Partial Purification and Characterisation of the Membrane-Associated DAG Kinase of Rat Brain.

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Dedicated to Rab (1942 - 1988).

Still with us in so many ways.

Also, in memory of Andrew and Agnes.

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Abbreviations.

ATP	Adenosine 5'-triphosphate.
$[Ca^{2+}]_i$	Intracellular calcium concentration.
cDNA	Complimentary deoxyribonucleic acid.
CDP-choline	Cytidine 5'-diphosphocholine.
c.m.c.	Critical micellar concentraton.
CMP	Cytidine 5'-monophosphate.
CMP-phosphatidic acid	Cytidine 5'-monophosphate-phosphatidic acid.
cpm.	Counts per minute.
CRF	Corticotrophin releasing factor.
CTP	Cytidine 5'-triphosphate.
DAG	*sn*-1,2-Diacylglycerol.
DAG kinase	Diacylglycerol kinase.
DETPAC	Diethylenetriaminepentaacetic acid.
E 64	*trans*-Epoxysuccinyl-L-leucylamido(4-guanidino)butane.
EDTA	Ethylenediaminetetraacteic acid.
EGF	Epidermal growth factor.
EGTA	[Ethylenebis(oxyethylenenitril)]-tetraacetic acid.
fMet-Leu-Phe	*N*-Formylmethionylleucylphenylalanine.
GLC	Gas/liquid chromatography.
GTP	Guanosine 5'-triphosphate.
GTPγS	Guanosine 5'-*O*-(3-thiotriphosphate).
h	Hour
HPLC	High pressure liquid chromaography.
Ins(1)P	D-*myo*-inositol 1-monophosphate.
Ins(3)P	D-*myo*-inositol 3-monophosphate.
Ins(4)P	D-*myo*-inositol 4-monophosphate.

$Ins(1,3)P_2$	D-*myo*-inositol 1,3-bisphosphate.
$Ins(1,4)P_2$	D-*myo*-inositol 1,4-bisphosphate.
$Ins(3,4)P_2$	D-*myo*-inositol 3,4-bisphosphate.
$Ins(1,3,4)P_3$	D-*myo*-inositol 1,3,4-trisphospahte.
$Ins(1,4,5)P_3$	D-*myo*-inositol 1,4,5-trisphosphate.
$Ins(1,4,5)P_3$	D-*myo*-inositol 1,3,4,5-tetrakisphosphate.
MARCKS protein	Myristoylated alanine-rich C-kinase substrate protein
min	Minute.
OD	Optical Density.
$^{32}P_i$	[^{32}P]orthophosphate.
PA.	Phosphatidic acid.
PDBu	Phorbol 12,13-dibutyrate.
PDGF	Platelet-derived growth factor.
pH_i	Intracellular pH.
PKC	Protein kinase C.
PMA	Phorbol 12-myristate 13-acetate.
PMSF	Phenylmethylsulfonyl fluoride.
PPH	Phsophatidic acid phosphohydrolase.
PtdCho	Phosphatidylcholine.
PtdEtn	Phosphatidylethanolamine.
PtdIns	Phosphatidylinositiol.
PtdIns(4)Ins	Phosphatidylinositol-4-phosphate.
$PtdIns(4,5)P_2$	Phosphatidylinositol-4,5-bisphosphate.
PtdIns-PLC	Phosphoinositide specific phospholipase C.
PtdOH	Phosphatidic acid.
PtdSer	Phosphatidylserine.
SAG	*sn*-1-Stearoyl-2-arachidonylglycerol.
SD	Standard deviation.
sec	Second.

t.l.c.	Thin layer chromatography.
TRH	Thyrotropin-releasing hormone

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Summary

The *sn*-1,2-Diacylglycerol produced on receptor-stimulated hydrolysis of PtdIns(4,5)P_2 hydrolysis is metabolised primarily by phosphorylation in a reaction catalysed by DAG kinase. The DAG kinase active in this role has been localised to the membrane-associated compartment and has been shown to display catalytic selectivity towards different *sn*-1,2-diacylglycerols. This study examines the purification and characterisation of membrane-associated DAG kinase from rat brain.

DAG kinase activity was predominantly associated with the soluble component of rat brain homogenate. Incubation of the homogenate with phospholipase C (*B. cereus*) affected a redistribution of the soluble DAG kinase activity toward the particulate fraction, thus providing a higher specific activity starting material for the purification of the membrane-associated enzyme. Incubation of such phospholipase C-treated membranes with hypertonic concentrations of KCl resulted in the efficient solubilisation of the DAG kinase activity. The protein preparation solubilised in this manner was found to contain two resolvable enzymes: (1) a KCl-independent DAG kinase which remains soluble in the absence of KCl, and (2) a KCl-soluble DAG kinase which requires the presence of at least 300 mM KCl for continued solubility.

The purification of the KCl-soluble enzyme was further investigated. It was possible to purify this enzyme, in the presence of 300 mM KCl, by successive chromatographic separation on AffiGel blue, butyl-Sepharose 4B/phenyl-Sepharose CL-4B, Sephacryl S-300 SF, heparin-agarose, and hydroxyapatite. SDS-PAGE revealed the final protein preparation to be heterogeneous. Furthermore, it was not possible to identify any proteins which co-migrate with DAG kinase activity on hydroxyapatite chromatography of the partially purified protein preparation.. Although, the KCl-soluble DAG kinase remains to be purified to homogeneity, it migrates on gel filtration with an apparent native molecular mass ozf 160 kDa..

The substrate dependence of the partially purified enzyme was characterised by mixed micellar assay methodology. The post gel filtration enzyme preparation was found to have a V_{max} of 29.9 nmol/min/mg and K_m values of 1.7 mol% and 330 μM for *sn*-1-stearoyl-2-arachidonylglycerol and ATP, respectively. Furthermore, when the K_m and V_{max} of the KCl-soluble DAG kinase was determined with *sn*-1,2-dioleoylglycerol and *sn*-1,2-dilaurylglycerol they were found not to be significantly altered from the values determined with *sn*-1-stearoyl-2-arachidonylglycerol. Therefore, the KCl-soluble enzyme was not found to be selective amongst *sn*-1,2-diacylglycerol substrates. The reaction velocity of the KCl-soluble DAG kinase is independent of Ca^{2+} and PtdSer, but the enzyme would appear to require 7.0 mM, or greater, free Mg^{2+} for maximal catalytic rates. Finally, although the V_{max} of the DAG kinase did not alter, its K_m for ATP was observed to decrease by greater than 4-fold in the presence of 20 mM potassium phosphate.

Chapter 1

Introduction.

1.1. Agonist-Stimulated Turnover of Phosphoinositides.

1.1.1. The Position of Diacylglycerol Kinase in Agonist-Stimulated Turnover of Phosphoinositides.

Agonist-stimulated inositol phospholipid hydrolysis was originally observed as the stimulated incorporation of $^{32}P_i$ into phospholipids on cholinergic stimulation of avian pancreas slices (Hokin & Hokin, 1953). The stimulated incorporation of $^{32}P_i$ into phospholipid was found to be confined specifically to the phosphoinositides and phosphatidic acid components (Hokin & Hokin, 1958b). Phosphatidic acid had only recently been identified in animal cells *in vivo* (Hokin & Hokin, 1958a), yet agonist-stimulated $^{32}P_i$ incorporation into this lipid was observed in parallel with stimulated incorporation of $^{32}P_i$ into the phosphoinositides in both CRF-stimulated rat hypothalamus (Hokin *et al.*, 1958a) and cholinergic-stimulated guinea pig adrenal medulla (Hokin *et al.*, 1958b). Moreover, stimulated incorporation of $^{32}P_i$ and [^{3}H]inositol into the 'monophosphoinositide' pool was observed to occur with near 1:1 stoichiometry and thus independent of *de novo* synthesis of the glycerolipid backbone in cholinergic-stimulated guinea pig brain slices (Hokin & Hokin, 1958c).

The effect of these agonists on the synthesis of phosphatidic acid and phosphatidylinositol was suggested by Durell *et al.* (1969) to be secondary to the receptor-mediated hydrolysis of phosphoinositides. These workers suggested hydrolysis of phosphoinositide in a phospholipase C-catalysed reaction as the initial receptor-mediated event. The previously observed incorporation of $^{32}P_i$ and [^{3}H]inositol merely identifying the resynthesis of the phosphoinositides via phosphorylation of *sn*-1,2-diacylglycerol and subsequent incorporation of free inositol. The nature of the 'PI-cycle' had been established and, within it, the key role of DAG kinase in the resynthesis of phosphoinositide had also been identified.

Agonist-stimulated reduction of the phosphatidylinositol content of cell membranes provided further evidence that hydrolysis of phosphoinositide was, indeed, the initial receptor-mediated event (Hokin-Neaverson, 1974; Jones &

Michell, 1974), whilst the role of DAG kinase within the cycle was corroborated by quantitative production of phosphatidic acid prior to resynthesis of phosphatidylinositol. Lapetina *et al.* (1981) and Rebecchi *et al.* (1983) observed the formation of phosphatidic acid prior to the synthesis of phosphatidylinositol in thrombin-stimulated platelets and TRH-stimulated GH_3 mammotropic pituitary cells, respectively. Furthermore, these workers observed the synthetic events to occur after the decrease in cellular phosphatidylinositol content. Additionally, Rebecchi *et al.* (1983) observed a transient accumulation of *sn*-1,2-diacylglycerol 15 sec after stimulation; a time equivalent to the initiation of increased rates of phosphatidic acid production. The 'PI-cycle' had, thus, been confirmed: the initial event proving to be the agonist-stimulated hydrolysis of phosphatidylinositol to *sn*-1,2-diacylglycerol and inositol phosphate. Thereafter, the cycle represents the biosynthetic pathway employed to resynthesise PtdIns, with DAG kinase initiating the metabolism of the*sn*-1,2-diacylglycerol product (Section 1.1.4).

Implicated in the phosphorylation of agonist-stimulated *sn*-1,2-diacylglycerol, DAG kinase was to be directly identified in this role. Employing a partially purified prepartion of porcine cytosolic DAG kinase, Bishop *et al.* (1986) identified two analogues of *sn*-1,2-dioctanoylglycerol active as competitive inhibitors of the kinase *in vitro*, i.e. dioctanylethylene glycol and 1-monooleoylglycerol. Both inhibitors resulted in longer-lived *sn*-1,2-diacylglycerol accumulation in thrombin-stimulated human platelets. Dioctanoylethylene glycol was also observed to elicit decreased production of phosphatidic acid. These findings were consistent with an inhibition of DAG kinase-catalysed phosphorylation of *sn*-1,2-diacylglycerol produced in response to agonist stimulation.

Direct identification of a cellular DAG kinase activity was performed by MacDonald *et al.* (1988a). Prelabelling Swiss 3T3 fibroblasts with $^{32}P_i$, these workers demonstrated the production of [^{32}P]phosphatidic acid on PDGF-stimulation. Additionally, the enzyme catalysing this phosphorylation event was

found to be selective for the *sn*-1,2-diacylglycerol produced endogenously on receptor-stimulation. PDGF-stimulation of Swiss 3T3 cells preincubated with *sn*-1,2-didecanoylglycerol, a cell-permeable *sn*-1,2-diacylglycerol, resulted in the selective phosphorylation of the endogenous lipid (MacDonald *et al.*, 1988a). This was also found to be the case when lysates prepared from PDGF-stimulated Swiss 3T3 fibroblasts that had been pretreated with *sn*-1,2-didecanoylglycerol were incubated with [γ-^{32}P]ATP (MacDonald *et al.*, 1988a). Therefore, *in vitro* evidence was now available to confirm that DAG kinase catalysed the phosphorylation of the *sn*-1,2-diacylglycerol produced on receptor-stimulation. Furthermore, the enzyme active in this role could be identified by its catalytic selectivity towards the *sn*-1,2-diacylglycerols produced endogenously on receptor-stimulation.

1.1.2. Agonist-Stimulated Hydrolysis of Phosphatidyl-4,5-Bisphosphate: The Initial Step of the 'PI-Cycle'.

Many hormones, neurotransmitters and growth factors are associated with receptor-stimulated phosphatidylinositol turnover and elevation of intracellular calcium concentrations ($[Ca^{2+}]_i$). Rather than being induced by the receptor-mediated elevation of $[Ca^{2+}]_i$, Michell (1975) proposed that agonist-stimulated phosphatidylinositol turnover was itself responsible for raising the $[Ca^{2+}]_i$. Indeed, stimulation of phosphatidylinositol turnover could be achieved in the absence of a rise in $[Ca^{2+}]_i$ after stimulation of Ca^{2+}-deprived parotid glands (Jones & Michell, 1975; Oron *et al.*, 1975). The absolute requirement for phosphatidylinositol metabolism in Ca^{2+} mobilisation was further demonstrated by the abrogation of intracellular Ca^{2+} mobilisation in 5-hydroxytryptamine-stimulated blowfly salivary glands in inositol-free medium (Fain & Berridge, 1979a,b).

The phosphoinositides are minor cell phospholipids in eukaryotic cells and together constitute only 2 % to 8 % of the cell membrane phospholipid (Majerus *et al.*, 1986). Phosphatidylinositol [PtdIns], phosphatidylinositol 4-monophosphate [PtdIns(4)P] and phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] exist in

equilibrium and are freely interconvertible by sequential phosphorylation and dephosphorylation reactions catalysed by discrete kinase and phosphatase activities (reviewed in Berridge & Irvine, 1989). However, the concentrations of the individual lipids are tightly regulated within the phosphoinositide pool.

In response to agonist-stimulation, PtdIns(4,5)P_2 is specifically hydrolysed in a reaction catalysed by phospholipase C, whereas PtdIns and PtdIns(4)P are not substrates for this reaction (Kirk *et al.*, 1981; Michell *et al.*, 1981; Michell & Kirk, 1981). This is despite PtdIns(4,5)P_2 existing as a minor component of the phosphoinositide pool, occupying only 1 - 10 % of the phosphoinositide pool (Majerus *et al.*, 1986). Yet, such agonist-stimulated hydrolysis of PtdIns(4,5)P_2 rather than the more abundant phosphoinositides was further confirmed by Arganoff *et al.* (1983) who observed a thrombin-induced reduction of PtdIns(4,5)P_2 without reduction of PtdIns content in $^{32}P_i$-labelled human platelets. Similarly, the PtdIns(4,5)P_2 content of hepatocytes was also observed to decrease on vasopressin-stimulation (Litosch *et al.*, 1983). However, rather than eliciting an agonist-stimulated decrease in PtdIns levels, vasopressin-stimulation of hepatocytes was found to be associated with increased synthesis of PtdIns. Such vasopressin-stimulated PtdIns synthesis was observed in parallel with resynthesis of PtdIns(4,5)P_2 and subsequent to the transient accumulation of phosphatidic acid (Litosch *et al.*, 1983). The previously observed reduction of PtdIns in response to agonists has since been attributed to its position as a precursor of PtdIns(4,5)P_2, although, this has yet to be widely demonstrated.

The agonist-mediated hydrolysis of PtdIns(4,5)P_2 has proven to be associated with the initial cellular action of many hormones, neurotransmitters and growth factors in a wide range of tissue and cell types, and has been extensively reviewed (Downes and Michell, 1985; Berridge, 1987; Berridge & Irvine, 1989; and Whitman & Cantley, 1988). Phosphoinositide-specific phospholipase C (phosphoinositidase C; suggested by Downes & Michell, 1985) has proven to consist of a family of related activities (reviewed by Rhee *et al.*, 1989; Meldrum *et*

al., 1991). Within the family there are four classes: α, β, γ and δ. It should be noted that the α classification is based on the estimated molecular weight of several PtdIns-PLC activities with apparent molecular mass of 60-70 kDa (Rhee *et al.*, 1989). Only one PtdIns-PLCα has been cloned to date, although its integrity is in doubt as the cDNA clone was unable to support catalytic activity in an expression system (Bennet & Crooke, 1987). Additionally, PtdIns-PLCα has been found to possess little structure or sequence similarity with cloned PtdIn-PLCβ, γ, or δ (Meldrum *et al.*, 1991). Of the other member classes (i.e. β, γ & δ), Rhee (1989) suggested the following classification for the PtdIns-PLC activities purified from bovine brain: β_1 to correspond to the the 154 kDa activity, γ_1 to the 145 kDa activity, and δ_1 to refer to the 85 kDa activity. Further purification and cloning has revealed yet more members of these three families. Comparison of their deduced primary structure and immunoreactivity allows their classification as β_2, γ_2, δ_2 and δ_3. Although little sequence identity is shared between the members of the β, γ and δ classes of PtdIns-PLC, comparison of the primary structures of β_1, γ_1 and δ_1 has revealed two highly conserved regions. PtdIns-PLCβ_1, -γ_1 and -δ_1 share 43 % identity in region I and 33 % identity in region II (Meldrum *et al.*, 1991). The degree of homology in these conserved regions between individual members of a single class is much higher, e.g. β_1 and β_2 share 79% identity in region I and 71% identity in region II (Meldrum *et al.*, 1991).

Receptor-stimulated PtdIns(4,5)P_2 hydrolysis, catalysed by PtdIns-PLC activity, is modulated across the plasma membrane by a guanine-nucleotide binding protein (reviewed by Cockcroft, 1987). Such guanine-nucleotide binding proteins (G-proteins) represent a family of heterotrimeric proteins that regulate the activity of a range of intracellular effector enzymes (see Simon *et al.*, 1991). The heterotrimeric G-proteins are comprised of α-, β- and γ-subunits and have been arranged into four families based on the primary sequence identity of their α-subunits (Simon *et al.*, 1991). From this classification, the Gα_q family members, Gα_{11} and Gα_q, were initially identified as the G-proteins active in the modulation

of PtdIns-PLC activity (Gutowski *et al.*, 1991; Taylor *et al.*, 1991; Wu *et al.*, 1992). Taylor and co-workers (1990) purified a mixture of $G\alpha_{11}/G\alpha_q$, on the basis of its ability to activate partially purified PtdIns-PLC, from GTPγS-treated bovine liver membranes. When reconstituted with specific PtdIns-PLC isoenzymes in the presence of GTPγS, the $G\alpha_{11}/G\alpha_q$ mixture specifically activated PtdIns-PLCβ_1, but not PtdIns-PLCγ_1 nor PtdIns-PLCδ_1 (Taylor *et al.*, 1991). Similarly, the $G\alpha_q$ ($G\alpha_{11}$) homologue purified from turkey erythrocytes has been shown to activate the PtdIns-PLC purified from the same source (PtdIns-PLCβ_1 homologue) (Waldo *et al.*, 1991). Further investigation of the individual $G\alpha_q$ family members' abilities to activate PtdIns-PLC isoenzymes has revealed a functional division between the family members not previously observed. Membranes prepared from Cos-7 cells transfected with cDNAs corresponding to all four $G\alpha_q$ family members, i.e. $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$ and $G\alpha_{16}$, have been found to stimulate PtdIns-PLCβ_1 on reconstitution with GTPγS (Rhee & Choi, 1992). Membranes prepared from cells expressing $G\alpha_q$ and $G\alpha_{11}$ were most effective in this role . However, a different pattern was observed when PtdIns-PLCβ_2 was reconstituted with similarly transfected Cos-7 cell membranes in the presence of GTPγS. In this instance, $G\alpha_{16}$ was the most potent activator of PtdIns-PLCβ_2 activity, with $G\alpha_q$, $G\alpha_{11}$ and $G\alpha_{14}$ far less stimulatory (Rhee & Choi,.1992).

The receptors for certain polypeptide growth factors, for example EGF and PDGF, stimulate PtdInsPLC activity by a mechanism distinct from that described above (reviewed Boyer *et al.*, 1989; Rhee, 1991; Rhee & Choi, 1992). Occupation of the growth factor receptor results in increased activity of the receptor tyrosine kinase (RTK), which is accompanied by increased phosphorylation of PtdIns-PLCγ_1. Associated with the increased phosphorylation of PtdIns-PLCγ_1 is an elevated rate of PtdIns(4,5)P_2 hydrolysis catalysed by this enzyme. Thus, receptor stimulated PtdIns-PLC activity is modulated by a pathway independent of independent of G-protein involvement.

To date, neither the receptors nor the transmembrane transduction system coupled with the PtdIns-PLCδ family members have been identified.

Of the *bona fide* cloned PtdIns-PLC family members: PtdInsPLCβ_1 and -β_2 possess catalytic specificity for the polyphosphoinositides, catalysing the hydrolysis of PtdIns(4)P and PtdIns(4,5)P_2 with greater reaction velocities than PtdIns. Elevation of the Ca^{2+} concentration to mM does not result in alteration of the catalytic selectivity towards polyphosphoinositides. Similar to the PtdIns-PLCβ family members, PtdIns-PLCδ_1 and -δ_2 catalyse the hydrolysis of polyphosphoinositides at greater reaction rates than measured with PtdIns at physiological or low Ca^{2+} concentrations. However, distinct from the PtdIns-PLCβ family members, PtdIns-PLCδ_1 and δ_2 will hydrolyse PtdIns with increased reaction velocity at mM Ca^{2+}, losing their specificity for polyphosphoinositides. Finally, PtdIns-PLCγ_1 has not been observed to possess catalytic selectivity at physiological Ca^{2+} concentrations, catalysing the hydrolysis of PtdIns(4,5)P_2 and PtdIns with equal rates (reviewed by Meldrum *et al.*, 1991).

1.1.3. The Products of PtdIns-PLC-Catalysed Hydrolysis of PtdIns(4,5)P_2 Are Active as Intracellular Messengers.

The products of PtdIns-PLC-catalysed hydrolysis of PtdIns(4,5)P_2 are *sn*-1,2-diacylglycerol and inositol-1,4,5-trisphosphate [Ins(1,4,5)P_3]. They are both active as second messengers. Ins(1,4,5)P_3 has been identified as the agent responsible for evoking the rise in intracellular Ca^{2+} (Streb *et al.*, 1983), characteristic of the agonists active in stimulating PtdIns(4,5)P_2 turnover. Binding a specific intracellular receptor, Ins(1,4,5)P_3 evokes release of calcium from the hormone-sensitive Ca^{2+} store located in the smooth endoplasmic reticulum (reviewed by Berridge & Irvine, 1984; Berridge & Irvine, 1989). On the other hand, acting synergistically with phosphatidylserine and Ca^{2+}, *sn*-1,2-diacylglycerol has been identified as the physiological activator of protein kinase C [PKC] (Nishizuka, 1988a; Parker *et al.*, 1989). *sn*-1,2-Diacylglycerol is, thus,

responsible for mediating the increased activity of PKC associated with Ca^{2+}-mobilising agonists.

Additionally, Ins(1,4,5)P_3 is phosphorylated by Ins(1,4,5)P_3 3-kinase to produce inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P_4] (see Section 1.1.4), an inositol phosphate active in elevating intracellular Ca^{2+} concentrations by promoting entry of extracellular Ca^{2+} in an Ins(1,4,5)P_3-dependent process (Irvine & Moor, 1986; reviewed by Berridge & Irvine, 1989).

1.1.4. Metabolism of Ins(1,4,5)P_3 and *sn*-1,2-Diacylglycerol in Agonist-Stimulated Cells.

Both second messengers, Ins(1,4,5)P_3 and *sn*-1,2-diacylglycerol, are rapidly metabolised to allow resynthesis of the parent, agonist-sensitive phosphoinositide; PtdIns(4,5)P_2. Ins(1,4,5)P_3 is exclusively dephosphorylated by the Ins(1,4,5)P_3/Ins(1,3,4,5)P_4 5-phosphatase to Ins(1,4)P_2. The same catalytic activity is able to dephosphorylate Ins(1,3,4,5)P_4 to Ins(1,3,4)P_3. The combined presence of Ins(1,4,5)P_3 3-kinase and Ins(1,4,5)P_3/Ins(1,3,4,5)P_4 5-phosphatase, thus, explains the observed increases in Ins(1,3,4,5)P_4 and Ins(1,4)P_2 following agonist stimulation. Ins(1,4)P_2 and Ins(1,3,4)P_3 are dephosphorylated by the Ins(1,3,4)P_3/Ins(1,4)P_2 1-phosphatase to produce Ins(4)P and Ins(3,4)P_2, respectively. Additionally, Ins(1,3,4)P_3 can be dephosphorylated by a 4-phosphatase activity that is also active in the dephosphorylation of the Ins(3,4)P_2 product of Ins(1,3,4)P_3/Ins(1,4)P_2 1-phosphatase activity. The Ins(1,3)P_2 product of the 4-phosphatase activity is metabolised by a 3-phosphatase activity to yield Ins(1)P. Collectively, the three monophosphate products, Ins(1)P, Ins(3)P and Ins(4)P are substrates for inositol monophosphatase and are dephosphorylated to free inositol base. The integrated phosphorylation and dephosphorylation events involved in the metabolism of the inositol phosphates and release of free inositol prior to resynthesis of PtdIns(4,5)P_2 have been summarised and reviewed elsewhere (Berridge & Irvine, 1989; Shears, 1991).

sn-1,2-Diacylglycerol, the glycerolipid product of the PtdIns-PLC-catalysed hydrolysis of PtdIns(4,5)P_2, is metabolised initially by phosphorylation to phosphatidic acid (*sn*-1,2-diacylglycero-3-phosphate). Phosphorylation by DAG kinase is the initial and arguably the commited step in the recycling of the intact glycerolipid backbone towards the parent phospholipid, PtdIns(4,5)P_2. Phosphatidic acid is condensed with CTP to form CMP-phosphatidate in a reaction catalysed by CMP-phosphatidic acid synthetase. Reaction of CMP-phosphatidate with the inositol released from the inositol phosphate pool produces the phospholipid, PtdIns, in a reaction catalysed by PtdIns synthetase. This reaction supplies the PtdIns precursor of the polyphosphoinositides while liberating CMP from the lipid pool. The steady-state levels of PtdIns, PtdIns(4)P and PtdIns(4,5)P_2 are then governed by the integrated action of several activities; PtdIns 4-kinase, PtdIns(4)P phosphomonoesterase, PtdIns(4)P 5-kinase, and PtdIns(4,5)P_2 monophosphoesterase (summarised by Majerus *et al.*, 1986; Berridge & Irvine, 1989). Thus, the 'PI-cycle' is complete and predicts both the removal of Ins(1,4,5)P_3 and *sn*-1,2-diacylglycerol to metabolic intermediates that are inactive as second messengers and also the resynthesis of the phosphoinositides.

