1978) J. Biol. Chem. 253, 6275-6280.

823. Walter, U., Uno, I., Liu, A. Y.-C. and Greengard, P. (1977) J. Biol. Chem. 252, 6494-6500.

824. Walter, U., Uno, I., Liu, A. Y-C. and Greengard, P. (1977) J. Biol. Chem. 252, 6588-6590.

825. Walton, G.M. and Gill, G.N. (1983) J. Biol. Chem. 258, 4440-4446.

826. Wang, J.H. and Desai, R. (1976) Biochem. Biophys. Res. Commun. 72, 926-932.

827. Watkins, P.A., Tarlow, D.M. and Lane, M.D. (1977) Proc. Natl. Acad. Sci. USA 74, 1497-1501.

828. Watters, C.D. (1984) Biochem. J. 224, 39-45.

829. Waymack, P.P., De Buyser, M.S. and Olsen, M.S. (1980) J. Biol. Chem. 255, 9773-9781.

830. Weber, H.W. and Appleman, M.M. (1982) J. Biol. Chem. 257, 5339-5341.

831. Weber, W. and Hilz, H. (1979) Biochem. Biophys. Res. Commun. 90, 1073-1081.

832. Wells, J.N., Baird, C.E., Wu, Y.J. and Hardman, J.G. (1975) Biochim. Biophys. Acta 384, 430-442.

833. Wells, J.N. and Hardman, J.G. (1977) Adv. Cyclic Nucleotide Res. Vol. 8, pp. 119-144 (Greengard, P. and Robison, G.A. Eds.) Raven Press, New York.

834. Werth, D.K., Niedel, J.E. and Pastan, I. (1983) J. Biol. Chem. 258, 11423-11426.

835. Westwood, S.A., Luzio, J.P., Flockhart, D.A. and Siddle, K. (1979) Biochim. Biophys. Acta 583, 454-466.

836. Wettenhall, R.E.H., Chesterman, C.N., Walker, T. and Morgan, F.J. (1983) FEBS Lett. 162, 171-176.

837. Wettenhall, R.E.H. and Cohen, P. (1982) FEBS Lett. 140, 263-269.

838. Wettenhall, R.E.H., Cohen, P., Caudwell, B. and Holland, R. (1982) FEBS Lett. 148, 207-213.

839. Wettenhall, R.E.H. and Morgan, F.J. (1984) J. Biol. Chem. 259, 2084-2091.

840. White, M.F., Haring, H.U., Kasuga, M. and Kahn, C.R. (1984) J. Biol. Chem. 259, 255-264.

841. Whitson, R.H. and Appleman, M.M. (1982) Biochim. Biophys. Acta 714, 279-291.

842. Wickson, R.D., Boudreau, R.J. and Drummond, G.I. (1975) Biochemistry 14, 669-675.

842a. Wilde, C.J. and Kuhn, N.J. (1979) Biochem. J. 182, 287-294.

843. Williams-Ashman, H.G. and Canellakis, Z. (1979) Perspect. Biol. Med. 22, 421-453.

843a. Williamson, D.H. (1980) FEBS Lett. 117, suppl. K93-K105.