Three exceptions to the accepted dogma of the 'PI-cycle' should be noted; the hydrolysis of PtdIns and PtdIns(4)P catalysed by PtdIns-PLC; the accumulation of cylic inositol phosphates in receptor-stimulated cells; and the production of PtdIns(3)P by PtdIns 3-kinase. As discussed earlier, both PtdIns-PLCδ_1 and -δ_2 will hydrolyse PtdIns and PtdIns(4,5)P_2 with similar reaction velocities in the presence of mM Ca^{2+}, despite showing marked specificity for the polyphosphoinositides at physiological or low Ca^{2+} concentrations (reviewed Meldrum *et al.*, 1991). In parallel it has been suggested that stimulated PtdIns-PLC activity has resulted in the hydrolysis of PtdIns and PtdIns(4)P, most likely secondary to the elevation of intracellular calcium in response to agonist-stimulation (Brammer & Weaver, 1989; Baird & Nahorski, 1990). Therefore, hydrolysis of

phosphoinositides distinct from PtdIns(4,5)P_2 does occur, albeit secondary to the receptor-mediated mobilisation of intracellular Ca^{2+} stores. The physiological significance of this hydrolytic event is not known as Ins(1,4)P_2 and Ins(1)P are not active in the role of mobilising intracellular calcium stores. However, the *sn*-1,2-diacylglycerol produced could potentially stimulate PKC activity.

Additionally, the cyclic inositol phosphates, Ins(1:2cyc,4,5)P_3, Ins(1:2cyc,4)P_2 and Ins(1:2cyc)P, have been found to accumulate in response to agonist stimulation (reviewed Berridge & Irvine, 1989). Formed by PtdIns-PLC-catalysed hydrolysis of the phosphoinositides, they constitute only a minor component of the inositol phosphate products. Only 1 - 2 % of phospholipase C-catalysed hydrolysis of PtdIns(4,5)P_2 results in the production of Ins(1:2cyc,4,5,)P_3 (Hawkins *et al.*, 1987; Wong *et al.*, 1988). It is unlikely that the cylic inositol phosphates are physiologically active as Ca^{2+} mobilising agents, since Ins(1:2cyc,4,5)P_3 possesses 10-fold less efficacy than Ins(1,4,5)P_3 in this role (Willcocks *et al.*, 1989). The *sn*-1,2-diacylglycerol product would still, however, be active in its physiological role.

Finally, Whitman *et al.* (1988) and Traynor-Kaplan *et al.* (1988) made the novel observation of inositol phospholipids phosphorylated in the 3-position of the inositol headgroup. The 3-phosphate containing phosphatidylinositols represent only 1 - 2 % of the other phosphatidylinositol polyphosphates (Stephens *et al.*, 1989). However, preliminary characterisation has shown that PtdIns(3)P and PtdIns(3,4)P_2 are only poor substrates for PtdIns-PLC while PtdIns(3,4,5)P_3 is not a substrate at all (Serunian *et al.*, 1989; Lips *et al.*, 1989). It is unlikely that these newly discovered lipids will make a significant contribution to agonist-stimulated levels of inositol phosphates or *sn*-1,2-diacylglycerols.

1.2. The Role of *sn*-1,2-Diacylglycerol in Agonist-Stimulated Cells: Physiological Activator of PKC.

PKC, the diacylglycerol-activated/Ca^{2+} and phospholipid-dependent protein kinase, is a critical component of the signal transduction system defined by the accelerated hydrolysis of PtdIns(4,5)P_2 (Nishizuka, 1984, 1986). As such, PKC directly catalyses many of the intracellular protein phosphorylation events associated with cellular activation by Ca^{2+}-mobilising agonists. In this role it is directly responsible for the modulation of the intracellular reponse after receptor occupation.

In a model recently proposed by Bell & Burns (1991), accumulation of *sn*-1,2-diacylglycerol in the membranes of agonist-stimulated cells results in the activation of membrane-associated PKC. PKC resides on the cell membrane by interaction with phospholipids, specifically phosphatidylserine, in a calcium-dependent manner and is, thus, juxtaposed to the membrane-intercalated *sn*-1,2-diacylglycerol that will modulate its rate of reaction. Apropos to this, PKC resident in the cytosol represents an inactive pool, conformationally unable to phosphorylate substrate proteins, but with full ability to associate with the membrane in response to elevation of intracellular Ca^{2+} and consequently be activated by increased levels of *sn*-1,2-diacylglycerol. Similarly, activation of PKC by phorbol 12-myristate 13-acetate (PMA), a tumour-promoting phorbol ester, is observed as an alteration of the enzyme's intracellular distribution (Nishizuka, 1984, 1986). Inactive cytosolic PKC redistributes to the membrane in response to PMA treatment, as would be predicted from the above model for a potent activator of PKC. Direct interaction of PMA with the *sn*-1,2-diacylglycerol binding site has been demonstrated, showing PMA acts as a mimetic of the physiological lipid (Castagna *et al.*, 1982; Sharkey *et al.*, 1984).

Originally cloned from bovine brain, PKC was found to be represented by a family of distinct isoenzymes sharing a high degree of homology in regions of their primary structure (reviewed by Parker *et al.*, 1989). Isolation of the cDNAs

corresponding to bovine brain PKC revealed four distinct isotypes: α, β_1, β_2 and γ (see Parker *et al.*, 1989). Huang *et al.* (1986) had observed that a purified preparation of PKC activity from bovine brain could be resolved to three immunologically distinct peaks of activity on hydroxyapatite chromatography. On the basis of both immunoreactivity of the antisera raised against these resolved activities and also comparison of cell-type specific expression of immunoreactive protein in rat brain, the putative isoenzymes were identified as PKC-α, PKC-β_1/β_2 and PKC-γ (Huang *et al.*, 1987). Finally, resolved PKC-α, -β_1/β_2 and -γ activities were found to be biochemically distinguishable with respect to (a) their phospholipid requirements, (b) *sn*-1,2-diacylglycerol and phorbol ester activation, and (c) K_m for different protein substrates, confirming their existence as distinct isoenzymes (Huang *et al.*, 1988a).

The deduced primary sequence of PKC-α, -β_1/β_2 and -γ reveals considerable sequence homology. Direct sequence comparison has identified four highly conserved regions (C1 - C4) surrounded by five regions of variable sequence (V1 - V5) (see Parker *et al.*, 1989; Parker 1992). Oligonucleotide probes constructed from the regions of conserved sequence have been employed to screen cDNA libraries. This approach has allowed the identification and isolation of several cDNA corresponding to novel PKC isoenzymes. As a result, the PKC family now consists of 10 members: α, β_1, β_2, γ, δ, ε, ζ, η, θ and L (Bell & Burns, 1991).

Although characterisation of all the putative members is incomplete it is clear that the members possess widely variant tissue distribution and biochemical characteristics with respect to Ca^{2+}-sensitivity, phospholipid activation and substrate specificity (Nishizuka 1988a, b; Marias *et al.*, 1990; Olivier & Parker, 1991; Schaap & Parker, 1990). However, it should be noted that the isotypes originally characterised from bovine brain, i.e. PKC-α, -β_1, -β_2 and -γ, have proved distinct from the other putative members of the PKC family as they represent a Ca^{2+}-sensitive sub-division within the family. On the other hand,

PKC-δ, -ε, -ζ, -θ, -η and -L lack the C2 region identified as conferring Ca^{2+}-sensitivity to PKC-α, -β_1, -β_2 and -γ. However, PKC-δ, -ε, -ζ, -θ, -η and -L display both phorbol ester binding and kinase activities independent of Ca^{2+} (Parker *et al.*, 1989; Bell & Burns, 1991; Parker *et al.*, 1992). The absence of a Ca^{2+}-requirement for phorbol ester binding and protein kinase activities is still encompassed by the model of PKC activation suggested by Bell & Burns (1991). The possibility of PKC activity binding the phospholipid membrane in a Ca^{2+}-independent manner was reconciled and, indeed, PKC-δ has been shown to associate with phospholipid in a Ca^{2+}-independent manner (Olivier & Parker, 1991). Furthermore, PKC-ζ does not translocate to the cell membrane in response to PMA-treatment, i.e. PKC-ζ does not accumulate in the catalytically active conformation on the cell membrane after treatment with either PMA. Typically, chronic exposure of cells to PMA results in association of PKC with the cell membrane in a catalytically active conformation that is susceptible to proteolytic degradation (Rodriguez-Pena & Rozengurt, 1984; Young *et al.*, 1987). However, chronic treatment of U937 cells with PMA neither results in translocation of the constitutively exressed PKC-ζ to the cell membrane nor increased susceptibility of the enzyme to proteolysis (Parker *et al.*, 1992). Conversely, these workers did observe translocation and down-regulation of PKC-α,-β, and -ε in U937 cells treated with PMA. In conclusion, PKC has been defined as a family of structurally related protein kinase activities that can be divided by their fundamental dependence on Ca^{2+} for activity. Until further studies are concluded, the physiological requirement for more than one Ca^{2+}-sensitive and one Ca^{2+}-insensitive PKC activity will remain unknown. However, it may possible to speculate that tissue-specific patterns of expression and also sub-cellular localisation of the divergent protein substrates have required the evolution of this growing family of proteins.

1.3. DAG Kinase Activity in Receptor-Stimulated PtdIns(4,5)P_2 Turnover.

1.3.1. Role of DAG Kinase In Stimulated PtdIns(4,5)P_2 Turnover: A Reiteration.

PMA is not readily metabolised in the cell and its use, with the exception of PKC-ζ, produces a chronic activation of PKC leading to proteolytic down-regulation (see Section 1.2.1). *sn*-1,2-Diacylglycerol is, on the other hand, readily metabolised and represents an activator species present only transiently within the cell, closely coupling PKC activation to receptor-mediated hydrolysis of PtdIns(4,5)P_2 by PtdIns-PLC (see Section 1.4.1). The activity responsible for removal the *sn*-1,2-diacylglycerol will, in effect, control the longevity of PKC activation and the phosphorylation events catalysed by the protein kinase. The cellular responses mediated by PKC-catalysed phosphorylation events are controlled by the activity responsible for the metabolism and, thus, removal of the *sn*-1,2-diacylglycerol second messenger.

The tight coupling of PKC-catalysed protein phosphorylation to the transient presence of *sn*-1,2-diacylglycerol was demonstrated in thrombin-stimulated platelets (Kawahara *et al.*, 1980; Sano *et al.*, 1983; Ieyasu *et al.*, 1982). Additionally, the synthetic cell-permeable *sn*-1,2-diacylglycerol analogue, *sn*-1-oleoyl-2-acetylglycerol, was found to promote phosphorylation of the 40 kDa PKC-substrate protein in intact platelets (Kaibuchi *et al.*, 1983). Thus, DAG kinase was implicated in the regulation of PKC activity when *sn*-1-oleoyl-2-acetylglycerol was found to be rapidly metabolised by phosphorylation to its phosphatidic acid derivative (Kaibuchi *et al.*, 1983). It is now widely accepted that PKC is regulated by DAG kinase-catalysed phosphorylation of its lipid activator (Nishizuka, 1984, 1986). Therefore, DAG kinase plays a key role in modulating the receptor-stimulated events mediated by PKC.

The dual role of DAG kinase in the cell signalling pathway associated with 'PI-cycle' is now clear: (a) it phosphorylates the *sn*-1,2-diacylglycerol produced in

response to receptor occupation, allowing resynthesis of the parent inositol phospholipids (see Section 1.1.4), and (b) it initiates the metabolism of PKC's physiological activator to metabolites inactive in this role.

1.3.2. The Metabolism of *sn*-1,2-Diacylglycerol Produced on Stimulated PtdIns(4,5)P_2 Turnover: A Caveat.

Diacylglycerol lipase (DAG lipase) has also been implicated in the metabolism of *sn*-1,2-diacylglycerol produced in response to receptor-occupation in platelets (Prescott & Majerus, 1983) and Swiss 3T3 fibroblasts (Habenicht *et al.*, 1981). Bell *et al.* (1979) originally observed that DAG lipase catalysed the release of arachidonate from the *sn*-2 position of PtdIns(4,5)P_2-derived *sn*-1,2-diacylglycerol in human platelets. In addition, they argued that the release of the acyl-group in the *sn*-1 position of the 1-monoacylglycerol product was also catalysed by DAG lipase. The ambiguity of DAG lipase possibly catalysing the hydrolysis of both acyl-ester bonds was resolved by Prescott & Majerus (1983). These workers demonstrated the initial hydrolytic event to be confined to the *sn*-1 position. The previously observed release of arachidonic acid from the *sn*-2 position of *sn*-1,2-diacylglycerol was the result of an endogenous 2-monoacylglycerol lipase, distinct from DAG lipase, that catalysed the release of free fatty acid and glycerol from 2-monoacylglycerol (Prescott & Majerus, 1983).

The presence of phospholipase A_2 activity, responsive to receptor-mediated events, has interfered with measurement of DAG lipase activity within cells pre-labelled with radioactive fatty acids, e.g. [^{3}H]arachidonic acid or [^{3}H]oleic acid. Rather than catalysing the release of the fatty-acid esterified at the *sn*-1 position of *sn*-1,2-diacylglycerol, phospholipase A_2 catalyses the release of the *sn*-2 fatty-acyl group from phospholipids. Interpretation of fatty-acid release experiments in prelabelled cells in the study of concerted DAG lipase/MAG lipase activity is compromised by the presence of phospholipase A_2. Although these enzymes act on distinct substrates, they catalyse the release of the same fatty acid, i.e. the fatty acyl

group esterified at the *sn*-2 position of either phospholipid or the *sn*-1,2-diacylglycerol derived from it. Therefore, interpretation of fatty acid release experiments must be accompanied by identification of the non-fatty acid product and the enzyme activity responsible for catalysing the hydrolytic event.

Bell *et al.* (1979) postulated that DAG lipase catalysed the release of arachidonate from *sn*-1-stearoyl-2-arachidonylglycerol, the major diglyceride product of receptor-mediated PtdIns-PLC activity in thrombin-stimulated platelets. However, Lapetina *et al.* (1981) concluded that the release of arachidonic acid in thrombin-stimulated platelets was the result of an event secondary to the production of phosphatidic acid, i.e. secondary to the action of DAG kinase and not a product of DAG lipase activity. Furthermore, Billah & Lapetina (1982) observed the parallel production of arachidonic acid and lysophosphatidic acid in thrombin-stimulated platelets, strongly indicating the existence of receptor-modulated phospholipase A_2 activity.

PDGF-stimulation of Swiss 3T3 fibroblasts results in the accumulation of free arachidonic acid subsequent to *sn*-1,2-diacylglycerol production (Habenicht *et al.*, 1981). Although this would implicate DAG lipase in the metabolism of *sn*-1,2-diacylglycerol in Swiss 3T3 fibroblasts, Currie *et al.* (1992) observed a phospholipase A_2-catalysed release of [^{3}H]arachidonate in pre-labelled Swiss 3T3 fibroblasts stimulated with bombesin. Furthermore, this bombesin-stimulated accumulation is maximal after 10 sec of stimulation (Currie *et al.*, 1992), suggesting PLA_2-catalysed release maybe secondary to Ins(1,4,5)P_3 and *sn*-1,2-diacylglycerol production in these cells (Cook *et al.*, 1990).

Therefore, in the case of platelets and Swiss 3T3 fibroblasts, the contributions of both DAG lipase and agonist-stimulted phospholipase A_2 must be considered when interpretationg fatty-acid release from pre-labelled cells. Nevertheless, the information suggests that receptor-stimulated levels of *sn*-1,2-diacylglycerol can be metabolised, in part, by DAG lipase. However, the route of *sn*-1,2-diacylglycerol metabolism is most likely split between the DAG lipase and

DAG kinase pathways. Sagawa *et al.* (1982) found that both DAG kinase and DAG lipase activities were present in human amniotic tissue homogenates, with the ratio of kinase:lipase activities altering with both Ca^{2+} and *sn*-1,2-diacylglycerol concentrations. Despite the requirement for supra-physiological Ca^{2+} concentrations *in vitro*, the possibility of alteration in the direction of *sn*-1,2-diacylglycerol metabolism in response to receptor-mediated events is evident. Bishop & Bell (1986) observed that [^{3}H]*sn*-1,2-dioctanoylglycerol, a cell permeable *sn*-1,2-diacylglycerol analogue, was metabolised primarily by DAG kinase in human platelets, although conserted DAG lipase/MAG lipase activity was still observed. Inhibition of platelet DAG kinase by either *sn*-1,2-dioctanoylethylene glycol or 1-monoacylglycerol (Bishop *et al.*, 1986) resulted in inhibition of phospholipid synthesis and increased [^{3}H]*sn*-1,2-dioctanoylglycerol longevity, consistent with inhibition of DAG kinase (Bishop & Bell, 1986). Compensatory elevation of the rate of *sn*-1,2-diacylglycerol metabolism by the DAG lipase/MAG lipase pathway was not observed on inhibition of DAG kinase, yet an increased production of phospholipid was observed on inhibition of DAG lipase by RHC 80267 (Bishop & Bell, 1986). This would serve to confirm the accepted view that the DAG kinase route is the major pathway for removal of the *sn*-1,2-diacylglycerol product of receptor-mediated PtdIns(4,5)P_2 hydrolysis.

1.3.3. The Physiological Importance of Diacylglycerol Kinase Activity: Diacylglycerol Kinase in the Diseased State.

Several *ras*-transformed fibroblast cell lines possess elevated levels of *sn*-1,2-diacylglycerol compared to non-transformed cells (Preiss *et al.*, 1986; Fleischman *et al.*, 1986; Kato *et al.*, 1988; Huang *et al.*, 1988b). As such, sustained elevation of *sn*-1,2-diacylglycerol levels has been suggested to be important in the maintenance of the transformed state (Macara, 1985).

Functional PKC activity has been shown to be required for maintenance, but not initiation of the *ras*-transformed state (Lacal *et al.*, 1987; Lloyd *et al.*,

1989). As may be expected for cells possessing elevated levels of *sn*-1,2-diacylglycerol, *ras*-transformed fibroblasts exhibit increased cellular PKC activity associated with the cell membranes, coincident with depleted cytosolic activity (Kamata, 1987; Huang *et al.*, 1988b). The translocation of PKC to the membrane in unstimulated transformed cells is also associated with elevated levels of PKC-catalysed phosphorylation compared to non-transformed cells (Wolfman & Macara, 1987). Predictably, such chronic activation results in down-regulation of PKC. Thus, *ras*-transformed cells exhibit decreased levels of PMA-stimulated PKC activity and reduced PDBu-binding (Wolfman & Macara, 1987; Wolfman *et al.*, 1987).

Consensus has still to be reached on whether elevated rates of phosphoinositide turnover initiates the elevation of *sn*-1,2-diacylglycerol levels in the membranes of *ras*-transformed cells (Wolfman & Macara, 1987; Huang *et al.*, 1988b), however the rate of its metabolism by DAG kinase would appear to be altered in the transformed state. Although total DAG kinase activity is not altered on transformation of rat fibroblasts, decreased membrane-associated and increased cytosolic activity is observed in the transformed state (Huang *et al.*, 1988b; Kato *et al.*, 1988). It was postulated that disruption of the mechanism by which soluble DAG kinase translocates to the membrane, in response to accumulation of *sn*-1,2-diacylglycerol, results in decreased membrane-association in transformed cells (Kato *et al.*, 1988). As translocation of cytosolic DAG kinase to the membrane-associated compartment has been suggested as a regulatory event in the metabolism of *sn*-1,2-diacylglycerol (see Section 1.8.4), loss of the ability to translocate may explain the elevated levels of *sn*-1,2-diacylglycerol in *ras*-transformed cells. This could indicate the importance of DAG kinase in maintaining the non-transformed state, with disfunction of the enzyme possibly leading to the activation-induced down-regulation of PKC observed in *ras*-transformed fibroblasts (Wolfman & Macara, 1987; Wolfman *et al.*, 1987).

Another example of DAG kinase dysfunction in the diseased state is the *Drosophila* retinal degradation mutant, *rdgA* (Inoue *et al.*, 1989). *rdgA* mutant flies experience age-dependent retinal degradation in adult life and their retinas possess abnormally low levels of PtdOH and DAG kinase activity compared to wild-type flies (Yoshioka *et al.*, 1983, 1984). Kinetic analysis of DAG kinase activity in heterozygotes of various *rdgA* mutants revealed that the K_m for *sn*-1,2-dioleoylglycerol was unaltered compared to the wild-type, yet the V_{max} was reduced by 40 % (Inoue *et al.*, 1989). Moreover, the observed DAG kinase activity was found to compare with the gene dosage of wild-type *rgdA* gene with a deletion mutant, spanning the *rdgA* locus, also unable to display DAG kinase activity (Inoue *et al.*, 1989). After eclosion from the pupal case, the retinas of mutant flies have reduced levels of DAG kinase activity and PtdOH, regardless of age. The development of their visual apparatus is also limited at eclosion, with complete degeneration ocurring within a few days. A possible causitive role of reduced DAG kinase activity in the mechanism of retinal degeneration is indicated.

1.3.4. The Significance of DAG kinase May be Indicated by the Specific Inhibitor, R59022.

R59022 was originally described as a DAG kinase inhibitor by Chaffoy de Courcelles *et al.* (1985). It inhibited the DAG kinase-catalysed phosphorylation of both endogenous *sn*-1,2-diacylglycerol and exogenous *sn*-1-oleoyl-2-acetylglycerol in human red blood cell membranes with an IC_{50} value of approximately 3.0 μM. Furthermore, when employed at 10 μM, these workers observed R59022 to be without effect on the of PtdIns 4-kinase, PtdIns(4)P 5-kinase or the Ca^{2+}-stimulated PtdIns-PLC activities associated with red blood cell membranes. Thrombin stimulation of intact platelets in the presence of R59022 elicits a 4-fold greater accumulation of *sn*-1,2-diacylglycerol than thrombin treatment alone (Chaffoy de Courcelles *et al.*, 1985). As might be expected, thrombin-stimulation in the presence of R59022 was associated with reduced levels of PtdOH compared

to thrombin treatment alone. Finally, the dose-dependency of thrombin-induced 40 kDa protein phosphorylation was shifted to the left in the presence of R59022, although the magnitude of phosphorylation under maximally stimulating concentrations of thrombin was unaltered by the presence of R59022 (Chaffoy de Courcelles *et al.*, 1985). In summary, R59022 can be employed in intact platelets as a specific inhibitor of DAG kinase to elicit elevation of *sn*-1,2-diacylglycerol levels under conditions of sub-maximal stimulation with thrombin. Such an elevation clearly resulted in elevated PKC activity and increased phosphorylation of its cellular substrates. Additionally, the inhibitor has further identified the role of DAG kinase in modulating PKC activity within the cell. Under sub-maximal stimulatory conditions, inhibition of DAG kinase elicits inceased PKC activity; this clearly implicates DAG kinase in regulating receptor-stimulated *sn*-1,2-diacylglycerol levels immediately after they are produced. Thus, the magnitude of stimulated PKC activity is not solely the product of PtdIns(4,5)P_2 mass hydrolysed. It is, however, the product of equilibrium concentrations of *sn*-1,2-diacylglycerol within the cell and these are determined jointly by the rates of PtdIns(4,5)P_2 hydrolysis and *sn*-1,2-diacylglycerol metabolism. Nevertheless, the equilibrium concentration of *sn*-1,2-diacylglycerol can rise sufficiently to become saturating, eliciting a maximal rate of PKC activity. Further elevation of *sn*-1,2-diacylglycerol above these levels, by inhibition of DAG kinase, does not result in additional protein phosphorylation.

Many examples of R59022 use in the analysis of DAG kinase and PKC activity in the agonist-stimulated state have been reported (Nunn & Watson, 1987; Muid *et al.*, 1987; Gomez-Cambronero *et al.*, 1987; Morris *et al.*, 1987). Caution may, however, need to be excercised in the interpretation of R59022 inhibition studies. Yada and co-workers (1990) reported differential inhibition of DAG kinase isoenzymes by R59022. The workers described the resolution and purification of three cytosolic DAG kinases from human platelets. *In vitro*, R59022 inhibited the DAG kinase activity with apparent molecular mass of 75 kDa, partially

inhibited the 58 kDa activity and was without effect on the 152 kDa enzyme. Similarly, Sakane *et al.* (1989) reported the activity of the 150 kDa porcine thymus cytosolic activity to be unaffected by R59022, while the 80 kDa activity was inhibited with an IC_{50} value of 10 μM. The differential inhibition of isoenymes may suggest that R59022 inhibitor studies cannot be unilaterally interpreted, without prior investigation of the effect of the inhibitor on the individual isoenzymes present. Alternatively, the potentiating effect of R59022 on PKC activity in thrombin-stimulated platelets (Chaffoy de Coucelles *et al.*, 1985) may indicate that an R59022-sensitive DAG kinase isoenzyme is responsible for the metabolism of receptor-generated *sn*-1,2-diacylglycerol.

1.4. Receptor-Generated *sn*-1,2-Diacylglycerol from Non-PtdIns(4,5)P_2 Sources.

1.4.1. Alternative Sources of *sn* -1,2-Diacylglycerol in Agonist-Stimulated Cells.

The possibility of stimulated production of *sn*-1,2-diacylglycerol from sources other than PtdIns(4,5)P_2 has recently been realised. Receptor-mediated hydrolysis of PtdIns(4,5)P_2 is of transient nature, limited to the immediate events after receptor occupation (Griendling *et al.*, (1986); Plevin *et al.*, 1991a; Plevin & Wakelam, 1992). Measurement of Ins(1,4,5)P_3 formation has identified only transient hydrolysis of PtdIns(4,5)P_2 in response to a variety of agonists in Swiss 3T3 fibroblasts, Rat-1 fibroblasts and A10 vascular smooth muscle cells (Plevin *et al.*, 1990, 1991a,b; Plevin & Wakelam, 1992). However, measurement of *sn*-1,2-diacylglycerol levels in α-thrombin-stimulated IIC9 fibroblasts (Wright *et al.*, 1988) and bombesin-stimulated Swiss 3T3 fibroblasts (Cook *et al.*, 1990) has revealed a biphasic pattern; an initial transient peak followed by a sustained increase in *sn*-1,2-diacylglycerol above control levels for up to 4 h (Takuwa *et al.*, 1991). The transient formation of *sn*-1,2-diacylglycerol and Ins(1,4,5)P_3 was immediately identified as corresponding to the brief receptor-mediated hydrolysis of

PtdIns(4,5)P_2, while the secondary phase of elevated *sn*-1,2-diacylglycerol levels has been associated to receptor-mediated hydrolysis of a phospholipid distinct from PtdIns(4,5)P_2. It was soon appreciated that this sustained phase of *sn*-1,2-diacylglycerol formation corresponded to the release of choline and phosphocholine, identifying the stimulated hydrolysis of PtdCho in a range of agonist-stimulated cells (Besteman *et al.*, 1986a; Cabot *et al.*, 1988; Martin & Michaelis, 1988, 1989; Cook & Wakelam, 1989). Comparison of the time-course of elevated levels of *sn*-1,2-diacylglycerol to the time-course of choline and phosphocholine mobilisation, although a cogent argument, did not unequivocally identify the precursor phospholipid. However, analysis of the acyl-chain molecular species present in agonist-stimulated levels of *sn*-1,2-diacylglycerol and comparison to those found in individual cellular phospholipids would provide a far more convincing identification.

In addition to observing the sole release of choline from IIC9 fibroblasts labelled with [^{3}H]choline, [^{3}H]ethanolamine or [^{3}H]serine in response to α-thrombin stimulation, Pessin & Raben (1989) employed an elegant derivatisation/gas liquid chromatography (GLC) separation method to analyse the different acyl-chain species of *sn*-1,2-diacylglycerols present in α-thrombin-stimulated IIC9 fibroblasts. Comparison of the stimulated *sn*-1,2-diacylglycerol acyl-chain profile to that of PtdIns, PtdSer, PtdCho and PtdEtn isolated from unstimulated cells allowed identification of the precursor phospholipid. These workers found phosphoinositides to contribute a large proportion of the *sn*-1,2-diacylglycerol present 15 sec after stimulation, as would be expected. However, PtdCho was identified as the major source of stimulated *sn*-1,2-diacylglycerol after 5 min and 1h of thrombin-stimulation. PtdCho was also found to be the source of stimulated *sn*-1,2-diacylglycerol levels after stimulation of IIC9 fibrobalsts with either EGF or PDGF for 5 min or 1 h (Pessin *et al.*, 1990). Although receptor-mediated hydrolysis of PtdCho is strongly indicated by such analysis, the comparison of acyl-chain species of *sn*-1,2-diacylglycerol and correlation to the

acyl-chain distribution of the putative precursor phospholipids is limited by its 'best match' nature. The role of phospholipids distinct from PtdCho can not be overlooked, and their possible contribution to the observed sustained levels of *sn*-1,2-diacylglycerol must be borne in mind (see Section 1.4.3).

Receptor-mediated hydrolysis of PtdCho to produce *sn*-1,2-diacylglycerol has been reviewed extensively (Pelech & Vance, 1989; Billah & Anthes, 1990; Cook & Wakelam, 1991, 1992a). The hydrolysis of PtdCho to produce *sn*-1,2-diacylglycerol can proceed by two routes: directly, by a phospholipase C-catalysed reaction; or indirectly, by dephosphorylation of the PtdOH product of phospholipase D-catalysed hydrolysis of PtdCho. This dephosphorylation reaction being catalysed by phosphatidic acid phosphohydrolase (PPH).

The receptor-mediated hydrolysis of phosphatidylethanolamine (PtdEtn) has been reported (reviewed by Billah & Anthes, 1990). However, only a small contribution to phosphatidic acid and *sn*-1,2-diacylglycerol formation has been attributed to accelerated PtdEtn turnover in agonist-stimulated cells. Indeed no *sn*-1,2-diacylglycerol molecular species typical of PtdEtn hydrolysis have been detected following bombesin- or PDGF-stimulation of Swiss 3T3 fibroblasts (Pettit & Wakelam, 1993; T.R. Pettit, personal communication).

1.4.2. Regulation of Receptor-Mediated PtdCho Hydrolysis.

Entry of extracellular Ca^{2+} has been implicated in receptor-mediated phospholipase D activation in neutrophils (Pai *et al.*, 1988; Billah *et al.*, 1989a). Also, depletion of extracellular Ca^{2+} inhibits 50 % of the phospholipase D response in bombesin-stimulated Swiss 3T3 cells (Cook *et al.*, 1991). Although this indicates that elevation of $[Ca^{2+}]_i$ regulates PtdCho hydrolysis, it is entry of extracellular Ca^{2+} and not the mobilisation of intracellular Ins(1,4,5)P_3-sensitive stores that is responsible. Agonists such as EGF, interleukin-1 and interleukin-3 have demonstrated receptor-mediated hydrolysis of PtdCho in the absence of stimulated turnover of phosphoinositides (Cook & Wakelam, 1992b; Rosoff *et al.*,

1988; Wright *et al.*, 1988), suggesting that Ins(1,4,5)P_3 production is not a prerequisite for PtdCho hydrolysis. Treatment of cells with thapsgaragin, a Ca^{2+}-ATPase inhibitor, interrupts the Ca^{2+} cycle between cytosol and endoplasmic reticulum and results in elevation of the $[Ca^{2+}]_i$ with concomitant depletion of the Ins(1,4,5)P_3-sensitive pool (Thastrup *et al.*, 1990). Thapsigargin treatment was unable to either stimulate phospholipase D activity, or modulate the bombesin-stimulated phospholipase D activity of Swiss 3T3 fibrolasts (Cook *et al.*, 1991). Additionally, phosphoinositide hydrolysis can be abolished without attenuating PtdCho hydrolysis in angiotensin-stimulated vascular smooth muscle cells, α-thrombin-stimulated IIC9 fibroblasts, and adrenaline-stimulated MDCK-D1 cells (Greindling *et al.*, 1986; Wright *et al.*, 1988; Slivka *et al.*, 1988).

PKC has been implicated in the modulation of receptor-mediated PtdCho hydrolysis. Phorbol esters have been found to act as potent stimulators of PtdCho hydrolysis by either phospholipase C- or phospholipase D-catalysed pathways (reviewed by Billah and Anthes, 1990). Activation of PtdCho hydrolysis by cell-permeable diacylglycerols in granulocytes and fibroblasts has also been observed (Billah *et al.*, 1989a; Muir & Murray, 1987). Activation-induced down-regulation of PKC by chronic PMA treatment or inhibition of PKC by the selective inhibitors H7, K252a and Ro-31-8220 has been shown to partially inhibit agonist-stimulated PtdCho hydrolysis in some cells (Billah *et al.*, 1989a; Besterman *et al.*, 1986a; Cook *et al.*, 1991), and inhibit it fully in others (Liscovitch, 1989; Martinson *et al.*, 1989; Muir & Murray, 1987). In conclusion, the involvement of PKC is clear, the extent and, indeed, the hydrolytic activity it modulates would, however, seem to follow a cell type-specific pattern. Receptor-mediated PtdCho hydrolysis would seem to proceed both by PKC-dependent and -independent pathways. Additionally, it would appear that the phospholipase C- and phospholipase D-catalysed pathways of PtdCho hydrolysis are subject to differential modulation by PKC.

G-proteins have been implicated in the transmembrane modulation of PtdCho hydrolysis in response to receptor occupation. Non-hydrolysable analogues of GTP, for example GTPγS, stimulate the activity of phospholipase D-catalysed PtdCho hydrolysis in broken cell preparations of rat hepatocytes and granulocytes (Boccking *et al.*, 1987; Anthes *et al.*, 1989). This has also been found to be the case for phospholipase C-catalysed PtdCho hydrolysis in rat hepatocyte membranes (Irving & Exton, 1987; Boccking *et al.*, 1987). Direct modulation of the enzyme catalysing PtdCho hydrolysis by a G-protein/s is implied. This would be analagous to the modulation of PtdIns-PLC activity by G-proteins in receptor-stimulated cells (see Section 1.1.2). However, it is most likely that the rate of PtdCho hydrolysis is controlled both by direct and indirect G-protein regulation of the phospholipase catalysing the hydrolytic event. The indirect pathway would proceed via G-protein stimulation of PtdIns-PLC, with the subsequent activation of PKC modulating the phospholipase (see above).

1.4.3. Alternative Sources of Sustained Levels of *sn*-1,2-Diacylglycerol.

PtdCho hydrolysis, like PtdIns(4,5)P_2 hydrolysis, has been found to be rapid and transient, limited to the events occuring shortly after receptor occupation (Besterman *et al.*, 1986a; Pai *et al.*, 1988; Martin & Michaelis, 1989). The source and nature of sustained, elevated *sn*-1,2-diacylglycerol levels in stimulated cells is, therefore, called into doubt. Two possibilities could immediately rationalise these observations: either these sustained levels originate from slow metabolism of the *sn*-1,2-diacylglycerol produced from transient hydrolysis of PtdCho; or they are derived, at least in part, from a source distinct from PtdCho.

Contribution to the sustained phase of *sn*-1,2-diacylglycerol from a further phospholipid source has recently gained attention. Imai & Gershengorn (1986) originally argued for the existence of sustained PtdIns hydrolysis after the initial transient hydrolysis of PtdIns(4,5)P_2 in TRH-stimulated GH_3 pituitary cells. In

cells that had been incubated with $^{32}P_i$ for 1 min prior to stimulation, these workers observed continued labelling of PtdIns(4,5)P_2 for only 2 min after agonist challenge. Continued labelling of PtdIns and PtdOH was, however, sustained. In cells that had been stimulated with TRH for 4 min prior to addition of $^{32}P_i$, Imai & Gershengorn (1986) observed that label was incorporated into PtdIns and PtdOH, but not PtdIns(4,5)P_2. These results indicated the transient hydrolysis and resynthesis of PtdIns(4,5)P_2 was complete after 4 min, whilst after this time the hydrolysis and resynthesis of PtdIns continues.

Several workers have suggested that continued hydrolysis of PtdIns and PtdIns(4)P after cessation of PtdIns(4,5)P_2 hydrolysis contributes to the sustained phase of *sn*-1,2-diacylglycerol (Griendling *et al.*, 1986, 1987; Wilson *et al.*, 1985; Plevin & Wakelam, 1992; Cook *et al.*, 1991). The use of LiCl to inhibit the inositol monophosphatase (Hallcher & Sherman, 1980; Gee *et al.*, 1988) has been employed to prevent the reincorporation of liberated inositol into the phosphoinositide pool (see Section 1.1.4). Pre-labelling cells with [^{3}H]inositol in conjunction with LiCl can identify the on-going hydrolysis of any phosphoinositide which yields an inositol phosphate product fated to metabolism via inositol monophosphatase. Cook *et al.* (1991) observed a biphasic accumulation of inositol phosphates in Swiss 3T3 fibroblasts stimulated with bombesin in the presence of LiCl. A very rapid initial accumulation of inositol phosphates in the first minute after stimulation was observed, over a time-course equivalent to that of the elevated Ins(1,4,5)P_3 levels in bombesin-stimulated Swiss 3T3 fibroblasts (Plevin *et al.*, 1990; Cook *et al.*, 1990). However, after 1 min of stimulation the rate of inositol phosphate accumulation was observed to be one eighth of that observed in the initial minute of agonist challenge (Cook *et al.*, 1991). After termination of the initial receptor-mediated PtdIns(4,5)P_2 hydrolysis, it would seem that an alternative inositol phospholipid is undergoing sustained hydrolysis. Similarly, Plevin & Wakelam (1992) indicate the sustained hydrolysis of an inositol phospholipid at

timepoints after the termination of PtdIns(4,5)P_2 hydrolysis in vasopressin-stimulated A10 cultured vascular smooth muscle cells.

Although this interpretation of the involvement of phosphoinositides apart from PtdIns(4,5)P_2 would seem correct, it assumes that continued hydrolysis of PtdIns(4,5)P_2 is not occurring. If the accumulation of Ins(1,4,5)P_3 on receptor-stimulation indicates that the initial step in its metabolism is the rate-limiting step, the formation of Ins(1,4,5)P_3 at a slower rate may not result in such accumulation. This is a possibility, provided that the concentration of Ins (1,4,5)P_3 does not rise to a level where the rate of metabolism becomes limiting. Although PtdIns(4,5)P_2 levels do not fall in the sustained phase of *sn*-1,2-diacylglycerol (Plevin & Wakelam, 1992), the possibility of continued PtdIns(4,5)P_2 hydrolysis exists. This problem is augmented by the difficulty in identifying the flux of phospholipid between PtdIns, PtdIns(4)P and PtdIns(4,5)P_2 in [^{3}H]inositol-labelled cells. A steady-state concentration of PtdIns(4,5)P_2 could conceivably be maintained, masking a flux state of increased hydrolysis and resynthesis during the sustained phase of agonist-stimulated *sn*-1,2-diacylglycerol generation.

In addition to the phospholipase-catalysed liberation of *sn*-1,2-diacylglycerol from PtdIns(4,5)P_2, PtdCho and other phospholipids, for example PtdEtn, phospholipase-catalysed hydrolysis of phosphatidylinositol glycan has been suggested to be a source of *sn*-1,2-diacylglycerol in insulin-stimulated BC_3H1 myocytes (Saltiel *et al.*, 1987). Insulin-stimulation of [^{3}H]myristic acid-labelled BC_3H1 cells results in the generation of an *sn*-1,2-diacylglycerol population distinct from that observed on α-adrenergic-stimulation (Saltiel *et al.*, 1987). Farese and co-workers (1985) have also suggested that the *sn*-1,2-diacylglycerol observed to accumulate on insulin-stimulation of BC_3H1 myocytes was the result of agonist-enhanced *de novo* synthesis. Similarly, *de novo* synthesis was observed to be the major source of agonist-stimulated *sn*-1,2-diacylglycerol in phagocytic neutrophils, with PtdIns(4,5)P_2 and PtdCho barely contributing to the accumulation of the messenger (Rossi et al., 1991). Additionally, although the acyl-chain distribution

of the sustained phase of *sn*-1,2-diacylglycerol in fibroblasts indicates that it is a product of PtdCho hydrolysis (Pessin & Raben, 1989; Pessin *et al.*, 1990), the sustained phase could equally be a product of *de novo* synthesis with only a minor contribution from PtdCho.

1.4.4. The Sustained Phase of Elevated *sn*-1,2-Diacylglycerol Levels: Effect on PKC Activity.

It is clearly established that *sn*-1,2-diacylglycerol is present at elevated levels for some time after agonist stimulation, even after the termination of PtdIns(4,5)P_2 hydrolysis. It is not clear, however, if these sustained levels play a role distinct from the PtdIns(4,5)P_2-derived messenger.

Comparison of the acyl-chain molecular species of *sn*-1,2-diacylglycerol present after 5 min stimulation of IIC9 fibroblasts with 500 ng/ml α-thrombin to those observed in the cellular phospholipids revealed that the agonist-elevated levels of *sn*-1,2-diacylglycerol were most likely derived from PtdCho (Pessin & Raben, 1989). This was also found to be the case when IIC9 fibroblasts were stimulated for 5 min with the following mitogens: 500 ng/ml α-thrombin following chymotrypsin pretreatment of the cells, 100 pg/ml α-thrombin, EGF, or PDGF (Pessin *et al.*, 1990). Early studies of PKC activation by differing molecular species of *sn*-1,2-diacylglycerols, although not complete, would suggest that PKC can, indeed, be activated by many of the *sn*-1,2-diacylglycerols encountered on receptor-stimulated hydrolysis of PtdCho (Mori *et al.*, 1982; Go *et al.*, 1987). Furthermore, *sn*-1,2-diacylglycerol derived from IIC9 fibroblast cellular PtdCho by phospholipase C digestion was found to effectively activate cytosolic PKC fractionated from these cells (Leach *et al.*, 1991). Prolonged stimulation of PKC activity at time-points beyond the transient hydrolysis of PtdIns(4,5)P_2 would seem feasible. Identification of the continued presence of a putative *sn*-1,2-diacylglycerol activator would undoubtably enhance the argument.

However, observation of PKC activity would suggest that its activation is a transient event, dissociated from the prolonged presence of elevated *sn*-1,2-diacylglycerol levels. In IIC9 fibroblasts, which express PKC-α and not PKC-β or -γ, treatment with 100 pg/ml of α-thrombin did not result in an increase in membrane-associated PKC-α (Leach *et al.*, 1991). Thus, the translocation of the cytosolic PKC, an event often employed as diagnostic of PKC activation, is not observed in IIC9 fibroblasts stimulated with 100 pg/ml α-thrombin. Such treatment of IIC9 cells, although still mitogenic, is associated with PtdCho but not not phosphoinositide hydrolysis. Incubation of these cells with 500 ng/ml α-thrombin, a treatment that elicits transient phosphoinositide hydrolysis in addition to PtdCho hydrolysis, did result in a depletion of cytosolic PKC-α with concomitant increase in both membrane-associated PKC-α and membrane-associated PKC activity. This classical PKC translocation/activation event is transient with membrane-associated activity reaching maximal values after 30 sec and declining to basal levels after 15 min (Leach *et al.*, 1991). Although PKC activation was transient, sustained *sn*-1,2-diacylglycerol levels were found to continue to increase over this time-course. Additionally, these workers observed that the decline of PKC activity observed after 15 sec was not the result of a activation-induced down-regulation but rather the redistribution of PKC back to the inactive cytosolic pool. Furthermore, 500 ng/ml α-thrombin stimulated phosphorylation of the 80 kDa MARCKS protein in digitonin-permeabilised IIC9 cells, with similar time-course to the activation of cellular PKC activity. 100 pg/ml α-thrombin, a treatment that stimulates PtdCho hydrolysis without receptor-mediated PtdIns(4,5)P_2, elicited no stimulated phosphorylation of the MARCKS protein (Leach *et al.*, 1991).

Bombesin and PDGF have been shown to stimulate PtdIns(4,5)P_2 and PtdCho hydrolysis in Swiss 3T3 fibroblasts (Cook & Wakelam, 1989; Cook *et al.*, 1990; Plevin *et al.*, 1991a; Pettit & Wakelam, 1993). Additionally, both these agonists stimulate PKC activity in these cells (Isacke *et al.*, 1986). Increased PKC-catalysed phosphorylation of the endogenous MARCKS protein was observed in

these cells 15 min after stimulation with either bombesin or PDGF (Isacke *et al.*, 1986). Yet, alteration of the MARCKS protein's phosphorylation state in response to EGF was not observed (Isacke *et al.*, 1986). Interestingly, EGF does elevate *sn*-1,2-diacylglycerol levels for a sustained period of time (60 min) in Swiss 3T3 cells (Cook & Wakelam, 1992b). However, as with other cell types, EGF stimulation of Swiss 3T3 fibroblasts is not associated with stimulated hydrolysis of PtdIns(4,5)P_2 (Cook & Wakelam, 1992b).

Together with the findings of Leach *et al.*, (1991), do these observations rule out the prolonged activation of PKC with a longevity equal to the sustained phase of *sn*-1,2-diacylglycerol ? The continued presence after 1 min of *sn*-1,2-diacylglycerol derived, at least in part, from PtdCho is not associated with prolonged PKC activation, despite possessing the ability to do so *in vitro*. Moreover, the absence of PKC activation in the presence of *sn*-1,2-diacylglycerol is not the result of activation-induced down-regulation. Leach *et al.* (1991) observed that IIC9 fibroblasts still possess an inactive cytosolic pool of PKC-α, a Ca^{2+}-dependent PKC (see Section 1.2.1), during the α-thrombin-stimulated sustained phase of *sn*-1,2-diacylglycerol. Therefore, it can be concluded that PKC activation in response to Ca^{2+}-mobilising agonists is transient, with activation closely following the stimulated hydrolysis of PtdIns(4,5)P_2. It is likely that only the *sn*-1,2-diacylglycerol produced on PtdIns(4,5)P_2 hydrolysis is active in modulating cellular PKC activity and that the *sn*-1,2-diacylglycerol which constitutes the sustained phase is inactive in this role. This conclusion must, however, be qualified: the PKC family has recently expanded to some 10 members, whilst the characterisation of PKC activation on cell-stimulation was conducted with immunological probes and assay methods developed for the PKC family members originally purified from bovine brain, i.e. PKC-α, -β_1/β_2 and -γ. Recently, it has become apparent that PKC-δ, -ε and -ζ are widely expressed and differ from the originally characterised PKC family members in their Ca^{2+} dependency, phospholipid requirements and reaction velocities towards different protein/peptide

substrates (see Parker *et al.*, 1989; Schaap *et al.*, 1989; Olivier & Parker, 1991; Parker *et al.*, 1992). If the activity of any PKC family member is elevated during the sustained phase of *sn*-1,2-diacylglycerol, it remains to be detected and the physiological substrates identified.

Previously, correlation of the biphasic increase of *sn*-1,2-diacylglycerol in bombesin stimulated Swiss 3T3 fibroblasts (Cook *et al.*, 1990) with the biphasic increase in pH_i observed under similar stimulatory conditions (Bierman *et al.*, 1990) has been employed as a diagnostic indication of sustained PKC activation by the sustained phase of *sn*-1,2-diacylglycerol (Cook & Wakelam, 1992b). Early elevation of intracellular pH by activation of the Na^+/H^+ antiporter has been suggested to be a necessary mitogenic signal (Pouyssegur *et al.*, 1984, 1985). In the presence of HCO_3^-, however, mitogenic stimulation fails to elevate pH_i (Ganz *et al.*, 1989). Therefore, elevation of intracellular pH may not be necessary for mitogenesis, but stimulation of the Na^+/ H^+ antiporter would seem to be required and elevation of pH_i in HCO_3^--free medium may be employed as a suitable assay for activation of the antiporter.

Although PKC-catalysed phosphorylation is implicated in the activation of the Na^+/ H^+ antiporter (Moolenar *et al.*, 1984), continued activation of PKC may not be required for the observed biphasic activation of antiporter activity. EGF-stimulated Swiss 3T3 fibroblasts still undergo a sustained alkalinisation (Bierman *et al.*, 1990). Yet, EGF stimulation is not associated with accelerated hydrolysis of PtdIns(4,5)P_2 or agonist-stimulated PKC activity (Cook & Wakelam, 1992a; Isacke *et al.*, 1986). Activation-induced down-regulation of PKC by pretreatment with PDBu (Rodriguez-Pena & Rozengurt, 1984) abrogated all bombesin-stimulated elevation of pH_i (Bierman *et al.*, 1990), whereas EGF continued to elicit alkalinisation in both PDBu-pretreated and control Swiss 3T3 cells (Bierman *et al.*, 1990). Both, EGF and bombesin elicit alteration of pH_i by activation of the Na^+/H^+ antiporter in Swiss 3T3 cells, yet PKC-catalysed activation is not implicated in the regulation of the antiporter in the case of EGF. It would seem fair

to accept that PKC does modulate the initial transient elevation of pH_i observed within 2 min of bombesin stimulation, as suggested by Moolenar and co-workers (1984). However, there is no evidence of PKC directly modulating the activity of the antiporter after this time. Indeed, the sustained increase in pH_i is outwith the time-course of bombesin-stimulated PKC activity as judged by phosphorylation of the endogenous MARCKS protein substrate in Swiss 3T3 fibroblasts (Isacke *et al.*, 1986). This does not rule out PKC playing some modulatory role on the activity of the antiporter as, in fact, PKC down-regulation did also prevent the secondary, sustained elevation of pH_i in addition to the initial transient increase (Bierman *et al.*, 1990). It is most likely that the sustained phase of antiporter activity is regulated by an event downstream of the transient activation of PKC observed in the first minute of stimulation with bombesin. Correspondingly, Sardet *et al.* (1991) have observed that α-thrombin, EGF, and okadaic acid activate the Na^+/H^+ antiporter by increased phosphorylation of a common set of sites by an unidentified serine kinase(s). This unidentified kinase(s) controls the activity of the antiporter on integration of signals from receptor tyrosine kinases and G protein-coupled receptors (Sardet *et al.*, 1991). This immediately reconciles the differential modulation of sustained antiporter activity by PKC in EGF- and bombesin-stimulated cells.