844. Wilson, S.R. and Houslay, M.D. (1983) Biochem. J. 213, 99-105.

845. Wilson, S.R., Wallace, A.V. and Houslay, M.D. (1983) Biochem. J. 216, 245-248.

846. Wise, B.C., Glass, D.B., Chou, C.-H.J., Raynor, R.L. and Katoh, N. (1982) J. Biol. Chem. 257, 8489-8495.

847. Wise, B.C., Guidotti, A. and Costa, E. (1983) Proc. Natl. Acad. Sci. USA 80, 886-890.

848. Witters, L.A. (1981) Biochem. Biophys. Res. Commun. 100, 872-878.

849. Witters, L.A., Kowloff, E.M. and Avruch, J. (1979) J. Biol. Chem. 254, 245-248.

850. Witters, L.A., Tipper, J.P. and Bacon, G.W. (1983) J. Biol. Chem. 258, 5643-5648.

851. Wolf, D.J. and Brostrom, C.O. (1979) Adv. Cyclic Nucleotide Res. 11, 27-88.

852. Wong, E.H.A. and Loten, E.G. (1981) Eur. J. Biochem. 115, 17-22.

853. Wong, E.H.A., Loten, E.G., Exton, J.H. and Park, C.R. (1981) FEBS Lett. 127, 217-220.

854. Wong, T.-W. and Goldberg, A.R. (1983) Proc. Natl. Acad. Sci. USA 80, 2529-2533.

855. Woodgett, J.R., Cohen, P., Yamauchi, T. and Fujisawa, H. (1984) FEBS Lett. 170, 49-54.

856. Woodgett, J.R., Davison, M.T. and Cohen, P. (1983) Eur. J. Biochem. 136, 481-487.

857. Wrenn, R.W., Katoh, N. and Kuo, J.F. (1981) Biochim. Biophys. Acta 676, 266-269.

858. Wrenn, R.W., Katoh, N., Wise, B.C. and Kuo, J.F. (1980) J. Biol. Chem. 255, 12042-12046.

859. Wrenn, R.W. and Wooten, M.W. (1984) Life Sci. 35, 267-276.

860. Yagi, K., Yazawa, M., Kakiuchi, S., Ohshima, M. and Uenishi, K. (1978) J. Biol. Chem. 253, 1338-1340.

861. Yamamoto, H., Fukunaga, K., Tanaka, K. and Miyamoto, E. (1985) J. Neurochem. 41, 1119-1125.

862. Yamauchi, T. and Fujisawa, H. (1982) Biochem. Biophys. Res. Commun. 109, 975-981.

863. Yamauchi, T. and Fujisawa, H. (1983) Eur. J. Biochem. 132, 15-20.

864. Yang, S.-D., Vandenheede, J.R. and Merlevede, W. (1980) J. Biol. Chem. 255, 11759-11767.

865. Yang, S.-D., Vandenheede, J.R. and Merlevede, W. (1981) J. Biol. Chem. 256, 10231-10234.

866. Yasuda, H., Matsumoto, Y., Mita, S., Marunouchi, T. and Yamada, M. (1981) Biochemistry 20, 4414-4419.

867. Yeaman, S.J. (1985) Abstr. 8th International Conference on Metabolic Interconversions of Enzymes, Pitlochry, Scotland.

868. Yeaman, S.J. and Cohen, P. (1975) Eur. J. Biochem. 51, 93-104.

868a. Yeaman, S.J., Cook, K.G., Boyd, R.W. and Lawson, R. (1984) FEBS Lett. 172, 38-42.

869 Yeaman, S.J., Hutcheson, E.T., Roche, T.E., Pettit, F.H., Brown, J.R., Reed, L.J., Watson, D.C. and Dixon, G.H. (1978) Biochemistry 17, 2364-2370.

870. Yearna, M.-J., Hartshorne, D.J. and Goldman, R.D. (1979) Biochemistry 18, 673-678.

871. Yokoyama, M., Furuya, E. and Uyeda, K. (1982) Biochem. Biophys. Res. Commun. 105, 264-270.

872. Yu, K.-T. and Czech, M.P. (1984) J. Biol. Chem. 259, 5277-5286.

872a. Zammit, V.A. (1985) Biochem. Soc. Trans. 13, 831-833.

873. Zammit, V.A. and Corstorphine, C.G. (1982) Biochem. J. 204, 757-764.

874. Zammit, V.A. and Corstorphine, C.G. (1982) Biochem. J. 208, 783-788.

875. Zick, Y., Kasuga, M., Kahn, C.R. and Roth, J. (1983) J. Biol. Chem. 258, 75-80.

876. Zick, Y., Rees-Jones, R.W., Taylor, S., Gorden, I. and Roth, J. (1984) J. Biol. Chem. 259, 4396-4400.

877. Zick, Y., Whittaker, J. and Roth, J. (1983) J. Biol. Chem. 258, 3431-3434.

877a. Zinder, O., Hamosh, M., Fleck, T.R.C. and Scow, R. (1974) Am. J. Physiol. 226, 744-748.

878. Zinman, B. and Hollenberg, C.H. (1974) J. Biol. Chem. 249, 2182-2187.

879. Zoller, M.J., Kerlavage, A.R. and Taylor, S.S. (1979) J. Biol. Chem. 254, 2408-2412.