1.4.5. The Sustained Phase of Elevated *sn* -1,2-Diacylglycerol Levels: Contribution of *sn*-1-*O*-Alkyl -2-acylglycerol and *sn*-1-*O*-Alkenyl-2-acylglycerol to the Sustained Phase ?

Investigation of the phospholipid source of stimulated levels of *sn*-1,2-diacylglycerol in α-thrombin-stimulated IIC9 fibroblasts clearly illustrated the difference between *sn*-1,2-diacylglycerol derived from PtdIns and PtdCho (Pessin & Raben, 1989; Pessin *et al.*, 1990). It should also be recognised that cellular PtdCho further differs from PtdIns in the nature of the bond at the *sn*-1 position of the phospholipid.

In addition to *sn*-1,2-diacylglycero-3-phosphocholine, cellular phosphatidylcholine posesses *sn*-1-*O*-alkyl-2-acylglycerol-3-phosphocholine and *sn*-1-alkenyl-2-acylglycerol-3-phosphocholine components. As glycerolipid messengers distinct from *sn*-1,2-diacylglycerol could be generated on receptor-stimulated hydrolysis, the role of *sn*-1-*O*-alkyl-2-acylglycerol-3-phosphocholine and *sn*-1-alkenyl-2-acylglycerol-3-phosphocholine must be considered further. *sn*-1-*O*-Alkyl-2-acylglycerol-3-phosphocholine has been found to constitute 30 - 70 % of PtdCho in neutrophils, eosinophils and macrophages while *sn*-1-alkenyl-2-acylglycerol-3-phosphocholine only represents a very minor component of the PtdCho pool (Mueller *et al.*, 1982; Ojima-Uchiyama *et al.*, 1988). Conversely, *sn*-1-alkenyl-2-acylglycerol-3-phosphocholine is the major ether-linked component in heart tissues, comprising 30 % of the PtdCho (Arthur *et al.*, 1985).

Specific labelling of *sn*-1-*O*-alkyl-2-acylglycerol-3-phosphocholine by incubation of cells with alkyl-[^{32}P]lysoPtdCho ([^{32}P]*sn*-1-*O*-alkyl-2-lysoglycerol-3-phoshocholine) has been employed to unequivocally identify phospholipase D-catalysed hydrolysis of PtdCho in fMet-Leu-Phe-stimulated HL-60 cells (Pai *et al.*, 1988). Similarly, phorbol esters, the cell-permeable diacylglycerol OAG, and the calcium ionophore A23187 have been shown to increase the rate of PtdCho hydrolysis in alkyl-[^{32}P]lysoPtdCho-labelled HL-60 granulocytes by the action of phospholipase D alone (Billah *et al.*, 1989a). It would appear that receptor-stimulated phospholipase D catalyses the hydrolysis of the PtdCho pool as a whole and does not catalyse specifically the hydrolysis of either *sn*-1,2-diacylglycero-3-phosphocholine or *sn*-1-*O*-alkyl-2-acylglycero-3-phosphocholine. Indeed, Agwu *et al.* (1989) and Tyagi *et al.* (1989) demonstrated both the production of *sn*-1,2-diacylglycerol and *sn*-1-*O*-alkyl-2-acylglycerol in human neutrophils stimulated with fMet-Leu-Phe, PMA and the calcium ionophore A23187. However, investigation of phospholipase D activity by labelling the *sn*-1-*O*-alkyl-2-acylglycerol-3-phosphocholine component is relevant only in cell-types where this phospholipid is present in the PtdCho pool to significant levels. In Swiss 3T3

fibroblasts, for example, PtdCho is wholly of the *sn*-1,2-diacylglycerol-3-phosphocholine species and neither *sn*-1-*O*-alkyl-2-acylglycerol nor *sn*-1-*O*-alkenyl-2-acylglycerol are found to accumulate on bombesin-stimulation (Pettit & Wakelam, 1993). Caution must, therefore, be exercised in the interpretation of PtdCho hydrolysis in alkyl-[^{32}P]lysoPtdCho-labelled cells and its use in the identification of the role of phospholipase D. Nevertheless, labelling of the cellular PtdCho pool by specific labelling of the *sn*-1-*O*-alkyl-2-acylglycerol-3-phosphocholine component has been adopted in the study of phospholipase D-catalysed hydrolysis of PtdCho in widely variant cell models, e.g. human platelets (Huang *et al.*, 1991), sea urchin spermatozoa (Domino *et al.*, 1989), and rat brain synaptic plasma membranes (Mohn *et al.*, 1992).

The significance of *sn*-1-*O*-alkyl-2-acylglycerol and *sn*-1-alkenyl-2-acylglycerol production concomitant with the sustained phase of *sn*-1,2-diacylglycerol has still to be defined. The physiological action of the vinyl-ether diglyceride, *sn*-1-alkenyl-2-acylglycerol is still poorly understood. Evidence of the ability of *sn*-1-*O*-alkyl-2-acylglycerol to modulate PKC activity is ambiguous. Ganong *et al.* (1986) and Daniel *et al.* (1988) concluded that *sn*-1-*O*-alkyl-2-acylglycerol inhibits rather than activates PKC activity *in vitro*. However, Ford *et al.* (1989) reported activation of PKC by *sn*-1-*O*-alkyl-2-acylglycerol *in vitro*, but at higher Ca^{2+} concentrations than those required for *sn*-1,2-diacylglycerol. As discussed previously for sustained *sn*-1,2-diacylglycerol production from PtdCho in fibroblasts, the ability to modulate PKC activity *in vitro* does not predispose the cell to continued, elevated PKC activity during the time-course of *sn*-1-*O*-alkyl-2-acylglycerol generation. The potential for *sn*-1-*O*-alkyl-2-acylglycerol and *sn*-1-alkenyl-2-acylglycerol to act within the cell as a second messenger in a manner analagous to *sn*-1,2-diacylglycerol will be restricted to a cell-type specific pattern. Potentially, *sn*-1-*O*-alkyl-2-acylglycerol may play a signalling role in cells of myeloid origin. However, stimulated levels of *sn*-1-*O*-alkyl-2-acylglycerol have not been observed in α-thrombin-stimulated IIC9 fibroblasts (Pessin & Raben,

1989), nor have stimulated levels of either *sn*-1-*O*-alkyl-2-acylglycerol or *sn*-1-alkenyl-2-acylglycerol been observed in bombesin-stimulated Swiss 3T3 fibroblasts (Pettit & Wakelam, 1993).

1.4.6. The Sustained Phase of Elevated *sn*-1,2-Diacylglycerol Levels: A Redefinition.

Recently, problems associated with the quantitation of *sn* -1,2-diacylglycerol levels in cell extracts have come to light. The enzymatic quantitative method originally described by Preiss *et al.* (1986) and modified by Paterson *et al.* (1991) has been found to measure *sn*-1,2-diradylglycerols collectively. Under the conditions employed, *sn*-1-*O*-alkyl-2-acylglycerol and *sn*-1-alkenyl-2-acylglycerol in addition to *sn*-1,2-diacylglycerol are phosphorylated and, thus, quantitated by this method (Tyagi *et al.*, 1989; Thompson *et al.*, 1990). It is possible to determine the individual contribution of each *sn*-1,2-diradylglycerol sub-class to the total *sn*-1,2-diradylglycerol concentration in cell lipid extracts (Tyagi *et al.*, 1989; Thompson *et al.*, 1990), but these procedures have not been widely applied. This illuminates the possibility of previous erroneous quantitation of collective *sn*-1,2-diradylglycerol concentration as *sn*-1,2-diacylglycerol when determined by the enzymatic method. This is especially pertinent in cells which produce elevated levels of *sn*-1-*O*-alkyl-2-acylglycerol with a time-course coincident with the sustained phase of *sn*-1,2-diacylglycerol associated with PtdCho hydrolysis, e.g. fMet-Leu-Phe-stimulated neutrophils (Billah *et al.*, 1989b). However, such erroneous quantitation of *sn*-1,2-diacylglycerol will be a cell-type specific phenomenon, as stimulated levels of *sn*-1-*O*-alkyl-2-acylglycerol and *sn*-1-alkenyl-2-acylglycerol have not been observed in α-thrombin-stimulated IIC9 fibroblasts (Pessin & Raben, 1989), bombesin-stimulated Swiss 3T3 fibroblasts (Pettit & Wakelam, 1993), and TRH-stimulated GH_3 pituitary cells (Martin *et al.*, 1990). For the continued purposes of the discussion within this thesis, in the absence of quantitation of individual *sn*-1,2-diradylglycerol components of total cellular *sn*-

1,2-diacylglycerol pool, no further distinction will be made between the *sn*-1,2-diacylglycerol and total *sn*-1,2-diradylglycerol content of cell lipids.

1.4.7. The Sustained Phase of Elevated *sn*-1,2-Diacylglycerol Levels: Route of Removal.

The sustained phase of elevated *sn*-1,2-diacylglycerol levels has now been attributed, in part, to the transient hydrolysis of PtdCho, with possible contribution from the sustained hydrolysis of other phospholipids (see Section 1.4.3). Although it has been clearly established that DAG kinase and DAG lipase are responsible for initiating the metabolism of the *sn*-1,2-diacylglycerol produced on PtdIns(4,5)P_2 hydrolysis (Sections 1.1.1 & 1.3.2), the metabolism of the sustained phase *sn*-1,2-diacylglycerol is less clear. Transient receptor-mediated hydrolysis of PtdCho requires a very slow rate of *sn*-1,2-diacylglycerol metabolism to sustain the elevated levels of *sn*-1,2-diacylglycerol. This is certainly true when the sustained phase of *sn*-1,2-diacylglycerol has been solely attributed to PtdCho hydrolysis, e.g. α-thrombin-stimulated IIC9 fibroblasts (Pessin & Raben, 1989) and bombesin-stimulated Swiss 3T3 fibroblasts (Pettit & Wakelam, 1992).

The possibility of inositol phospholipids contributing to the sustained phase of *sn*-1,2-diacylglycerol further complicates the issue. Imai and Gershengorn (1986) suggested that the hydrolysis of PtdIns in TRH-stimulated GH_3 cells continues after the termination of receptor-mediated PtdIns(4,5)P_2 hydrolysis. PtdIns hydrolysis therefore contributed to the sustained phase of *sn*-1,2-diacylglycerol accumulation in these cells. Similarly, TRH-stimulated PtdOH production in these cells continued after the cessation of PtdIns(4,5)P_2 hydrolysis (Imai & Gershengorn, 1986). This would indicate that the *sn*-1,2-diacylglycerol derived from PtdIns is phosphorylated by DAG kinase and follows a similar metabolic fate to the *sn*-1,2-diacylglycerol produced transiently from PtdIns(4,5)P_2. However, the sustained phase of *sn*-1,2-diacylglycerol in TRH-stimulated GH_3 cells is only partly derived from PtdIns hydrolysis, as Martin *et al.*

(1990) have also identified a contribution from stimulated PtdCho hydrolysis. The route of PtdCho-derived *sn*-1,2-diacylglycerol metabolism remains to be clearly defined, although increased synthesis of PtdCho via incorporation of CDP-choline has been observed in TRH-stimulated GH_3 cells (Kolesnick, 1987).

Further distinction between the metabolic fates of *sn*-1,2-diacylglycerol derived from PtdIns or PtdCho can be observed on PMA stimulation of PtdCho hydrolysis. In agonist-stimulated cells, increased degradation and subsequent synthesis of both PtdIns and PtdCho is observed (reviewed Pelech & Vance, 1989; Michell, 1975). However, Muir & Murray (1987) observed that PMA-stimulated hydrolysis of PtdCho resulted in resynthesis of PtdCho alone, without concomitant degradation and resynthesis of PtdIns. It would seem clear that the *sn*-1,2-diacylglycerol derived from stimulated PtdCho hydrolysis is not metabolised by a pathway identical to PtdIns(4,5)P_2-derived *sn*-1,2-diacylglycerol, nor is it incorporated into the phosphoinositide pool. Instead, the glycerolipid backbone is utilised in the resynthesis of PtdCho via CDP-choline (reviewed Pelech & Vance, 1989; Shears, 1991).

Diacylglycerol choline phosphotransferase catalyses transfer of the phosphocholine moiety of CDP-choline to *sn*-1,2-diacylglycerol, resulting in production of PtdCho, thus completing the synthesis of PtdCho from the products of receptor-stimulated PtdCho hydrolysis. The water-soluble substrate for this enzyme, CDP-choline, is the product of condensation of phosphocholine and CTP in a reaction catalysed by CTP:phosphocholine cytidyltransferase. Phosphocholine could be produced direct from phospholipase C-catalysed hydrolysis of PtdCho. Alternatively, it could be produced from the action of choline kinase on the water-soluble product of phospholipase D-catalysed hydrolysis of PtdCho.

Diacylglycerol choline phosphotransferase efficiently catalyses the conversion of *sn*-1,2-diacylgycerol, 1-*O*-alkyl-2-acyl-*sn*-glycerol and 1-alkenyl-2-acyl-*sn*-glycerol to PtdCho *in vitro* (Ford & Gross, 1988, 1990). The physiological substrate specificity of this enzyme has still to be determined.

Interestingly, the ether-linked diradylglycerols are not significantly metabolised by DAG kinase or DAG lipase *in vitro* (Kanoh & Åkesson, 1978; Ford & Gross, 1990). It would seem that the cell-type specific production of 1-*O*-alkyl-2-acyl-*sn*-glycerol and 1-alkenyl-2-acyl-*sn*-glycerol in the sustained phase of *sn*-1,2-diacylglycerol is metabolised solely by diacylglycerol choline phosphotransferase. This may indicate that the *sn*-1,2-diradylglycerol products of PtdCho hydrolysis are metabolised collectively via diacylglycerol choline phosphotransferase. Further metabolic compartmentalisation of the *sn*-1,2-diradylglycerol present in the sustained phase from the product of transient receptor-mediated PtdIns(4,5)P_2 hydrolysis is indicated.

Diacylglycerol choline phosphotransferase is restricted to the endoplasmic reticulum of rat liver (Kanoh & Ohno, 1976; Jelsema & Morre, 1978). CTP: phosphocholine cytidyltransferase, the activity catalysing the rate limiting step of PtdCho synthesis, partitions between the cytosol and endoplasmic reticulum (reviewed: Vance & Pelech, 1984; Pelech & Vance, 1989). However, CTP: phosphocholine cytidyltransferase is activated by *sn*-1,2-diacylglycerol (Kolesnick, 1987, 1990), and it has been suggested that such activation is manifested by an accumulation of membrane-bound enzyme and depletion of the soluble reservoir (Cornell & Vance, 1987).

Further dissociation between the metabolism of PtdCho- and PtdIns(4,5)P_2-derived *sn*-1,2-diacylglycerol is identified by localisation of the PtdCho synthetic apparatus on the endoplasmic reticulum. Plasma membrane-associated DAG kinase (see Section 1.8.2) and plasma membrane-associated DAG lipase (Farooqui *et al.*, 1984; Mauco *et al.*, 1984; Ide *et al.*, 1990) are suitably juxtaposed to the site of *sn*-1,2-diacylglycerol production to initiate its metabolism. Similarly, receptor-mediated hydrolysis of PtdCho is localised to the plasma membrane (Irving & Exton, 1987; Martin, 1988; Gelas *et al.*, 1989) and *sn*-1,2-diacylglycerol, thus formed, requires transfer to the membranes of the endoplasmic reticulum. For example, TRH-stimulated GH_3 pituitary cells experience induction of PtdCho

synthesis subsequent to stimulated PtdCho hydrolysis (Kolesnick, 1987). However, TRH-stimulation of these cells results in the sustained accumulation of *sn*-1,2-diacylglycerol specifically in the intracellular membranes, with only transient accumulation in the plasma membrane (Martin *et al.*, 1990). Therefore, PtdCho-derived *sn*-1,2-diacylglycerol would appear to be rapidly transferred to intracellular membranes, probably those of the endoplasmic reticulum. Indeed, fluorescent *sn*-1,2-diacylglycerol analogues present in the plasma membrane have been observed to translocate to intracellular membranes (Pagano & Longmuir, 1985).

The hydrolysis of PtdCho at a location distinct from the plasma membrane cannot be overlooked. Augert *et al.* (1989) observed that a substantial contribution to agonist-stimulated PtdCho hydrolysis in hepatocytes was the result of Ca^{2+}-activated phospholipase activity located on intracellular membranes. The recent report of Ca^{2+}-stimulated base-exchange activity in the microsomes of rat liver (Siddiqui & Exton, 1992) therefore indicates the need for cautious interpretation of choline release studies in the identification of a phospholipase-catalysed hydrolysis of PtdCho.

1.5. Phosphatidic Acid as a Second Messenger.

DAG kinase and phospholipase D both catalyse reactions that produce phosphatidic acid (PtdOH). It has been suggested that PtdOH performs a signalling role quite distinct from that of *sn*-1,2-diacylglycerol. Originally, it was suggested that PtdOH acted as a Ca^{2+} ionophore, mediating the entry of extracellular Ca^{2+} across the plasma membrane of parotid gland cells (Putney *et al.*, 1980). However, the ability of PtdOH to act as a Ca^{2+} ionophore in artificial phospholipid membranes is still a matter of contention (Serhan *et al.*, 1981; Holmes & Yoss, 1983). Additionally, it has recently been postulated that PtdOH mediates Ca^{2+} influx by binding a specific extracellular receptor, with ion transport occurring as a secondary event (Murayama & Ui, 1987).

The possibility that PtdOH and its lyso-derivative, lyso-PtdOH, act as mitogenic agonists has recently received much attention. Incubation of fibroblasts with exogenous PtdOH or lyso-PtdOH results in PtdIns(4,5)P_2 hydrolysis, stimulation of PKC activity, inhibition of adenylyl cyclase, and stimulation of DNA synthesis (Moolenar *et al.*, 1986; Murayama & Ui, 1987; Van Corven *et al.*, 1989; Plevin *et al.* 1991b). Lyso-PtdOH was found to be 25 times more potent a mitogen than lyso-PtdOH-free PtdOH in Balb/c 3T3 cells, being active at 2 μg/ml (Fukami & Takenawa, 1992). Moreover, a specific receptor for such extracellular lyso-PtdOH has recently been identified (van der Bend *et al.*, 1992). Although the exact mechanism for release of PtdOH or its lyso-derivative has still to be defined, it is clear that PtdOH does accumulate in the extracellular medium on treatment of rabbit platelets with the Ca^{2+} ionophore A23187, or thrombin (Murayama & Ui, 1987). This underscores the possibility of PtdOH/lyso-PtdOH acting as an autocrine or paracrine messenger molecule.

In addition PtdOH-dependent protein phosphorylation has been demonstrated in the soluble fractions of rat liver, brain, lung and testis (Bocckino *et al.*, 1991). These workers observed the PtdOH-dependent phosphorylation to be Ca^{2+}-insensitive in heart and stimulated by 360 - 800 nM free Ca^{2+} in liver . Furthermore, the phosphorylation profiles elicited by PtdOH were found to be distinct from those ellicited by PtdSer/*sn*-1,2-dioleoylglycerol indicating the putative PtdOH-dependent protein kinase to be distinct from PKC (Bocckino *et al.*, 1991). However, the possibility of a novel PKC activity with phospholipid requirements distinct from PKC-α, -β_1, -β_2 or -γ was not discounted. PtdOH has previously been reported to activate PKC *in vitro*. (Kaibuchi *et al.*, 1981; Sekiguchi *et al.*, 1988; Burns *et al.*, 1990). This discrepancy may be explained by the recent reports that PKC-ζ, a Ca^{2+}-independent PKC activity, can employ PtdOH as lipid cofactor (D.J. Burns, personal communication).

1.6. Phosphatidic Acid Phosphohydrolase: Functional Antagonist to Diacylglycerol Kinase Activity.

Phosphatidic acid phosphohydrolase (PPH) catalyses the dephosphorylation of phosphatidic acid (PtdOH) to *sn*-1,2-diacylglycerol and, as such, may participate in a futile cycle with DAG kinase. Additionally when receptor-stimulated PtdCho hydrolysis is catalysed by phospholipase D, PPH catalyses the dephosphorylation of the PtdOH product and thus may yield the *sn*-1,2-diacylglycerol associated with receptor-stimulated PtdCho hydrolysis (see Section 1.4.1).

Both hydrolytic events are situated on the plasma membrane, the most likely site of DAG kinase-catalysed phosphorylation of the *sn*-1,2-diacylglycerol product of PtdIns(4,5)P_2 hydrolysis (see Section 6.3). Alternatively, the *sn*-1,2-diacylglycerol produced by dephosphorylation of the PtdOH product of PLD-catalysed PtdCho hydrolysis accumulates in intracellular membranes (Martin *et al.*, 1990). Although the intracellular location of PPH active in the dephosphorylation of PtdCho-derived PtdOH has still to be identified, the accumulation of PtdCho-derived *sn*-1,2-diacylglycerol at an intracellular site may possibly indicate that it resides on such intracellular membranes. Indeed, the majority of cellular PPH activity has been localised to the endoplasmic reticulum (Jelsema & Morre, 1978; Brindley, 1984). However, such endoplasmic reticulum-associated PPH activity may represent an isoenzyme/s solely active in the *de novo* synthesis of phospholipid and triacylglycerol and thus be distinct from the enzyme responsible for the dephosphorylation of PtdCho-derived PtdOH in agonist-stimulated cells. PPH activity, biochemically distinct from the cytosolic and microsomal-associated activity, has been identified in the plasma-membrane fraction of rat liver (Jamal *et al.*, 1991) and may possibly localise the PtdOH dephosphorylation event to the plasma membrane with intracellular accumulation resulting from subsequent *sn*-1,2-diacylglycerol translocation.

As discussed previously (Section 1.1.4), PtdIns(4.,5)P_2-derived *sn*-1,2-diacylglycerol is commited to the resynthesis of phosphoinositides initially by

phosphorylation in a reaction catalysed by DAG kinase, whereas PtdCho-derived *sn*-1,2-diacylglycerol is most likely committed to resynthesis of PtdCho via a reaction catalysed by *sn*-1,2-diacylglycerol choline phosphotransferase. In consideration of the direction of flux through these separate metabolic pathways of *sn*-1,2-diacylglycerol, PPH is apparently specific for the PtdOH produced from PLD-catalysed PtdCho hydrolysis and not the PtdOH product of the DAG kinase activity associated with PtdIns(4,5)P_2 turnover. Therefore, the action of PPH may further indicate the metabolic compartmentalisation of the distinct *sn*-1,2-diacylglycerol products of receptor-stimulated PtdIns-PLC and PLD hydrolysis (see Section 1.4.1).

The existence of DAG kinase:PPH futile cycles in both pathways of phospholipid resynthesis from the *sn*-1,2-diacylglycerol produced from PtdCho and PtdIns(4,5)P_2 hydrolytic events cannot be overlooked. Both, DAG kinase and PPH isoenzymes are widely distributed throughout the cell and would seem suitably juxtaposed to operate futile cycling of the *sn*-1,2-diacylglycerol/PtdOH products of PtdCho and PtdIns(4,5)P_2 hydrolysis. As yet, however, such futile cycling has yet to be reported and the reason for any such metabolically expensive processing within this physiological context is not clear.

In conclusion, although PPH functionally antagonises DAG kinase activity in broken cell prepartions (see Section 3.1.3), the situation within the cell is less clear. The cell possibly holds the potential to operate futile cycles to carefully regulate the flux of glycerolipid through the PtdCho and PtdIns(4,5)P_2 resynthesis pathways but, as yet, there is little evidence of such. The cell would also seem, at first appearance, to possess the ability to reverse the direction of glycerolipid metabolism shortly after agonist-stimulation from phosphorylation of the *sn*-1,2-diacylglycerol produced on PtdIns(4,5)P_2 hydrolysis to dephosphorylation of the PtdOH product of PtdCho hydrolysis. Yet, the evidence, although not conclusive, would suggest that both pathways operate, with the glycerolipid products of

PtdIns(4,5)P_2 and PtdCho hydrolysis remaining metabolically distinct if not spatially separated within the cell.

1.7. Does DAG Kinase Participate in the Production of lyso-PtdOH?

The mitogenic stimulation by PtdOH and its lyso-derivative, lyso-PtdOH, has recently received much attention (see Section 1.5). Potentially, PtdOH can be produced from the DAG kinase-catalysed phosphorylation of *sn*-1,2-diacylglycerol, PLD-catalysed hydrolysis of phospholipid or *de novo* synthesis via acylation of glycerol -3-phosphate in a reaction catalysed by glycerol-3-phosphate acyltransferase. Additionally, lyso-PtdOH may potentially be produced by phosphorylation of *sn*-1-monoacylglycerol, deacylation of PtdOH at the *sn*-2 position in a reaction catalysed by phospholipase A_2 or by acylation of glycerol-3-phosphate.

The contribution of DAG kinase to the accumulation of PtdOH on cell-stimulation has been discussed previously (Section 1.1.1), its contribution to the accumulation of lyso-PtdOH has not. DAG kinase may function indirectly in the accumulation of lyso-PtdOH by catalysing the phosphorylation of *sn*-1,2-diacylglycerol, providing PtdOH that may be deacylated at the *sn*-2 position in a reaction catalysed by a phospholipase A_2. Such a PtdOH-specific phospholipase A_2 has been described in platelets (Billah *et al.*, 1982), a cell that experiences an accumulation of PtdOH and lyso-PtdOH on thrombin-stimulation (Mauco *et al.*, 1978; Lapetina *et al.*, 1981).

It is, however, unlikely that DAG kinase contributes directly to the accumulation of lyso-PtdOH by catalysing the phosphoryaltion of *sn*-1-monoacylglycerol. In addition to *sn*-1,2-diacylglycerol, the cytosolic 80kDa DAG kinase purified from pig brain was observed to catalyse the phosphorylation of 2-monoacylglycerol but only limited phosphorylation of *sn*-1-monoacylglycerol was observed (Kanoh *et al.*, 1986). Such shared identity between DAG kinase and 2-monoacylglycerol kinase activities has also been observed in both the particulate

and soluble fractions prepared from rat brain (Bishop & Strickland, 1980). Furthermore, the rat brain acylglycerol kinase activity was observed to catalyse the phosphorylation of acylglycerols with increasing reaction velocities: *sn*-1-monoacylglycerol, 2-monoacylglycerol, *sn*-1,2-diacylglycerol (Bishop & Strickland, 1980). This was not, however, found to be the case for the cytosolic 72 kDa DAG kinase purified to near homogeneity from bovine brain (Lin *et al.*, 1986). This enzyme was found to be devoid of 2-monoacylglycerol kinase activity with the ratio of DAG kinase/2-monoacylglycerol kinase activity increasing from 4.6 in brain homogenate to 1381 in the 475-fold purified DAG kinase preparation (Lin *et al.*, 1986). Subsequently, monoacylglycerol kinase activity was found to copurify with bovine cytosolic DAG kinase. It was possible to partially resolve the monoacylglycerol kinase and DAG kinase activities on hydroxyapatite chromatography (Shim *et al.*, 1989). The partially purified 2-monoacylglycerol kinase activity reported by these workers was found to catalyse the phosphorylation of *sn*-1-monoacylglycerol with a higher reaction velocity than measured for 2-monoacylglycerol (Shim *et al.*, 1989).

Although the information is limited mainly to the mammalian brain enzyme, cytosolic DAG kinase is capable of phosphorylating 2-monoacylglycerol but not *sn*-1-monoacylglycerol. The activity observed to catalyse the phosphorylation of *sn*-1-monoacylglycerol also catalyses the phosphorylation of 2-monoacylglycerol, although at slightly lower reaction velocities than the *sn*-1 isomer and would thus appear to be distinct from cytosolic DAG kinase. In conclusion, if lyso-PtdOH is produced in the cell by phosphorylation of *sn*-1-monoacylglycerol, it would appear that the *sn*-1-monacylglycerol/2-monoacylglycerol kinase catalyses the reaction and not DAG kinase. However, the physiological route of lyso-PtdOH production still remains to be established.

1.8. Properties of DAG Kinase.

1.8.1. Properties of the Diacylglycerol Kinase Involved in Stimulated PtdIns(4,5)P_2 Turnover.

The triple-faceted importance of DAG kinase in the metabolism of receptor-generated *sn*-1,2-diacylglycerol would seem to be clear. Firstly, it establishes the link between receptor-mediated phosphoinositide hydrolysis and the subsequent phospholipd resynthesis events by converting the lipid product of phosphoinositide hydrolysis to PtdOH, the substrate of the commited step in phosphoinositide resynthesis. Secondly, it rapidly phosphorylates PtdIns(4,5)P_2-derived *sn*-1,2-diacylglycerol, contributing to the tight coupling of PKC activation to the receptor occupation event. Finally, the product of the reaction catalysed by DAG kinase, phosphatidic acid, has been identified as having putative signalling activity itself (Section 1.5).

Given that DAG kinase is active in the above roles, what are the properties of such an activity? As discussed previously, PtdIns(4,5)P_2-derived *sn*-1,2-diacylglycerol is present only transiently, with a longevity similar to the time-course of PtdIns(4,5)P_2 hydrolysis (Section 1.4.1). Therefore, the DAG kinase responsible must catalyse the phosphorylation of PtdIns(4,5)P_2-derived *sn*-1,2-diacylglycerol with a reaction velocity that would result in the transience of *sn*-1,2-diacylglycerol in agonist-stimulated cells.

The DAG kinase active in the metabolism of PtdIns(4,5)P_2-derived *sn*-1,2-diacylglycerol would most likely be associated with the plasma membrane, i.e. juxtaposed to the site of PtdIns(4,5)P_2 hydrolysis. Direct access of DAG kinase to *sn*-1,2-diacylglycerol as it was produced would ensure tight coupling of PKC activation to receptor occupation. Rapid redistribution of *sn*-1,2-diacylglycerol from the plasma membrane to intracellular membranes, as demonstrated by Pagano & Longmuir (1985), might indicate such localisation of DAG kinase activity as a prerequisite for its role in defining the transient appearance of PtdIns(4,5)P_2-derived *sn*-1,2-diacylglycerol.

Finally, the DAG kinase responsible for catalysing the phosphorylation of PtdIns(4,5)P_2-derived *sn*-1,2-diacylglycerol would seem to exhibit some selectivity amongst *sn*-1,2-diacylglycerol molecular species. MacDonald *et al.* (1988a) reported the presence of a membrane-associated DAG kinase activity in lysates prepared from PDGF-stimulated Swiss 3T3 fibroblast which catalysed the phosphorylation of endogenous *sn*-1,2-diacylglycerol. PDGF stimulation of Swiss 3T3 fibroblasts in the presence of *sn*-1,2-didecanoylglycerol, a cell-permeable *sn*-1,2-diacylglycerol, resulted in the preferential phosphorylation of the endogenous lipid (MacDonald *et al.*, 1988a). Furthermore, the membrane-associated DAG kinase of Swiss 3T3 cells exhibited selectivity amongst several endogenous *sn*-1,2-diacylglycerols. It catalysed the phosphorylation of *sn*-1-stearoyl-2-arachidonylglycerol with a reaction rate 2- to 8-fold greater than other naturally occurring *sn*-1,2-diacylglycerols (MacDonald *et al.*, 1988b).

1.8.2. Diacylglycerol Kinase: Subcellular Distribution.

Subcellular fractionation has demonstrated DAG kinase as an activity expressed ubiquitously throughout the cell. DAG kinase activity is located in the cytosol, non-synaptic plama membranes, synaptic plasma membranes, synaptic vesicles, mitochondria, microsomes and synaptosomes in rat cerebral cortex (Stubbs *et al.*, 1988). Additionally, DAG kinase activity has been observed in the nuclear fraction of rat cerebral cortex (Lapetina & Hawthorne, 1971). Within this nuclear fraction, DAG kinase has further been localised both to rat liver nuclear envelope (Smith & Wells, 1983) and to the internal nuclear matrix of NIH 3T3 mouse fibroblasts (Payrastre *et al.*, 1992). In addition to the microsomal membrane-associated enzyme reported in rat cerebral cortex (Lapetina & Hawthorne, 1971; Stubbs *et al.*, 1988) and rat lung (Ide & Weinhold, 1982), microsomal-associated DAG kinase activity from rabbit brain and liver has also been studied (Ford & Gross, 1990). As well as the plasma membrane activity identified in rat cerebral cortex by Stubbs *et al.* (1988), plasma membrane-

associated DAG kinase has been demonstrated in human platelets (Chaffoy de Courcelles, 1985) and rat liver (Rider & Baquet, 1988). Freely soluble DAG kinase activity has been studied in the cytosolic fraction of rat liver (Kanoh & Ohno, 1981), rat brain (Bishop & Strickland, 1980; Kato & Takenawa, 1990), rat lung (Ide & Weinhold, 1982), bovine brain (Lin *et al.*, 1986), baboon brain (Lemaitre *et al.*, 1990), pig brain (Kanoh *et al.*, 1983; Kanoh & Ono, 1984), pig thymus (Sakane *et al.*, 1989), human white blood cells (Schaap *et al.*, 1990), human platelets (Yada *et al.*, 1990), Swiss 3T3 fibroblasts (McDonald *et al.*, 1988b; Coco-Maroney & Macara, 1989) and NIH 3T3 fibroblasts (Strathoupoulos *et al.*, 1990).

Despite the ubiquitous distribution of DAG kinase activity throughout the cell, most of the activity within the cell is typically associated with the cytosol. Cytosolic DAG kinase is the predominant DAG kinase activity in rat brain, thymus, spleen, liver, lung, kidney and uterus (Kato & Takenawa, 1990), proving to have greater total and specific activity than the particulate-associated enzyme. However, Kato & Takenawa (1990) found an altered distribution in rat heart, with the particulate-associated DAG kinase predominating although it did possess reduced specific activity compared to the cytosolic enzyme. Coco-Maroney and Macara (1989) also observed partitioning of DAG kinase activty towards the cytosol of Swiss 3T3 fibroblasts when employing a deoxycholate-based assay similar to that utilised by Kato and Takenawa (1990). However, Coco-Maroney and Macara (1989) also observed that alteration of the detergent and phospholipid cofactor conditions of the assay could affect the measured specific activity of both the cytosolic and membrane fraction-associated DAG kinase activity. Replacing deoxycholate with 51 mM *n*-octyl-β,D-glucopyranoside indicated that the kinase was predominantly cytosolic, as previously observed. However, the measured specific activities of the membrane-associated and cytosolic enzymes were similar when PtdSer was introduced to the *n*-octyl-β,D-glucopyranoside assay system to a final concentration of 2 mM [7 mol%] (Coco-Maroney & Macara, 1989). PtdSer

had previously been identified as a phospholipid cofactor for the membrane-associated DAG kinase of Swiss 3T3 fibroblasts (MacDonald *et al.*, 1988b). Finally, replacing the deoxycholate with 16 mM CHAPS resulted in membrane-associated DAG kinase displaying the greatest specific activity when the assay was performed in the presence of 2 mM (20 mol%) PtdSer (Coco-Maroney & Macara, 1989). Therefore, discussion of subcellular localisation and, indeed, the proportion of total cellular DAG kinase resident within each compartment must be carefully qualified with respect to the detergent and phospholipid requirements of the DAG kinase activity resident within each fraction.

1.8.3. Multiple Isoenzymes of Diacylglycerol Kinase.

Rabbit antisera, raised against the 80 kDa cytosolic DAG kinase previously purified from pig brain (Kanoh *et al.*, 1983), immunoprecipitated only 60 % of the DAG kinase activity from pig brain cytosol (Kanoh *et al.*, 1986). Furthermore, it was possible to resolve the immunoreactive and non-immunoreactive activities by ion-exchange chromatography (Kanoh *et al.*, 1986). Also, the antisera raised against the 80 kDa DAG kinase exhibited little or no cross-reactivity with the DAG kinase in cytosolic fractions prepared from chicken and rat brain and also from pig, chicken and rat liver (Kanoh *et al.*, 1986). Further investigation of the distribution of the immunoreactive and non-immunoreactive DAG kinases in different pig tissues revealed the 80 kDa immunoreactive activity to be limited to brain, thymus and spleen (Yamada & Kanoh, 1988). In addition to the non-immunoreactive DAG kinase activity previously reported in pig liver, Yamada and Kanoh (1988) observed non-immunoreactive activity in pig platelet cytosol with further possible non-immunoreactive activity in pig heart and kidney. However, the low specific activity of the heart and kidney preparations indicate possible difficulty in quantifiying the expression of the 80 kDa immunoreactive DAG kinase by immunoblot analysis.

Gel filtration of cytosolic preparations from pig brain and thymus revealed the presence of a 280 kDa DAG kinase activity in addition to the immunoreactive 80 kDa activity. In contrast, gel filtration of platelet cytosol identified the presence of a 120 kDa non-immunoreactive DAG kinase in addition to the 80 kDa and 280 kDa activities observed in other pig tissues (Yamada & Kanoh, 1988). In an attempt to study the nature of this heterogeneity, Sakane *et al.* (1989) purified the soluble DAG kinases from porcine thymus cytosol. Consistent with the earlier findings of Yamada and Kanoh (1988) they were able to purify an 80 kDa DAG kinase from pig thymus that displayed cross-reactivity to the antisera raised against the pig brain 80 kDa cytosolic activity. Additionally, they purified the non-immunoreactive DAG kinase activity which displayed an apparent native molecular weight of 150 kDa on gel filtration analysis of partially purified enzyme and 2 polypeptide bands of 50 and 75 kDa after SDS-PAGE analysis of the purified preparation (Sakane *et al.*, 1989). These workers were further able to distinguish these isoenzymes on the basis of their heat-lability, action of anionic activators, inhibition by sphingosine and inhibition by the DAG kinase inhibitor; R59022. In pig there would thus appear to be three resolvable cytosolic DAG kinases, two of which have been purified to homogeneity.

Further proof of the existence of isoenzymes has been provided by the identification of individual DAG kinase activities in homogeneous cell populations rather than in tissue homogenates. Yada *et al.* (1990) have purified three distinct isoenzymes from human platelets. The 152 kDa and 58 kDa isoenzymes were both purified to homogeneity from this source, while an additional activity with apparent molecular mass of 75 kDa was identified and partially purified (Yada *et al.*, 1990). These isoenzymes were further distinguishable by the different effects of detergents, phospholipids, and R59022 on their reaction velocity.

Similarly, two isoenzymes with different substrate, phospholipid cofactor, and detergent dependencies have been identified in the cytosol of rat brain and were successfully separated by heparin-agarose chromatography (Strathopoulos *et al.*,

1990). These workers were also able to identify two isoenzymes in the cytosol of NIH 3T3 fibroblast which could be separated by heparin-agarose chromatography (Strathopoulos *et al.*, 1990). Although the substrate, detergent and phospholipid cofactor dependencies of the NIH 3T3 isoenzymes were not further characterised, their separation on heparin-agarose was similar to that of the two DAG kinases identified in rat brain rat brain (Strathopoulos *et al.*, 1990). Thus, there is strong evidence to suggest that isoenzymes of DAG kinase co-exist within a single cell type.

1.8.4. Regulation of Diacylglycerol Kinase by 'Translocation'.

Vasopressin-treatment of rat liver for 5 min resulted in both an elevation of apparent K_m for *sn*-1,2-dioleoylglycerol and V_{max} of the plasma membrane-associated DAG kinase activity (Rider & Baquet, 1988). Total DAG kinase activity resident in homogenates of treated and untreated livers displayed no alteration in DAG kinase activity, although the activity recovered with the plasma membrane was found to have doubled.

Besterman *et al.* (1986b) demonstrated the redistribution of DAG kinase activity from the cytosol to the particulate fraction on incubation of rat brain homogenate with either *sn*-1,2-diacylglycerol or phospholipase C. After partial purification of DAG kinase from phospholipase C-treated rat brain homogenate, these workers were able to demonstrate binding of the purified kinase to phospholipase C-treated inside-out vesicles prepared from human red blood cells (Besterman *et al.*, 1986b). The translocation event is wholly dependent on prior incubation of the vesicles with phospholipase C, presumably indicating the requirement for elevation of *sn*-1,2-diacylglycerol levels in the membranes for DAG kinase binding to occur. Similarly, incubation of PMA-down regulated Swiss 3T3 fibroblasts with *sn*-1,2-dioctanoylglycerol results in translocation of soluble DAG kinase to the cell membranes (Coco-Maroney & Macara, 1989). Short-term (2 min) treatment of Swiss 3T3 fibroblasts with PMA also affected a translocation of DAG

kinase to the membrane (Coco-Maroney & Macara, 1989). The accumulation of DAG kinase activity on the cell membranes coincided with the PMA-stimulated elevation of *sn*-1,2-diacylglycerol within these cells. Also, PMA was found not to alter the total cellular DAG kinase activity, nor did it exert any direct effect on cytosolic or membrane-bound DAG kinase activity. Thus, the redistribution of DAG kinase towards the membranes was attributable solely to the PMA-stimulated production of *sn*-1,2-diacylglycerol within these cells (see also Cook *et al.*, 1991).

It is not clear if this translocation event does indeed represent an up-regulation of the DAG kinase activity, functioning to remove PtdIns(4,5)P_2-derived *sn*-1,2-diacylglycerol. PMA-stimulated elevation of cellular *sn*-1,2-diacylglycerol levels has been correlated with the stimulated hydrolysis of PtdCho, but not PtdIns(4,5)P_2 (Muir & Murray, 1987). Therefore, hydrolysis of a phospholipid other than PtdIns(4,5)P_2 could stimulate the redistribution of cytosolic DAG kinase observed on PMA treatment. Once resident on the membrane, it is not apparent if the DAG kinase is active in phosphorylating the *sn*-1,2-diacylglycerol that elicited its translocation. This is pertinent, as the *sn*-1,2-diacylglycerol accumulating in response to PMA-stimulated PtdCho hydrolysis would presumably be metabolised by a pathway other than DAG kinase-catalysed phosphorylation (see Section 1.4.7). The subcellular localisation of the DAG kinase recruited to the membrane compartment by PMA treatment has yet to be identified. This prevents definition of the translocation as an increased association of the cytosolic activity with either the plasma or intracellular membranes. The importance of such a finding is underscored by the observed sustained accumulation of *sn*-1,2-diacylglycerol in intracellular membranes compared to its transient appearance in the plasma membrane (Martin *et al.*, 1990).

The very nature of the translocation event is also called into doubt. Antisera specific for the cytosolic 80 kDa DAG kinase of pig brain have been employed in immunoblotting techniques to additionally further localise the 80 kDa DAG kinase to both the microsomal and synaptosomal fractions prepared from pig brain

homogenate (Kanoh *et al.*, 1986). Although originally purified from pig brain cytosol, the 80 kDa DAG kinase would seem to co-exist both in the soluble and membrane fractions of pig brain. Immunohistochemistry with anti-80 kDa DAG kinase antisera demonstrated intense staining of neuronal nuclei in pig brain, in addition to more diffuse staining of the cytosolic compartment in general (Kanoh *et al.*, 1986). The 80 kDa DAG kinase has also been demonstrated to phosphorylate the *sn*-1,2-diacylglycerol present in phospholipase C-treated microsomes prepared from rat liver (Kanoh & Ono, 1984). Therefore, it would seem that rigid definition of soluble DAG kinases as cytosolic proteins may be premature. However, it may be possible to redefine them as non-integral membrane-associated proteins which rapidly redistribute to the cytosolic fraction on cell disruption. Such redistribution can be prevented by increased levels of *sn*-1,2-diacylglycerol within the cellular membranes, e.g. on agonist stimulation. However, distinction of this process from the previously discussed *sn*-1,2-diacylglycerol-stimulated translocation of soluble DAG kinase activity cannot be made at this time.

1.8.5. Regulation of Diacylglycerol Kinase Activity by Covalent Modification.

Alteration of DAG kinase activity by covalent modification, in particular phosphorylation, has been suggested by many workers. Rigorous examination of the alteration of activity by such modification has yet to be performed.

Phosphorylation of the 80 kDa cytosolic DAG kinase of pig brain by an endogenous protein kinase was demonstrated *in vitro* by Kanoh & Ono (1986). Despite considerable purification of the DAG kinase (equivalent to 50- and 1050-fold, see Kanoh *et al.*, 1983), phosphorylation of the kinase was still observed on incubation of the heterogenous protein preparation with Mg^{2+} and [γ-^{32}P]ATP. Phosphorylation of the DAG kinase was not, however, observed after further purification of the protein to a homogeneous preparation (Kanoh & Ono, 1986). These workers reported the responsible kinase/s to be independent of Ca^{2+},

Ca^{2+}/calmodulin and cyclic nucleotides, and only capable of limited phosphorylation of the DAG kinase (maximally, phosphate was incorporated with a stoichiometry of 0.25 mol P_i/mol DAG kinase). It has also been demonstrated that PKC prepared from rat brain phosphorylates purified 80 kDa DAG kinase *in vitro* (Kanoh *et al.*, 1989). Under the conditions employed, the 80 kDa DAG kinase proved to be only a poor substrate for cAMP-dependent protein kinase, compared with protein kinase C which achieves 1:1 stoichiometries of P_i incorporation per mol of enzyme. Clear alteration of either the apparent K_m for *sn*-1,2-diacylglycerol or the enzyme's V_{max} by protein kinase C-catalysed phosphorylation was not reported. The phosphorylated 80 kDa DAG kinase did display increased affinity for PtdSer vesicles and phosphorylation was postulated to stimulate partitioning of the soluble enzyme to a membrane-bound compartment (Kanoh *et al.*, 1989). Moreover, the PKC-treated DAG kinase that remained soluble after incubation with PtdSer vesicles was found to be entirely non-phosphorylated. This may identify a mechanism for activation of DAG kinase by partitioning to a potentially *sn*-1,2-diacylglycerol-rich enviroment rather than by alteration of the V_{max} and reduction of its apparent K_m for *sn*-1,2-diacylglycerol.

In vitro the cAMP-dependent protein kinase phosphorylated the porcine 80 kDa DAG kinase with lower stoichiometry than protein kinase C (Kanoh *et al.*, 1989). In parotid cells cAMP-dependent protein kinase has been implicated in the activation of DAG kinase (Soling *et al.*, 1989). Activation of parotid gland DAG kinase activity has been demonstrated to be mediated by Ca^{2+}/calmodulin dependent protein kinase activity on muscarinic stimulation (Soling *et al.*, 1989). Yet, Kanoh & Ono (1986) reported that both Ca^{2+} and calmodulin were without effect on the rate of phosphorylation of the 80 kDa DAG kinase by an endogenous protein kinase. In conclusion, although the modulation of DAG kinase activity by protein phosphorylation events have, to date, been poorly examined, it is probably safe to conclude that such modulation will become evident with both a tissue- and agonist-specific pattern.

1.8.6. Regulation of Diacylglycerol Kinase Activity by Calcium.

Alteration of DAG kinase reaction rates by Ca^{2+} has received little attention. Modulation of DAG kinase reaction rates was originally observed for both the soluble 110 kDa, and membrane-associated 150 kDa DAG kinases purified from rat brain (Kato & Takenawa, 1990). Both activities displayed maximal rates of reaction at 1 mM Ca^{2+}, with rates rapidly decreasing as the Ca^{2+} concentration increased or decreased about the optimal. Isolation and sequencing of cDNA coding for the 80 kDa DAG kinase of porcine brain and thymus has revealed that the primary sequence of this protein possesses two E-F hand motifs (Sakane *et al.*, 1990). E-F hands are α-helix-loop-α-helix motifs which are highly conserved amongst the Ca^{2+}-binding sites of calmodulin, troponin C and parvalbumin (reviewed Moncrief *et al.*, 1990). Concomitant with the identification of these putative Ca^{2+}-binding domains was the finding that purified porcine thymus 80 kDa DAG kinase activity was enhanced by the presence of 10^{-7} - 10^{-4} M Ca^{2+}, higher concentrations resulted in the return to basal levels of activity (Sakane *et al.*, 1990). Sakane *et al.* (1991a) observed Ca^{2+} to bind the 80 kDa DAG kinase with both high affinity and a stoichiometry of 2 mol Ca^{2+} per mol. of enzyme. Furthermore, the Ca^{2+}-dependent activation of the 80 kDa DAG kinase was found to require the presence of phosphatidylserine for full stimulation of activity (Sakane *et al.*, 1991a). Physiologically, the requirement for phospholipid was found to represent a translocation event within the cell, from soluble to membrane-bound environment, in response to elevation of free Ca^{2+} concentrations (Sakane *et al.*, 1991a). Expression of truncation and deletion mutants of the 80 kDa DAG kinase identified the requirement of the E-F hand motifs for Ca^{2+} binding of the resulting DAG kinase mutant (Sakane *et al.*, 1991b), confirming Ca^{2+} does bind to the 80 kDa DAG kinase at a site equivalent to the E-F hands. Expression of a truncated mutant lacking E-F hand motifs in COS-7 cells resulted in greater Ca^{2+}-independent DAG kinase activity than that observed for the native enzyme expressed in the same

system. In the presence of 100 μM Ca^{2+}, however, the truncated mutant displayed similar rates of reaction to the native enzyme. Additionally, deletion of the E-F hand was found to alter the subcellular distribution towards the membrane-associated compartment, compared to the soluble native kinase. Sakane *et al.* (1991b) concluded that binding of Ca^{2+} to the E-F hand domain induces conformational change, dis-inhibiting the binding of the soluble enzyme to cellular membranes.

Similarly, the deduced amino-acid sequence of the human 80 kDa DAG kinase purified from white blood cells revealed a high level of homology between the cloned human and porcine DAG kinases (Schaap *et al.*, 1990). The sequences of these two proteins were found to differ by only 51 amino acid substitions and a single amino acid insertion in the human enzyme. The existence of two E-F hand motifs in the human enzyme with similar organisation to those observed in the porcine kinase would immediately identify the human DAG kinase a putative Ca^{2+}-binding enzyme. Alignment of the tandemly repeated E-F hand motifs of the human and porcine enzymes revealed a high level of identity between the repeated motifs (27/29 and 27/29 residues, respectively). However, this enzyme, whether expressed in COS-7 cells from cloned cDNA or purified from human white blood cells, was unresponsive to alteration of free Ca^{2+} concentrations (Schaap *et al.*, 1990). This anomaly could simply represent problems in achieving Ca^{2+} modulation of DAG kinase activity in the absence of phospholipid (Sakane *et al.*, 1991b).

1.8.7. Further Structure/Function Analysis of Porcine 80 kDa Diacylglycerol Kinase.

Definitive structure/function relationships for DAG kinase as a family of related enzyme activities cannot, as yet, be undertaken, since only limited sequence data is available to date. Sakane *et al.* (1990) isolated and sequenced a full-length cDNA coding for the 80 kDa DAG kinase of porcine thymus. Subsequent to this,

Schaap *et al.* (1990) sequenced a full-length cDNA coding for a soluble 80 kDa DAG kinase previously purified from human white blood cells. Comparison of the deduced amino-acid sequences revealed the human kinase to be a direct homologue of the porcine 80 kDa DAG kinase. A high degree of conservation between the aligned sequences was observed with 55 amino acid substitutions and only one insertion in the human sequence (Schaap *et al.*, 1990). Furthermore, all the structural motifs deduced from the primary structure of the porcine enzyme were observed in the deduced human sequence, i.e. two E-F hand motifs localised towards the amino terminal, two cysteine-rich regions with homology to the cysteine-rich zinc finger sequences associated with *sn*-1,2-diacylglycerol/phorbol ester binding in the C1 region of PKC-α (Parker *et al.*, 1989), and two putative ATP-binding sites. The high degree of homology between pig and human sequences does not aid interpretation of the structural requirements of a given DAG kinase. For clearer definition of the structural requirements for DAG kinase activity, comparison of the primary sequence of further, more divergent enzymes must be undertaken. Location of highly conserved sequences among polypeptides of little overall homology will allow functional motifs to be assigned with greater confidence.

Recently, a *Drosophila melangoster* DAG kinase homologue was isolated after screening of a *Drosophila* genomic library with the porcine DAG kinase cDNA under conditions of low stringency (Masai *et al.*, 1992). Masai and co-workers then employed the isolated genomic DNA fragment to screen a *Drosophila* cDNA library to identify and isolate a full-length cDNA corresponding to the *Drosophila* DAG kinase homologue. Comparison of the *Drosophila* DAG kinase homologue's deduced primary structure to the porcine sequence reveals only limited homology. The carboxy-terminal half of the porcine protein holds 50 % identity with the homologous sequence in *Drosophila*, although the homologous region in the insect sequence is bissected by an insert of some 288 amino acids. However, these separated domains of *Drosophila* do have 76 and 72 % conserved homology with

the correponding sequence of the porcine protein (Masai *et al.*, 1992). This would suggest that the catalytic region of porcine DAG kinase to be resident within the carboxy terminus of the porcine kinase. Except for a single putative ATP-binding domain, the other putative structural/functional motifs are resident in the amino-terminal half of the porcine sequence and are, thus, absent from the *Drosophila* sequence (Sakane *et al.*, 1990). This may suggest that these amino-terminal motifs are not involved in the catalytic site of DAG kinase and are restricted to the regulatory apparatus of the porcine/human 80 kDa DAG kinase isoenzyme. However, as yet, the *Drosophila* DAG kinase homologue has still to be expressed in an active form and despite its homology with the porcine protein may not actually possess any catalytic properties. This is pertinent, as the catalytic site of porcine diacylgycerol kinase has recently been located to the combined putative ATP-binding site/cysteine-rich zinc finger domain located between residues 218-252 of the porcine sequence (F. Sakane, personal communication), a structural motif that is lacking in the *Drosophila* homologue.

The cysteine-rich domain (CRD) of porcine 80kDa DAG kinase shares the motif $HX_{11/12}CX_2CX_nCX_2CX_4HX_2CX_{6/7}C$ (*n* varies between 12 and 14) with human 80 kDa DAG kinase (Schaap *et al*, 1990), human neuronal chimaerin (Hall *et al.*, 1990), PKC-α, -β, -γ, -δ, -ε and PKC-ζ (Parker *et al.*, 1989, 1992). Additionally, the DAG kinases and PKC-α, -β, -γ, -δ and -ε possess two copies of the cysteine-rich motif expressed in tandem. Human neuronal chimaerin and PKC-ζ, however, only possess a single CRD. Such cysteine-rich domains are indicative of metal binding-domains where coordination between the metal, usually zinc, and the cysteinyl residues results in the stabilisalion of the characteristic structural motif (Berg, 1986). The requirement for a CRD to confer Zn^{2+}-binding properties was demonstrated for neuronal chimaerin, human 80 kDa DAG kinase and human PKC-γ (Ahmed *et al.*, 1991). Ahmed and co-workers employed truncation mutants of neuronal chimaerin and PKC-γ to demonstrate the requirement for an intact CRD for $^{65}Zn^{2+}$-binding to the recombinant protein. These workers also demonstrated

that human DAG kinase also bound $^{65}Zn^{2+}$, presumably through its CRD (Ahmed *et al.*, 1991). Furthermore, identification of the CRD as the structural motif responsible for high-affinity phospholipid-dependent binding of phorbol esters to neuronal chimaerin and PKC (Ono *et al.*, 1989; Ahmed *et al.*, 1990) strongly implicates the coordination of Zn^{2+} in the phorbol ester binding and membrane localisation activity of PKC. Although the possession of a CRD and the ability to bind Zn^{2+} correlates with phorbol ester binding in neuronal chimaerin and PKC-γ, human DAG kinase was able to bind Zn^{2+} but not phorbol ester, despite the possession of two tandemly arranged cysteine-rich motifs (Ahmed *et al.*, 1991). Therefore, although the secondary structure formed by a CRD in the presence of Zn^{2+} would confer phorbol ester binding and, thus, membrane association for neuronal chimaerin and PKC-γ, it does not exclusively dictate phorbol ester and *sn*-1,2-diacylglycerol binding.

1.9. Aim of Project.

Receptor-stimulated hydrolysis and resynthesis of PtdIns(4,5)P_2 is a response common to many agonists. Within such, DAG kinase activity can be identified and is essential in initiating the reincorporation of the *sn*-1,2-diacylglycerol glycerolipid backbone to the phosphoinositides and ultimately PtdIns(4,5)P_2. Specifically, the DAG kinase involved in receptor-accelerated PtdIns(4,5)P_2 turnover can be identified as catalysing the phosphorylation of *sn*-1-acyl-2-arachidonylglycerol with reaction velocities greater than those observed with non-arachidonyl *sn*-1,2-diacylglycerols (see Sections 6.1 & 6.2). This catalytic selectivity towards *sn*-1-acyl-2-arachidonylglycerol is not shared with all the DAG kinase activities that can be monitored in cell and tissue homogenates (MacDonald *et al.*, 1988b; Lemaitre *et al.*, 1990). In consequence, *sn*-1-acyl-2-arachidonylglycerol-selectivity has been employed as a diagnostic test for such DAG kinase activities dedicated to catalysing the phosphorylation of *sn*-1,2-

diacylglycerol generated by receptor-stimulated PtdIns(4,5)P_2 hydrolysis (MacDonald *et al.*, 1988b).

The aim of the work towards this thesis was to purify from rat brain the DAG kinase involved in receptor-stimulated PtdIns(4,5)P_2 turnover. From the information gained on the location of the *sn*-1-acyl-2-arachidonylglycerol-selective DAG kinase in Swiss 3T3 mouse fibroblasts (MacDonald *et al.*, 1988a, b) and the apparent inability of the previously purified cytosolic DAG kinases to catalyse the phosphorylation of *sn*-1-acyl-2-arachidonylglycerol with reaction velocities greater than those observed with other *sn*-1,2-diacylglycerols (Kanoh & Ohno, 1981; Kanoh *et al.*, 1983; Lin *et al.*, 1986), the particulate fraction of rat brain was selected as a suitable tissue source for the purification of the *sn*-1-acyl-2-arachidonylglycerol-selective enzyme.

sn-1-Stearoyl-2-arachidonylglycerol would also be employed as a substrate throughout the development of the purification so that the *sn*-1-acyl-2-arachidonylglycerol-selective enzyme may be resolved from other non-selective DAG kinase enzymes. Such, *sn*-1-acyl-2-arachidonylglycerol selectivity of the purified enzyme would be confirmed by comparison of the reaction velocities measured when incubated with non-arachidonyl *sn*-1,2-diacylglycerols and *sn*-1-acyl-2-arachidonylglycerol under reaction conditions identical to those developed for the assessment of the Swiss 3T3 fibroblast membrane-associated enzyme (MacDonald *et al.*, 1988a, b).

Chapter 2

Materials and Methods.

2.1. Materials.

All reagents were of analytical or similar grade. They were purchased from the following suppliers as follows.

Amersham International p.l.c. Aylesbury, U.K.

[γ-^{32}P]ATP (3 mmol/Ci), and [^{14}C]*sn*-1-stearoyl-2-arachidonylglycerol.

Amicon Ltd. Stonehouse, U.K.

Centriprep 30 concentrators.

B.D.H. Ltd. Poole, U.K.

EDTA, ethanediol, formaldehyde, glutaraldehyde, imidazole, and β-mercaptoethanol.

Bachem Feinchemikalien AG, Bubendorf, Switzerland.

sn-1,2-dilaurylglycerol.

B.D.H.

10 x 20 cm silica SG60 t.l.c. plates, and 20 x 20 cm silica SG 60 t.l.c. plates (Both are Merck products.

BioRad Laboratories Ltd. Hemel Hempstead, U.K.

Affigel Blue, BioGel HTP (hydroxyapatite), and Protein Assay Dye Reagent Concentrate.

Boehringer Manheim. Lewes, U.K.

ATP (high purity, from horse muscle), DTT, and Tris.

CalbiochemNovabiochem. Leicester, U.K.

Diacylglycerol kinase (*E. coli*), *n*-octyl-β,D-glucopyranoside, and Zwitteregent 3-14.

Fisons Scientific Equipment. Loughborough, U.K.

Absolute alcohol, acetic acid (glacial), acrylamide, chloroform, citric acid, $CuSO_4$, Folin-Coicalteau solution, glycerol, glycine, hydrochloric acid, KCl, K_2HPO_4, KH_2PO_4, KOH, methanol, *N,N'*-methylenebisacrylamide, $MgCl_2.6(H_2O)$, MES, MOPS, Na_2CO_3, perchloric acid, polyethyleneglycol 6000,and *iso*-propylalcohol.

Lipid Products. Redhill, U.K.

Phosphatidylserine (bovine brain).

May and Baker Ltd. Dagenham, U.K.

Ammonium persulphate.

Pharmacia. Milton Keynes, U.K.

Butyl-Sepharose 4B, phenyl-Sepharose CL-4B, octyl-Sepharose CL-4B, Sephacryl S-300 SF, QAE-Sephadex A-50, and SP-Sephadex C-50.

Scientific Instrument Centre Ltd. Eastleigh, U.K.

Dialysis tubing (Visking).

Scientific Marketing Associates. Barnet, U.K.

E64 [L-*trans*-epoxysuccinyl-leucylamide-(4-guanidino)-butane], and leupeptin.

Sigma. Poole, U.K.

ATP-agarose (immobilised via N^6-amino group), butyl-agarose, methyl-agarose, bovine serum albumin (fraction V), cardiolipin, Coomassie Brilliant Blue R-250,

DETAPAC, heparin agarose (Type I), Lubrol PX, Nonidet P40, pepstatin A, phospholipase C (Type XIII, *B. cereus*), PMSF, sodium cholate, sodium deoxycholate, sodium fluoride, *sn*-1,2-dioleoylglycerol, *sn*-1-stearoyl-2-arachidonylglycerol, TEMED, andTriton X-100.

Whatman LabSales Ltd. Maidstone, U.K.

DE 52 (DEAE-cellulose), P11 (phosphocellulose), and PEI-cellulose.

2.2. Determination of Protein Concentration.

2.2.1. Copper/Tartrate Method.

The copper/tartrate method of Lowry *et al.* (1951) was employed as follows. Protein samples (5.0 - 50 μg protein) were corrected to a volume of 100 μl with buffer solution identical to that employed in the preparation of the sample. 900 μl of copper/tartrate solution was added with mixing and the mixture incubated at room temperature for 10 min [copper/tartrate solution was prepared by mixing 2.0 % (w/v) Na_2CO_3 in 100 mM NaOH, 2.0 % (w/v) sodium potassium tartrate and 1.0 % (w/v) $CuSO_4$ in the ratio 100:1:1, (v/v) respectively]. 100 μl of 20 % (v/v) Folin-Ciocalteu solution was added with mixing and the absorbance of the solution measured at 750 nm after 10 min incubation at room temperature. The concentration of the sample protein solution was determined from a standard curve (5.0 - 50 μg protein) constructed from 1.0 mg/ml bovine serum albumin (fraction V) and treated identically to the protein samples of unknown concentration. The buffer/salt conditions of the protein sample were duplicated in the standard curve.

2.2.2. Trichloroacetic Acid Precipitation Method.

A method adapted from Peterson *et al.* (1977) was employed to determine the protein concentrations of solutions with compositions known to interfere with the Coomassie Dye or copper/tartrate methods..

The volume of the protein sample (5.0 - 50 μg protein) was corrected to 100 μl with buffer solution identical to that employed in the preparation of the protein sample and brought to a final volume of 1.0 ml with H_2O. 100 μl of 0.15 % (w/v) sodium deoxycholate was added to the solution with brief mixing and followed by the addition of 100 μl of 72 % (w/v) trichloroacetic acid. The resulting suspension was vortexed thoroughly and incubated at room temperature for 10 min before centrifugation (18,000 xg, 15 min, 4°C). The supernatant was carefully aspirated and 400 μl of water added to each pellet followed by the addition of 500 μl copper/SDS reagent with vigorous mixing [Copper/SDS reagent was prepared

immediately before assay as follows: 1.0 % (w/v) $CuSO_4$, 2.0 % (w/v) sodium potassium tartrate and 2 % (w/v) Na_2CO_3 in 100 mM NaOH were mixed in the ratio of 1:1:100, (v/v) respectively. This copper/tartrate/carbonate solution was mixed with H_2O, 0.8 M NaOH and 10 % (w/v) SDS at a ratio of 1:1:1:1, (v/v) respectively]. The mixture was incubated at room temperature for 10 min before addition of 100 µl of 50 % (v/v) Folin-Ciocalteu solution. The absorbance of the solution was measured at 750 nm after incubation at room temperature for 30 min. The protein concentration of the sample soution was determined by comparison to a standard curve (5.0 - 50 µg protein) constructed from 1.0 mg/ml bovine serum albumin (fraction V) and treated in an identical manner to the protein solution of unknown concentration. Prior to the TCA precipitation step, care was taken to duplicate the salt/buffer conditions of the protein samples in the standard curve.

2.2.3. Coomassie Dye Method.

A method adapted from Bradford *et al.* (1976) was employed to determine the protein concentration of pooled chromatography eluants. This method employs Protein Assay Dye Reagent Concentrate (BioRad) as described in the manufacturer's Micro assay protocol.

In brief, the volume of the protein sample (1.0 - 30 µg protein) was made to a volume of 100 µl with buffer solution identical to that employed in the preparation of the protein sample and the volume corrected to 800 µl with H_2O. 200 µl of the Assay Dye Reagent Concentrate was added with mixing and the optical density of the solution measured at 595 nm after incubation at room temperature for 5 min. The concentration of the protein sample was determined from a standard curve (0.5 - 30 µg protein) constructed from 1.0 mg/ml bovine serum albumin (fraction V) and treated identically to the protein samples of unknown concentration. Care was taken to duplicate the salt/buffer conditions of the sample protein solutions in the standard curve.

2.2.4. U.V. Absorbance Method.

The protein concentration of chromatography column eluants was determined by an 'on-line' Uvicord S spectrophotometer (LKB) connected in series with the column outlet. The absorbance of the eluant solution was monitored at 280 nm in a 3 mm quartz flow cell and recorded by a 2210 pen recorder (LKB).

2.3. SDS-PAGE Analysis of Proteins.

Discontinuous buffer SDS-PAGE analysis of protein solutions was performed according to the method of Laemmli *et al.* (1970) in 1.5 mm thick polyacrylamide gels employing a vertical 100 mm x 140 mm slab gel apparatus (BioRad) as described by the manufacturers. The protein samples to be analysed and the SDS-PAGE gels prepared as follows.

2.3.1. Sample Preparation.

The protein sample solution was corrected to 1.0 ml in an eppendorf tube with H_2O and 100 µl of 0.15 % (w/v) sodium deoxycholate added with mixing. The protein was precipitated by addition of 100 µl of 72 % (w/v) trichloroacetic acid and the suspension incubated at room temperature for 10 min after vigorous vortexing. The suspension was pelleted by centrifugation (18,000 xg, 15 min, 4°C) and the supernatant removed by aspiration. 20 µl of 1.0 M Tris base and 20 µl of Laemmli buffer were added to the pellet which was solubilised by incubation in a boiling water bath for 3 min [Laemmli buffer: 30 % (w/v) urea, 5 % (w/v) SDS, 6 % (w/v) DTT, 20 mM Tris/HCl pH 8.0, 0.2 % bromophenol blue].

2.3.2. SDS-PAGE Stock Gel Solutions.

The following solutions were prepared and stored at 4°C.

Acrylamide solution: 30 % (w/v) acrylamide, 0.8%(w/v) *N,N'*-methylene-bisacrylamide.

Resolving gel buffer: 0.4 % (w/v) SDS, 1.5 M Tris/HCl pH 8.8.

Stacking gel buffer: 0.4 % (w/v) SDS, 0.5 M Tris/HCl pH 6.8.

Glycerol solution: 50 % (v/v) glycerol.

SDS solution: 0.1 % (w/v) SDS.

2.3.3. SDS-PAGE Resolving Gel Preparation.

SDS-PAGE analysis of proteins was performed on 10 % acrylamide resolving gels prepared by mixing the stock gel solutions as follows:

8.0 ml	Acrylamide stock.
6.0 ml	Resolving gel buffer stock.
1.6 ml	Glycerol solution.
8.2 ml	H_2O
8 µl	TEMED.
90 µl	10 % (w/v) ammonium persulphate, freshly prepared.

Directly after preparation, the resolving gel solution solution was poured into the assembled electrophoresis apparatus and overlaid with a few ml of 0.1 % SDS solution and left at room temperature for 3 h to polymerise.

2.3.4. SDS-PAGE Stacking Gel Preparation.

3.0 % acrylamide stacking gels were prepared from the stock gel solutions by mixing the solutions as follows:

1.5 ml	Acrylamide stock.
3.75 ml	Stacking gel buffer stock.
9.75 ml	H_2O.
8 µl	TEMED.
150 µl	10 % (w/v) ammonium persulphate, freshly prepared.

Immediately after preparation, the 0.1% (w/v) SDS overlay was removed from the polymerised resolving gel and the gel surface washed with a few ml of stacking gel solution. The stacking gel solution was then poured into the electrophoresis

apparatus and the well comb positioned according to the manufacturer's instructions and the solution left at room temperature to polymerise.

2.3.5. SDS-PAGE Electrophoresis.

The polymerised gels were assembled into the buffer reservoir as described by the manufacturer's instructions, the well comb removed and the reservoirs filled with running buffer [25 mM Tris base, 125 mM glycine, 0.1 % (w/v) SDS]. Protein samples, prepared in Laemmli sample buffer (Section 2.3.1), were placed directly into the wells of the stacking gel with a Hamilton syringe. Electrophoresis was performed in the direction of the cathode at 110 v, 25 mA until the dye front reached the end of the resolving gel.

2.3.6. Fixing and Coomassie R-250 Staining of SDS-PAGE Gels.

After the completion of electrophoresis, the apparatus was disassembled and the polyacrylamide resolving gel removed to a dish for staining. The gel was fixed and stained by immersion in staining solution [10 % (v/v) glacial acetic acid, 20 % (v/v)*iso*-propylalcohol, 0.1 % (w/v) Coomassie Brilliant Blue R-250] for 2 h. The staining solution was removed to a bottle for re-use and the gel destained in 40 % (v/v) methanol, 10 % (v/v) glacial acetic acid until the desired level of destaining was achieved. The destained gel was transfered to 10 % (v/v) glacial acetic acid for storage at 4°C.

2.3.7. Silver Staining of SDS-PAGE Gels.

Gels were silver stained after the method of Morrissey (1981) either directly after electrophoresis or after staining with Coomassie Brilliant Blue R-250 (Section 2.3.6).

Gels were placed in a clean dish and fixed by washing for 30 min with 50 % (v/v) methanol, 10% (v/v) glacial acetic acid solution followed by washing in 5 % (v/v) methanol, 7.5% (v/v) glacial acetic acid solution for 30 min. The washed

gels were placed in 10 % (v/v) glutaraldehyde and incubated for 30 min. The glutaraldehyde was removed by washing the gels with repeated changing in distilled H_2O over 3 h. After washing, the gels were placed in an aqueous solution of 5 μg/ml DTT for 30 min followed by immersion in 0.1 % (w/v) silver nitrate for 30 min. The excess silver nitrate solution was removed by aspiration and the gels washed briefly in a small volume of distilled H_2O. The distilled H_2O was rapidly removed by aspiration and the gels washed briefly in a small volume of developer solution (3 % (w/v) Na_2CO_3, 0.019 % (v/v) formaldehyde). The developer solution was removed rapidly by aspiration and development continued by immersion of the gel in 100 ml of developer solution. When the desired level of staining was achieved the development reaction was stopped by the addition of 12 ml of 2.3 M citric acid to the developer solution and agitation of the gel in this solution for 10 min. The developed gels were placed in 10 % (v/v) glacial acetic acid and stored in the dark at 4°C.

2.4. DAG Kinase Assays.

2.4.1. *n*-Octyl-β,D-glucopyranoside/Cardiolipin Mixed Micellar Method.

The reaction velocity of rat brain DAG kinase was measured by adaptation of the *n*-octyl-β,D-glucopyranoside/cardiolipin mixed micellar assay employed by Preiss *et al.* (1986) and Walsh & Bell (1986) to measure the reaction velocity of *E. coli* DAG kinase.

The rat brain DAG kinase was incubated in the following reaction mixture: 1.5 % (w/v) *n*-octyl-β,D-glucopyranoside, 3.6 mol% (1.0 mM) cardiolipin, 0.36 mol % (100 μM) *sn*-1-stearoyl-2-arachidonylglycerol, 60 mM imidazole/HCl pH 6.6, 50 mM NaCl, 10 mM DTT, 12.5 mM $MgCl_2$, 1.0 mM EGTA, 0.1 mM DETAPAC, 0.5 mM ATP (20 μCi/μmol [γ-^{32}P]ATP). These components were supplied to the final reaction mixture from concentrated stock solutions as follows: 10 nmol of *sn*-1-stearoyl-2-arachidonylglycerol in chloroform stock solution (5

mM) was dried under vacuum in each reaction tube. The dried *sn*-1-stearoyl-2-arachidonylglycerol was solubilised by sonication for 30 min at 4°C in 20 µl of *n*-octyl-β,D-glucopyranoside/cardiolipin solution [prepared immediately prior to use by sonication of 100 nmol cardiolipin, previously dried down under vacuum from chloroform/methanol stock solution, in 1.0 ml 7.5 % (w/v) *n*-octyl-β,D-glucopyranoside, 1.0 mM DETAPAC at 4°C until the solution was optically clear]. 50 µl of 2x incubation buffer (100 mM imidazole/HCl pH 6.6, 100 mM NaCl, 25 mM $MgCl_2$, 2.0 mM EGTA) and 10 µl of 100 mM DTT were added to the solubilised *sn*-1-stearoyl-2-arachidonylglycerol solution. The DAG kinase reaction was initiated by the addition of 10 µl of rat brain DAG kinase preparation followed by 10 µl of 0.5 mM ATP (20 µCi/µmol [γ-^{32}P]ATP) in 100 mM imidazole/HCl pH 6.6, 1 mM DETAPAC and the reaction mixture vortexed briefly. The reaction mixture was incubated for 30 min at 30°C and the reaction terminated by the addition of 470 µl of $CHCl_3$/MeOH/1 % (v/v) perchloric acid (15:30:2, v/v). The mixture were incubated at room temperature for 15 min and the phases split by addition of 150 µl of $CHCl_3$ and 150 µl of H_2O. The aqueous phase was removed and the lower phase washed twice with 1.0 ml H_2O. The [^{32}P]phosphatidic acid present in the final organic phase was quantitated directly by Cerenkov counting and the [^{32}P]phosphatidic acid quantitated from the specific activity of [γ-^{32}P]ATP employed in the reaction mixture. The integrity of the [^{32}P]-labelled products present in the final organic phase were routinely assessed by t.l.c. The final organic phase was dried down under vacuum, solubilised in 20 µl of $CHCl_3$/MeOH (19:1) and spotted onto 10 cm x 20 cm silica SG 60 plates (the silica t.l.c. plates were preactivated prior to use by heating to 100°C in an oven for 1 h and allowed to cool to room temperature). The t.l.c. plates were developed in $CHCl_3$/MeOH/glacial acetic acid (65:15:5, v/v). The radiolabelled products were visualised by autoradiography and [^{32}P]phosphatidic acid was identified by the position of authentic non-radiolabelled phosphatidic acid on staining with iodine

vapour. The silica corresponding to [^{32}P]phosphatidic acid was scraped and scintillation counted in 4 ml HiSafe II scintillation fluid.

2.4.2. Deoxycholate/Phosphatidylserine Dispersion Assay.

The dispersed diacylglycerol assay method of Coco-Maroney & Macara (1989) was routinely employed to monitor the DAG kinase activity present in column chromatography eluants.

The DAG kinase activity was measured by incubation in a reaction mixture of 1.0 mM sodium deoxycholate, 130 μM PtdSer, 200 μM *sn*-1-stearoyl-2-arachidonylglycerol, 50 mM Tris/HCl pH 7.4, 10 mM $MgCl_2$, 20 mM NaF, 0.5 mM DTT, 1.0 mM ATP (10 μCi/μmol [γ-^{32}P]ATP). These components were delivered to the reaction mixture as follows. Each reaction vial received 20 nmol *sn*-1-stearoyl-2-arachidonylglycerol from 5.0 mM stock solution in $CHCl_3$ and the solvent removed under vacuum. The dried *sn*-1-stearoyl-2-arachidonylglycerol was dispersed by sonication in 40 μl of PtdSer/deoxycholate solution for 30 min at 4°C [the PtdSer/deoxycholate solution had been prepared prior to use by solubilisation of 650 nmol PtdSer, dried down from chloroform stock solution, in 2.0 ml of 112.5 mM Tris/HCl pH 7.4, 50 mM NaF, 2.5 mM sodium deoxycholate, 1.25 mM DTT and the mixture sonicated at 4°C in a glass vial until the solution became optically clear]. 50 μl of DAG kinase preparation was introduced to the dispersed *sn*-1-stearoyl-2-arachidonylglycerol mixture and the reaction initiated by addition of 10 μl of 10 mM ATP (10 μCi/μmol [γ-^{32}P]ATP) in 100 mM $MgCl_2$, 50 mM Tris/HCl pH 7.4. The reaction mixture was incubated at 30°C for 30 min and the reaction terminated by the addition of 470 μl of $CHCl_3$/MeOH/10 mM HCl (15:30:2, v/v) and the mixture allowed to extract for 15 min. Phases were split by the addition of 150 μl of $CHCl_3$ and 1.0 ml of H_2O. The aqueous phase was removed by aspiration and the organic phase washed with a further 1.0 ml of H_2O. The resulting lower phase was removed to a 2 ml polytube. The residue remaining in the glass vial was removed by extraction of the reaction

vial with 470 µl of $CHCl_3$/MeOH/10 mM HCl (15:30:2, v/v) and this residue pooled with the original organic phase in the polytube. Phases were split by the addition of 150 µl of $CHCl_3$ and 1.0 ml of H_2O and the upper phase removed by aspiration. The [^{32}P]-labelled products remaining in the final organic phase were quantitated by Cerenkov counting.

Routinely greater than 95 % of the [^{32}P]-labelled reaction products present in the final organic phase co-migrated with authentic phosphatidic acid when analysed by t.l.c. as described in Section 2.4.1 (data not shown).

This assay method was capable of measuring DAG kinase activity of between 10 and 90 pmol PtdOH formed/min. The relationship between measured enzyme activity and enzyme present in the incubation mixture was hyperbolic and found to saturate above 80 pmol/min. However, a linear relationship was obtained with enzyme activities below 50 pmol/min. DAG kinase activity measured by this method was found not to follow Michaelis-Menten kinetics with respect to its *sn*-1,2-diacylglycerol substrate.

2.4.3. *n*-Octyl-β,D-glucopyranoside/Phosphatidylserine Mixed Micellar Assay.

An *n*-octyl-β,D-glucopyranoside/PtdSer mixed micellar assay adapted from MacDonald *et al.* (1988a) was routinely employed in the kinetic analysis of the KCl-soluble enzyme.

Unless otherwise stated, the reaction velocity of DAG kinase was measured by incubation in a reaction mixture containing 73 mM *n*-octyl-β,D-glucopyranoside, 8.0 mol% (4.38 mM) PtdSer, 3.7 mol% (2.0 mM) *sn*-1-stearoyl-2-arachidonylglycerol, 50 mM MOPS/NaOH pH 7.2, 18 mM $MgCl_2$, 20 mM NaF, 1.0 mM DTT, 2.0 mM ATP (10 µCi/µmol [γ-^{32}P]ATP). The assay was performed as follows: 16.05 mg (21.9 µmol) PtdSer (18 mg/ml $CHCl_3$/MeOH stock solution) was dried down under a stream of N_2 in a 2 ml glass vial. The dried PtdSer was solubilised in 2.0 ml of 182.5 mM *n*-octyl-β,D-glucopyranoside, 112.5 mM

MOPS/NaOH pH 7.2, 50 mM NaF, 2.5 mM DTT by vortexing, followed by sonication at 4°C until the solution was optically clear. In each reaction vial 40 µl of the *n*-octyl-β,D-glucopyranoside/PtdSer solution was employed to solubilise 200 nmol of *sn*-1-stearoyl-2-arachidonylglycerol, previously dried down under vacuum from 5.0 mM $CHCl_3$/MeOH stock solution. Solubilisation was completed by sonication of the reaction vials for 30 min at 4°C. Immediately before initiation of the DAG kinase reaction, 50 µl of diluted DAG kinase preparation was added to each vial and the reaction initiated by addition of 10 µl of 20 mM ATP (10 µCi/µmol [γ-^{32}P] ATP) in 180 mM $MgCl_2$, 50 mM MOPS/NaOH pH 7.2. The reaction mixture was incubated for 3-15 min at 30°C and terminated by the addition of 940 µl of $CHCl_3$/MeOH/10 mM HCl (15:30:2, v/v) and the products allowed to extract for 15 min at room temperature. Phases were split by the addition of 300 µl of $CHCl_3$ and 400 µl of H_2O and the upper aqueous phase removed. The organic phase was washed twice with 1.0 ml of synthetic upper phase [produced as the upper phase when $CHCl_3$, MeOH and H_2O were mixed in the ratio 1:1:0.9, v/v]. The final organic phase was dried under vacuum and the [^{32}P]-labelled products separated by t.l.c. on 10 cm x 20 cm silica SG 60 plates [the t.l.c. plates were preactivated prior to use by heating to 100°C for 1 h and allowed to cool]. Routinely, half the organic [^{32}P]-labelled reaction products were spotted in 20 µl of $CHCl_3$/MeOH (2:1, v/v) and the t.l.c. plates developed over 10 cm in $CHCl_3$/MeOH/glacial acetic acid (26:6:3, v/v). The [^{32}P]-labelled products were visualised by autoradiography and the [^{32}P]phosphatidic acid identified by the migration of authentic non-radiolabelled phosphatidic acid after staining in iodine vapour. The silica corresponding to [^{32}P]phosphatidic acid was scraped and the [^{32}P]phosphatidic acid quantitated by scintillation counting in 4 ml HiSafe II scintillation fluid.

This assay of DAG kinase activity was found to be linear with increasing amounts of enzyme activity (0 - 80 pmol/min), protein (0 - 3.0 µg of the post HIC preparation) and time (0 - 15 min). Furthermore, the KCl-soluble DAG kinase was

observed to follow Michaelis-Menten kinetics with respect both to *sn*-1,2-diacylglycerol and ATP substrate.

The mean assay time for this method was 2 to 3 days. It has the advantage of measuring DAG kinase with first order rate kinetics for either substrate, time, and enzyme concentration. However, it is time consuming and was unsuitable for use in the assay of column fractions for DAG kinase activity. The deoxycholate/PtdSer method described above (Section 2.4.2) was employed for measuring the DAG kinase activity present in column fractions. Although it cannot be employed for kinetic analysis of the KCl-soluble enzyme, it is rapid (6 - 8 h) and provides a qualitative estimate of DAG kinase activity.

2.5. Preparation of the KCl-Soluble DAG Kinase.

2.5.1. Phospholipase C-Mediated Enrichment of Rat Brain Particulate Fraction in DAG Kinase Activity.

The particulate fraction of rat brain was enriched in DAG kinase activity by affecting a redistribution of DAG kinase activity within rat brain homogenate from the soluble to the membrane-associated compartment. This was achieved by incubation of rat brain homogenate with bacterial phospholipase C in a method adapted from that described previously by Besterman *et al.* (1986b).

Sprague-Dawley rats (250 g - 300 g) were killed by cervical fracture and the cerebral cortices removed to ice-cold buffer A (118 mM KCl, 20 mM Tris/HCl pH 7.0, 5.0 mM DTT, 1.0 mM $CaCl_2$, 0.5 mM EDTA). The cortices were homogenised in 5 vol. of buffer A supplemented with 0.1 mg/ml leupeptin and 6 U/ml phospholipase C (Type XIII, *B. cereus*) with six strokes in a teflon/glass homogeniser at full speed (1100 r.p.m.) and the homogenate incubated for 15 min at 37°C. The phospholipase C-catalysed reaction was teminated by addition of ice-cold buffer A supplemented with 10 mM EGTA until a final concentration of 5.0 mM was achieved and the mixture incubated on ice for 10 min. The enriched particulate fraction was pelleted by centrifugation (31,400 xg_{av}, 25 min, 4°C). The

supernatant (SN_1) was collected as a crude cytosolic preparation and the pellet resuspended in 10 vol. of buffer B (78 mM KCl, 20 mM Tris/HCl pH 7.0, 2.0 mM EDTA, 2.0 mM DTT, 20 % (v/v) glycerol, 0.2 mM PMSF, 1.0 μM E-64) and the suspension centrifuged (31,400 xg_{av}, 25 min, 4°C), the supernatant discarded and the DAG kinase-enriched particulate fraction resuspended in 10 vol. of buffer B.

2.5.2. Solubilisation of Membrane-Associated DAG Kinase Activity from the Particulate Fraction of Rat Brain.

DAG kinase-enriched particulate fraction resuspended in 9 vol. of buffer C (buffer B supplemented with 0.5 M KCl) and incubated at 4°C with slow stirring for 30 min before pelleting by centifugation (31,400 xg_{av}, 25 min, 4°C). The supernatant containing the KCl-extracted DAG kinase activity was removed and used for further purification of the membrane-associated DAG kinase.

2.5.3. Preparation of the KCl-Soluble DAG Kinase Activity of Rat Brain Particulate Fraction.

The KCl-solubilised protein preparation was dialysed extensively against 20 mM Tris/HCl pH 6.5, 2.0 mM EDTA, 2.0 mM β-mercaptoethanol, 20 % (v/v) glycerol, 0.2 mM PMSF, 1.0 μM E64, 1 μM pepstatin A. Dialysis was typically performed overnight at 4°C against 3 x 5 l volumes of the above buffer solution. The precipitate that formed on dialysis was pelleted by centrifugation (100,000 xg_{av}, 1 h, 4°C) and the supernatant discarded. The KCl-soluble DAG kinase was solubilised from the pellet by resuspension of the pellet in 2.0 ml buffer E/g of rat brain used to prepare the DAG kinase-enriched particulate fraction (see Section 2.5.1) [buffer E: 20 mM Tris/HCl pH 6.5, 300 mM KCl, 2.0 mM EDTA, 2.0 mM β-mercaptoethanol, 20 % (v/v) glycerol, 0.2 mM PMSF, 1.0 μM E64, 1.0 μM pepstatin A]. The insoluble material was removed by centrifugation (100,000 xg_{av}, 1 h, 4°C) and the supernatant collected as the KCl-soluble protein preparation and further employed for purification of the KCl-soluble DAG kinase.

N.B. Despite thae in appropriate use of Tris/HCl at pH 6.5, the dialysis buffer was found remain at pH 6.5 during dialysis. Similarly, the pH of the KCl-soluble protein preparation was measured as 6.5.

2.6. Preparation of Chromatography Media.

All the following procedures employed in the preparation of chromatography media were performed at 4°C. Despite the inappropriate use of Tris/HCl at pH 6.5 in the preparation of AffiGel blue and Hydrophobic Interaction Media, these matrices were found to be equilibrated at pH 6.5. Once prepared media was packed into columns, the pH of effluent buffer was found to have remained at 6.5.

2.6.1. AffiGel Blue.

Both new and previously used AffiGel blue were prepared for use by the following method. Settled AffiGel blue was suspended gently in 10 vol. of 20 % (v/v) ethanol and allowed to settle for 30 min. The supernatant and the gel finings were decanted and the process repeated. The settled gel was washed by suspension in 20 vol. of distilled H_2O and the gel allowed to settle. This process was repeated twice with fresh distilled H_2O. The washed gel was suspended in 20 vol. of 0.5 M NaOH and incubated for 30 min before the supernatant was decanted and the gel resuspended in a further 20 vol. of 0.5 M NaOH. After 30 min the supernatant was decanted and the gel washed with distilled H_2O as described above. The final distilled H_2O wash was decanted and the gel equilibrated to pH 6.5 at 4°C by incubation in 10 vol. of 200 mM Tris/HCl pH 6.5, 1.0 M KCl, 2.0 mM β-mercaptoethanol, 2.0 mM EDTA, 20 % (v/v) glycerol. After 30 min the solution was removed and the gel incubated in fresh 200 mM Tris/HCl pH 6.5, 1.0 M KCl, 2.0 mM β-mercaptoethanol, 2.0 mM EDTA , 20 % (v/v) glycerol as above. After 30 min the gel was resuspended and the column packed and equilibrated with the initial chromatography buffer [20 mM Tris/HCl pH 6.5, 300 mM KCl, 10 mM

$MgCl_2$, 2.0 mM β-mercaptoethanol, 2.0 mM EDTA, 20 % (v/v) glycerol]. Prior to use, the column was washed with at least 20 column vol. of initial chromatography buffer or until a steady baseline was observed when the column eluant was monitored at 280 nm.

2.6.2. Hydrophobic Interaction Chromatography Media.

Butyl-Sepharose 4B, phenyl-Sepharose CL-4B, octyl-Sepharose CL-4B, butyl-agarose and methyl-agarose were all prepared for column chromatography by the following method. An appropriate volume of gel suspension was placed in a small beaker and the gel allowed to settle. The solution was decanted and the gel suspended for column packing in an equal volume of equilibration buffer [20 mM Tris/HCl pH 6.5, 1.0 M KCl, 2.0 mM β-mercaptoethanol, 2.0 mM EDTA, 20 % (v/v) glycerol]. The column was packed with the above equilibration buffer and the packed column washed with at least 50 column vol. of the same buffer until the a steady baseline was observed when the column eluant was monitored at 280 nm.

2.6.3. Sephacryl S-300 SF Gel Filtration Media.

An appropriate volume of Sephacryl S-300 SF suspension was measured into a beaker and the gel allowed to settle. The solution was decanted and the settled gel resuspended in an equal volume of 20 mM Mes/KOH pH 6.5, 300 mM KCl, 10 mM $MgCl_2$, 2.0 mM EDTA, 2.0 mM β-mercaptoethanol, 20 % (v/v) glycerol, 0.02 % (w/v) Triton X-100. This suspension was degassed and used to pack the gel filtration column (1.6 cm x 90 cm) by the method described in the manufacturer's instructions. The above buffer was employed during column packing and subsequent washing of the packed column.

2.6.4. Heparin-Agarose.

The heparin-agarose suspension supplied by the manufacturers was allowed to settle and the solution decanted. 10 vol. of 200 mM Mes/KOH pH 6.5, 1.0 M

KCl were employed to resuspend the gel and the suspension incubated overnight. The supernatant was removed and the settled gel resuspended in 10 vol. of 200 mM Mes/KOH pH 6.5, 1.0 M KCl and the suspension incubated for 1 h and the gel allowed to settle. The settled gel was equilibrated by resuspension in the initial chromatography buffer [20 mM Mes/KOH pH 6.5, 300 mM KCl, 10 mM $MgCl_2$, 2.0 mM EDTA, 2.0 mM β-mercaptoethanol, 20 % (v/v) glycerol, 0.02 % (w/v) Triton X-100] and the suspension incubated for 1 h. The process was repeated with fresh buffer. After 1 h the solution was decanted and the settled gel was resuspended in an equal volume of initial chromatography buffer and the column packed.

2.6.5. Hydroxyapatite (BioGel HTP).

An appropriate amount of BioGel HTP was weighed into a beaker and the powder suspended in H_2O by gentle stirring. The BioGel HTP was 'fined' by allowing the hydroxyapatite to settle and the cloudy solution remaining decanted. The settled hydroxyapatite was resuspended in H_2O and the fining procedure repeated until a clear solution was obtained on settling of the hydroxyapatite. The fined BioGel HTP was equilibrated by resuspension in 20 vol. of 200 mM Mes/KOH pH 6.5, 1.0 M KCl, the mixture incubated for 1 h and the solution decanted from the settled hydroxyapatite. The settled hydroxyapatite was resuspended in a further 20 vol. of 200 mM Mes/KOH pH 6.5, 1.0 M KCl and the mixture incubated for 1 h. The hydroxyapatite was allowed to settle, the solution removed and the settled hydroxyapatite prepared for chromatography by resuspension in 20 vol. of initial chromatography buffer [20 mM Mes/KOH pH 6.5, 300 mM KCl, 2.0 mM β-mercaptoethanol, 20 % (v/v) glycerol, 0.02 % (w/v) Triton X-100]. After 1 h, the hydroxyapatite allowed to settle and the settled hydroxyapatite resuspended in a further 20 vol. of fresh initial chromatography buffer. The mixture was incubated for 1 h and the settled hydroxyapatite

resuspended in an equal volume of initial chromatography buffer and used for column packing.

2.7. Measurement of *sn*-1,2-Diradylglycerol Mass: The Triton X-100 Method.

sn-1,2-Diradylglycerol mass was determined by the Triton X-100/PtdSer method of Thompson *et al.* (1990), as adapted by Paterson *et al.* (1991).

The final reaction mixture contained 0.3% (w/v) Triton X-100, 144 μM PtdSer, 50 mM imidazole/HCl pH 6.6, 50 mM NaCl, 12.5 mM $MgCl_2$,1 mM EGTA, 10 mM DTT, 0.5 mM ATP (20 μCi/μmol [γ-^{32}P]ATP) and 5 mU DAG kinase (*E.coli*) in a volume of 100 μl. DRG/lipid extract was dried under vacuum in a 2 ml glass tube and solubilised in 50 μl of 0.6 %(w/v) Triton X-100/288 μM PtdSer by brief vortexing followed by sonication (30 min, 4°C). To this was added 10 μl of 100 mM DTT and 20 μl of 5x incubation buffer (225 mM imidazole/HCl ph 6.6, 250 mM NaCl, 62.5 mM $MgCl_2$, 5.0 mM EGTA). Prior to incubation, 10 μl (5 mU) DAG kinase was added and the reaction initiated by the addition of 10 μl 5.0 mM ATP (20 μCi/μmol [γ-^{32}P]ATP). The mixture was incubated at 30°C for 30 min and the reaction terminated by the addition of 470 μl $CHCl_3$:MeOH:10 mM HCl (15:30:2) and the tubes left to stand for 10 min at room temperature and the phases split by addition of 150 μl $CHCl_3$ and 1.0 ml H_2O. The resulting organic phase was washed twice with 1.0 ml H_2O before drying under vacuum and the radiolabelled products are separated by t.l.c on silica SG60 plates developed in $CHCl_3$:MeOH:Acetic Acid (130:30:15). The [^{32}P]phosphatidic acid was identified by autoradiography and comparison to unlabelled phosphatidic acid stained with iodine vapour. Silica corresponding to phosphatidic acid was scraped and quantitated by liquid scintillation counting.

2.8. Miscallaneous Procedures.

2.8.1. Preparation of Dialysis Tubing.

Dialysis tubing was immersed in 20 % (v/v) ethanol and incubated at room temperature for 1 h. The swollen dialysis tubing was removed from the ethanol solution and boiled in 10 mM EDTA for 30 min. The boiled dialysis tubing was removed to distilled H_2O and boiled for a further 30 min. The tubing was allowed to cool and either used immediately or stored in 20 % (v/v) ethanol.

2.8.2. Concentration of Protein Solutions.

Pooled chromatography eluants were concentrated by dialysis against polyethyleneglycol 6000 at 500 g/l in 20 mM Mes/KOH pH 6.5, 300 mM KCl, 2.0 mM β-mercaptoethanol, 2.0 mM EDTA, 20 % (v/v) glycerol, 0.02 % (w/v) Triton X-100, 200 μM PMSF, 1.0 μM E64, 1.0 μM pepstatin A.

Concentration was also performed with Centriprep 30 concentrators. The protein solution (1 - 15 ml) was placed in a Centriprep 30 concentrators and centrifuged (2000 xg, 4°C) until the appropriate volume obtained.

2.8.3. Critical Micelle Concentrations.

The critical micelle concentration (c.m.c.) values employed in this thesis were obtained from Neugebauer (1990).

2.8.4. Statistical Analysis.

Unless otherwise stated, the results presented within this thesis are representative of at least three experiments.

The DAG kinase reaction velocities measured in the fractions collected from chromatography columns are single determinations. Otherwise, the values stated within this thesis are expressed as the mean of a triplicate determination and the error expressed as ± 1 SD.

2.9. Buffer Solutions Employed in the Purification of the KCl-Soluble DAG Kinase.

Dialysis buffer: 20 mM Tris/HCl pH 7.0, 2.0 mM EDTA, 2.0 mM DDT, 1.0 mM KCl, 20 %(v/v) glycerol, 200 μM PMSF, 1.0 μM E64, 1.0 μM pepstatin A

Buffer B: 20 mM Tris/HCl pH 7.0, 2.0 mM EDTA, 2.0 mM DTT, 78 mM KCl, 20 % (v/v) glycerol, 200 μM PMSF, 1.0 μM E64, 1.0 μM pepstatin A

Buffer C: 20 mM Tris/HCl pH 7.0, 2.0 mM EDTA, 2.0 mM DTT, 578 mM KCl, 20 % (v/v) glycerol, 200 μM PMSF, 1.0 μM E64, 1.0 μM pepstatin A

Buffer D: 20 mM Tris/HCl pH 6.5, 2.0 mM EDTA, 2.0 mM DTT, 1.0 mM KCl, 20%(v/v) glycerol, 200 μM PMSF, 1.0 μM E64, 1.0 μM pepstatin A

Buffer E: 20 mM Tris/HCl pH 6.5, 2.0 mM EDTA, 2.0 mM DTT, 300 mM KCl, 20%(v/v) glycerol, 200 μM PMSF, 1.0 μM E64, 1.0 μM pepstatin A

Buffer F: 20mM Tris/HCl pH 6.5, 2.0 mM EDTA, 2.0 mM β-mercaptoethanol, 300 mM KCl, 10 mM $MgCl_2$, 20 % (v/v) glycerol, 0.02 % (w/v) Triton X-100, 200 μM PMSF, 1.0 μM E64, 1.0 μM pepstatin A

Buffer G: 20 mM Tris/HCl pH 6.5, 2.0 mM EDTA, 2.0 mM β-mercaptoethanol, 1.0 M KCl, 10 mM $MgCl_2$, 20 %(v/v) glycerol, 0.02 % (w/v) Triton X-100, 200 μM PMSF, 1.0 μM E64, 1.0 μM pepstatin A

Buffer H: 20 mM Mes/KOH pH 6.5, 300 mM KCl, 10 mM $MgCl_2$, 2.0 mM EDTA, 2.0 mM β-mercaptoethanol, 20 % (v/v) glycerol, 0.02 % (w/v) Triton X-100, 200 μM PMSF, 1.0 μM E64, 1.0 μM pepstatin A .

Buffer I: 20mM Mes/KOH pH 6.5, 300 mM KCl, 2.0 mM β-mercaptoethanol, 20 % (v/v) glycerol, 0.02 % (w/v) Triton X-100, 200 μM PMSF, 1.0 μM E64, 1.0 μM pepstatin A.

Buffer J: 20 mM potassium phosphate pH 6.5, 300 mM KCl, 2.0 mM β-mercaptoethanol, 20 % (v/v) glycerol, 0.02 % (w/v) Triton X-100, 200 μM PMSF, 1.0 μM E64, 1.0 μM pepstatin A.

Chapter 3

Purification of Membrane-Associated DAG Kinase From Rat Brain.

3.1. Preparation of DAG Kinase-Enriched Particulate Fraction from Rat Brain Homogenate.

The proportion of cellular DAG kinase activity which is resident within the membrane-associated compartment of rat brain remains a point of contention. Lapetina & Hawthorne (1971) reported that 77.7 % of rat cerebral cortex activity is located on the particulate fraction of the tissue homogenate. Subsequent to this, Besterman *et al.* (1986b) reported that the membrane-associated enzyme of rat brain represents only 25 % of the total homogenate activity. It is most likely that these observed differences are the result of the different assay methodologies employed in the above two studies. Nevertheless, it is clear that a considerable proportion of rat brain DAG kinase is associated with the particulate fraction. Furthermore it is possible to enrich the membranes of rat brain homogenate with DAG kinase by facilitating the translocation of soluble activity to the particulate fraction. This is achieved by incubating rat brain homogenate with phospholipase C from a bacterial source. Besterman *et al.* (1986b) reported that incubation of rat brain homogenate with phospholipase C from *Bacillus cereus* increased the DAG kinase activity recovered with the particulate fraction by nearly 3-fold, such that the percentage of the total homogenate associated enzyme recovered in the particulate fraction increased from 25 to 73 %. The use of bacterial phospholipase C to affect a redistribution of soluble DAG kinase activity towards the membrane associated component within the homogenate has been succesfully demonstrated within this laboraotory. In this case, however, the magnitude of redistribution was of lower magnitude than that reported previously, i.e. membrane-associated DAG kinase activity increased from 37 to 65 % on treatment (S. Palmer and S.J. Cook, personal communication).

It has now been demonstrated in several laboratories that incubation of a tissue homogenate with phospholipase C increases the proportion of DAG kinase activity which is recovered with the membrane-associated component. For example, incubation of inside-out vesicle prepared from human red blood cells with

phospholipase C (*B. cereus*) has been reported to increase the amount of rat brain DAG kinase which will bind to these membranes (Besterman *et al.*, 1986b). Similarly, 80 % of the DAG kinase activity within a crude cytosolic preparation of rat brain has been reported to bind to phospholipase C-treated membranes prepared from human red blood cells (Coco-Maroney & Macara, 1989). Clearly, incubation of rat brain homogenate with phospholipase C has an application to the preparation of an enriched source of membrane-associated DAG kinase which would prove a suitable source for purification of the enzyme. Indeed, this approach has been successfully employed. A membrane-associated DAG kinase has been 500- to 750-fold purified from phospholipase C-treated rat brain membranes (Besterman *et al.*, 1986b). Therefore, the procedure of Besterman *et al.* (1986b) was adopted to prepare a particulate fraction enriched in DAG kinase activity. This DAG kinase-enriched particulate fraction would then be employed as a source of enzyme for purification of the membrane-associated DAG kinase. Typically, the particulate fraction prepared from phospholipase C-treated rat brain homogenate (see Section 2.5.1) possessed a specific activity of 1210 ± 160 pmol/30 min/mg of protein (DAG kinase activity measured in the presence of 5.0 mM NaF by the method described in Section 2.4.1).

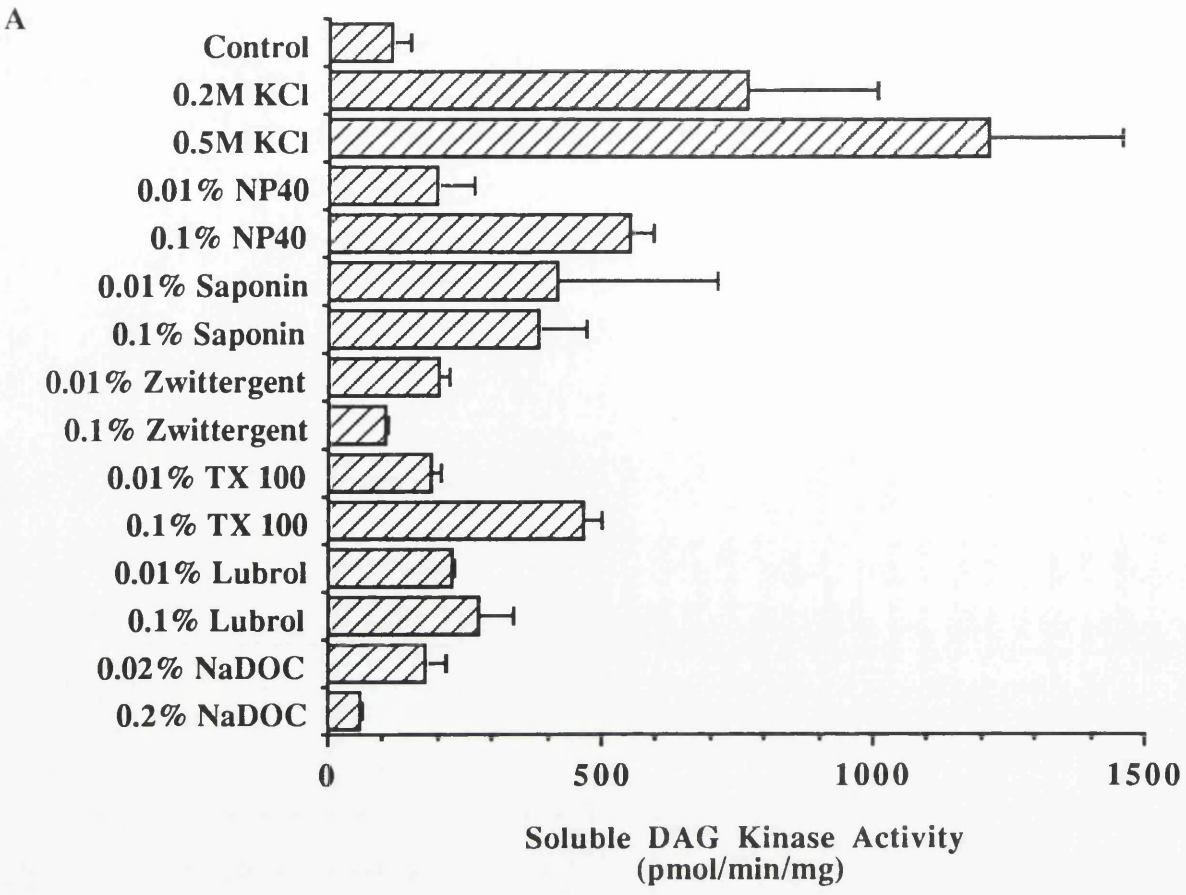
3.2.1. Solubilisation of Membrane-Associated Diacylglycerol Kinase.

The exact nature of the interaction between DAG kinase and the membranes of the phospholipase C-treated homogenate is not clear. The enzyme could exist as an integral membrane protein with transmembrane hydrophobic domains or as a non-integral membrane protein associated with the surface of the phospholipid bilayer. In addition, it remains possible that DAG kinase exists as a cytoskeleton-associated protein, with its localisation depending on the cytoskeletal proteins anchored to the membrane. In the absence of further information, solubilisation of

the membrane-associated DAG kinase both by detergent and hypertonic KCl extraction were examined.

Hypertonic KCl extraction was performed by resuspending the DAG kinase-enriched particulate fraction in buffer B supplemented with either 0.2 or 0.5 M KCl. Solubilisation by non-ionic, anionic and zwitterionic detergents was examined by resuspension of the DAG kinase-enriched particulate fraction in buffer B (Section 2.9) supplemented with detergent of appropriate concentration. The non-ionic detergents Triton X-100, Nonidet P40, Lubrol PX and Saponin were examined at final concentrations of both 0.1 and 0.01 % (w/v). Solubilisation by the zwitterionic detergent Zwittergent 3-14 was examined at final concentrations of both 0.1 and 0.01 % (w/v), whilst the anionic detergent sodium deoxycholate was employed at final concentrations of both 0.2 and 0.02 % (w/v).

Solubilisation of the membrane-associated enzyme by these treatments was assessed by their ability to release DAG kinase activity to the supernatant after centrifugation of the KCl- or detergent-treated particulate fraction (Fig 3.1A). At the above concentrations Zwittergent 3-14, Saponin and sodium deoxycholate reproducibly gave no significant decrease in the specific activity of the detergent-treated particulate fraction. Nor did they elicit a significant increase in the specific activity of the soluble fraction (Fig 3.1B). This indicated that these detergents were unsuitable for further use in the solubilisation of membrane-associated DAG kinase. However, 0.1 % (w/v) Nonidet P40, 0.1 % (w/v) Lubrol PX and 0.1 % (w/v) Triton X-100 did solubilise the membrane-associated DAG kinase. At a final concentration of 0.1 % (v/v) the above detergents affected a clear increase in the specific activity of the resulting soluble fractions. This increase was not observed at the lower concentration of 0.01 % (w/v) (Fig.3.1A). Incubation of the DAG kinase-enriched particulate fraction with 0.1 % (w/v) Nonidet P40, 0.1 % (w/v) Lubrol PX and 0.1 % (w/v) Triton X-100 also elicited a lowering of the specific activity of the post solubilisation particulate fraction, consistent with solubilisation of membrane-associated DAG kinase (Fig 3.1B). In the experiment represented in


A
Control
0.2M KCl
0.5M KCl
0.01% NP40
0.1% NP40
0.01% Saponin
0.1% Saponin
0.01% Zwittergent
0.1% Zwittergent
0.01% TX 100
0.1% TX 100
0.01% Lubrol
0.1% Lubrol
0.02% NaDOC
0.2% NaDOC
0
500
1000
1500
Soluble DAG Kinase Activity
(pmol/min/mg)

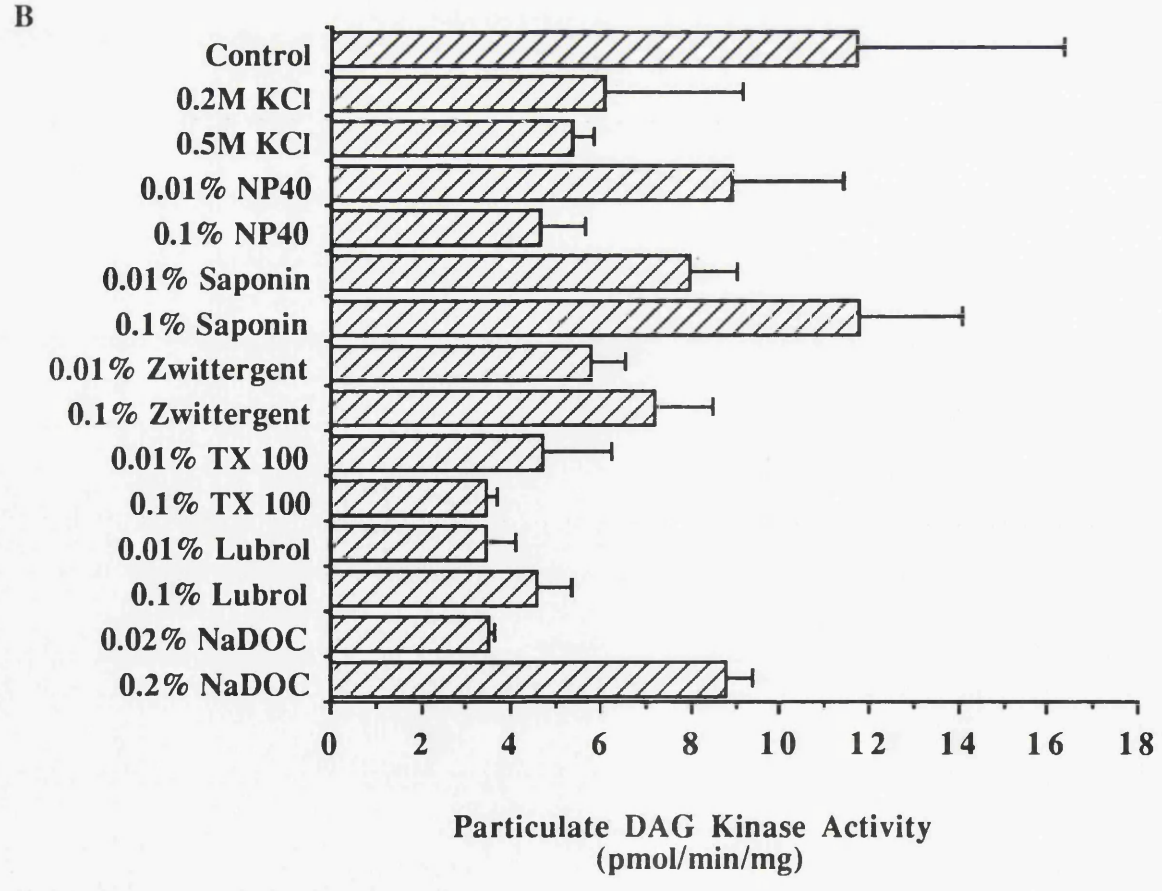

B
Control
0.2M KCl
0.5M KCl
0.01% NP40
0.1% NP40
0.01% Saponin
0.1% Saponin
0.01% Zwittergent
0.1% Zwittergent
0.01% TX 100
0.1% TX 100
0.01% Lubrol
0.1% Lubrol
0.02% NaDOC
0.2% NaDOC
0
2
4
6
8
10
12
14
16
18
Particulate DAG Kinase Activity
(pmol/min/mg)

Fig 3.1. Solubilisation of Membrane-Associated DAG Kinase from the DAG Kinase-Enriched Particulate Fraction of Rat Brain Homogenate.

A. DAG kinase activity released to the soluble fraction after incubation of DAG kinase-enriched particulate fraction with various detergents and hypertonic KCl.

B. DAG kinase activity remaining associated with the particulate fraction after incubation of DAG kinase-enriched particulate fraction with various detergents and hypertonic KCl.

DAG kinase-enriched particulate fraction was prepared as described in Section 2.5.1 and suspended to 15.0 ml with buffer B. 1.0 ml aliquots (13.65mg protein) of the suspension were pelleted by centrifugation (18,000 xg,15min, 4°C) and the supernatants discarded. The pellets were resuspended in 1.0 ml of buffer B (Control) or buffer B supplemented with detergent/KCl, as appropriate, and incubated on ice for 30 min. The suspensions were centrifuged (18,000 xg, 15 min, 4°C) and the solubilised DAG kinase collected in the supernatant. The pellet was resuspended in 1.0 ml of buffer B (no added detergent or KCl). 10 µl of post-solubilisation supernatants and suspended particulates were assayed for DAG kinase activity by the *n*-octyl-β,D-glucopyranoside method (Section 2.4.1). Protein concentrations were determined by the copper/tartrate method (Section 2.2.1).

Fig 3.1 both 0.1 and 0.01 % (w/v) Saponin would appear to have increased the specific activity of the DAG kinase recovered in the soluble fraction recovered after incubation of the DAG kinase-enriched particulate with detergent. However, this was not observed in two other experiments and Saponin was not observed to elicit a reduction in the specific activity of the DAG kinase activity remaining associated with the particulate fraction. It was concluded that Saponin at the concentrations employed was unsuitable for the solubilisation of the membrane-associated enzyme.

The membane-associated DAG kinase activity was also solubilised with hypertonic concentrations of KCl. Resuspension of the enriched particulate fraction in KCl resulted in the release of DAG kinase activity to the soluble fraction (Fig 3.1A). This release was concomitant with a reduction in the specific activity of the particulate-associated enzyme (Fig 3.1B). Supplementing buffer B (Section 2.9) with 0.5 M KCl reproducibly resulted in a solubilised DAG kinase preparation of greater specific activity than detergent-solubilised enzyme (Fig 3.1A).

The yield of DAG kinase activity solubilised from the phospholipase C-treated membranes cannot be calculated. The enzyme solubilised from the membranes on incubation with either detergents or hypertonic concentrations of KCl was assayed for DAG kinase activity in the absence of NaF, a general phosphatase inhibitor. The presence of phosphatidic acid phosphohydrolase (PPH) activity in both the crude cytosolic and membrane fraction prepared from phospholipase C-treated rat brain has been confirmed (data not shown). Additionally this enzyme activity has been shown to be inhibited by the presence of 5.0 mM or greater NaF (data not shown). This is relevant to the measurement of DAG kinase activity in enzyme preparations which also contain PPH activity as the product of the DAG kinase-catalysed reaction is also a substrate of PPH, i.e [^{32}P]phosphatidic acid. Thus, the presence of an uninhibited PPH activity will lead to the underestimation of of the DAG kinase activity present. It is therefore not possible to calculate the yield of DAG kinase solubilised from phospholipase C-treated membranes as the extent of co-solubilisation of PPH was not determined.

Similarly it is not possible to accurately detemine the fold-purification of the DAG kinase activity on solubilisation. However, subsequent to the completion of the above initial survey of solubilisation conditions, DAG kinase activity was measured in the presence of 10 mM NaF. Under these conditions it was found that 55.3 - 87.1 % (mean 69.5 %, n =4) of the DAG kinase activity associated with the phospholipase-treated particulate fraction was solubilised on suspension in buffer B supplemented with 0.5 M KCl (0.578 M KCl final concentration).

Suspension of the phospholipase C-treated particulate fraction in buffer B supplemented with 0.5 M KCl was selected as the preferred method of solubilising membrane-associated DAG kinase. As this investigation of detergent and KCl solubilisation was performed in the absence of NaF the results cannot be considered to be more than a qualitative indication of the extent of enzyme solubilisation by these treatments. It can only be concluded that 0.5 M KCl apparently solubilised the DAG kinase with greater selectivity than the detergents under these conditions. Subsequent to the completion of this work the yield of DAG kinase solubilised from the phospholipase C-treated membranes was determined in the presence of 10 mM NaF, a PPH inhibitor. Under these conditions it was found incubation of the phospholipase C-treated membranes with 0.5 M KCl in buffer B results in 69.5 % of the membrane-associated DAG kinase being solubilised (data not shown). Therefore, KCl at a final concentration of 578 mM was chosen as the method for the solubilisation of the membrane-associated DAG kinase. The use of KCl also had the advantage of avoiding any possible interference with the mixed-micellar assay which may have arisen with a detergent-solubilised enzyme preparation. Moreover, the elevated levels of KCl would be more easily removed from the enzyme preparation than detergent, should the need arise.

The use of hypertonic concentrations of salt is a common approach to the solubilisation of membane-associated DAG kinase. Besterman *et al.* (1986b) employed 1.0 M KCl to solubilise DAG kinase from the enriched particulate fraction prepared from phospholipase C-treated rat brain homogenate. Kato and

Takenawa (1990) solubilised a membrane-associated DAG kinase from rat brain by incubation with 2.0 M NaCl. Coco-Maroney and Macara (1989) reported that 0.5 M NaCl removed 70 % of the rat brain cytosolic DAG kinase activity that had previously been reconstituted with phospholipase C-treated human red blood cell membranes. Additionally, these workers also reported that 78 % of the DAG kinase activity associated with rat brain membranes could be extracted by a single 0.5 M NaCl wash (Coco-Maroney & Macara, 1989). Therefore, it was with some confidence that solubilisation of the membrane-associated DAG kinase by incubation with 578 mM KCl was adopted for continued use in the purification procedure.

For the purposes of this thesis, the protein solubilised from the DAG kinase-enriched particulate fraction by incubation with 578 mM KCl is defined as the 'KCl-extracted protein'. Apropos of this, the DAG kinase activity within this protein preparation is defined as the 'KCl-extracted DAG kinase'.

3.2.2. Purification of the KCl-Extracted DAG Kinase of Rat Brain: Overall Strategy.

As discussed in Section 3.1, the ability to solubilise a considerable proportion of the DAG kinase activity associated with the phospholipase C-treated particulate fraction is in agreement with the work of Besterman *et al.* (1986b). In addition to the solubilisation of membrane-associated DAG kinase by incubation of the phospholipase C-treated membranes with 1.0 M KCl they also reported the ability to further purify the solubilised enzyme by affinity chromatography on a column of immobilised *sn*-1,2-diacylglycerol and phosphatidylserine (Besterman *et al.*, 1986b). This procedure was reported to yield a protein preparation of DAG kinase which was purified 500- to 750-fold relative to the enzyme in the tissue homogenate and possessing only 4 - 5 proteins on analysis by SDS-PAGE. However, this procedure was not adopted in the work towards this thesis. Despite an obvious application of such affinity chromatography to the purification of DAG

kinases, workers within this laboratory have been unable to reproduce these results. It was found that DAG kinase prepared from rat brain did not bind a polyacrylamide-immobilised column of *sn*-1,2-diacylglycerol/PtdSer which had been prepared by the method described in Besterman *et al.* (1986b) [S. Palmer & M.J.O. Wakelam, personal communication]. It was considered more prudent to progress with more conventional chromatographic methods, e.g. ion-exchange chromatography, gel filtration and ATP-agarose chromatography. This may possibly avoid the stability problems experienced with the DAG kinase preparation recovered from the acrylamide-immobilised *sn*-1,2-diacylglycerol/PtdSer column (Besterman *et al.*, 1986b). This strongly suggests that it would be preferable to at least partially purify the KCl-extracted enzyme by more conventional chromatographic methods before introduction of the *sn*-1,2-diacylgycerol affinity column, should this affinity column become applicable in our hands. Additionally, characterisation of a purification strategy using more conventional methods may be succesful in the purification of the KCl-extracted DAG kinase to homogenity without recourse to chromatography on immobilised *sn*-1,2-diacylglycerol/PtdSer. Since the initiation of this project Kato and Takenawa (1990) reported the purification of a membrane-associated DAG kinase from rat brain to homogeneity by a strategy which did not employ polyacrylamide-immobilised *sn*-1,2-diacylglycerol/PtdSer. Rather, these workers purified the 2.0 M NaCl-solubilised enzyme by chromatography on Q-Sepharose (anion-exchange), phenyl-Superose, hydroxyapatite, and ATP-agarose.

3.3. A Proportion of the KCl-Extracted DAG Kinase Activity is Insoluble in The Absence of KCl.

Initially, the use of anion-exchange chromatography was considered for purification of the KCl-extracted DAG kinase. In preparation for this, the activity of the KCl-extracted DAG kinase was not found to alter significantly on exposure to pH ranging from 4.5 to 9.0 (data not shown). The pH of the KCl-extracted

protein preparation was altered to various pH values ranging from 4.5 to 9.0, maintained at this for 1 h and returned to the assay pH of 6.6 without significant alteration of the DAG kinase activity (data not shown). Therefore, investigation of the appropriate pH for ion-exchange chromatography can proceed as the KCl-extracted DAG kinase has proven stable to incubation for 1 h in buffer of pH ranging from 4.5 to 9.0 without detectable loss of activty.

In preparation for ion-exchange chromatography the KCl-extracted protein preparation was dialysed against buffer of reduced KCl concentration. Extensive dialysis of the KCl-extracted protein preparation against buffer containing 1.0 mM KCl (buffer D) resulted in considerable loss of DAG kinase activity from solution. Measurement of the DAG kinase activity remaining soluble after centrifugation (20,000 xg, , 30 min, 4°C) of the dialysate revealed that only 13.4 ± 7.0 % of the KCl-extracted enzyme remained in solution. The possibility that a proportion of the KCl-extracted DAG kinase had precipitated during dialysis was indicated by the recovery of 38.1 ± 16.2 % of the KCl-extracted activity in the insoluble material collected by centrifugation of the dialysate. Although this did not represent a quantitative recovery of the DAG kinase activity lost from solution, it did indicate that a significant proportion of the KCl-extracted enzyme was rendered insoluble by reduction of the KCl concentration from 578 to 1.0 mM. It is felt likely that greater than 38.1 % of the KCl-extracted DAG kinase is present within the soluble material but cannot be accurately quantitated when present within a resuspended precipitate. Nevertheless, even in the absence of further information on the accuracy of determining DAG kinase activity within this insoluble material it is still clear that a significant proportion of the KCl-extracted DAG kinase is rendered insoluble by reduction of the KCl concentration.

3.4. KCl Maintains the KCl-Extracted DAG Kinase in Solution: KCl Concentration Dependence.

The KCl-extracted protein preparation was dialysed against a range of KCl concentrations (1.0 mM - 0.6 M) and the DAG kinase activity remaining in solution measured. This range was selected as it would mimic the fall in KCl concentration experienced by the KCl-extracted protein in preparation for ion-exchange chromatography. Dialysis against 0.6 - 0.3 M KCl resulted in no KCl-dependent loss of DAG kinase activity from solution, although a KCl-independent loss was observed (Fig 3.2). Only 54.9 - 70.0 % of the KCl-extracted activity was recovered over the range 0.6 - 0.3 M KCl. Reduction of the KCl concentration below 0.3 M did, however, result in further loss of DAG kinase activity from solution in a KCl-dependent manner. Recovery of the KCl-extracted DAG kinase activity from dialysis decreased from 60.4 % at 0.3 M to 11.4 % at 1.0 mM KCl. Associated with this loss of activity from solution is an increase in the DAG kinase activity which can be measured in the insoluble material which forms on dialysis (Fig 3.2). It is therefore apparent that the KCl-extracted DAG kinase requires at least 300 mM KCl for continued solubility.

The nature of the KCl-independent loss of KCl-extracted DAG kinase activity during dialysis is unknown. This loss could possibly be due to the presence of proteolytic activity in the KCl-extracted protein preparation. Alternatively, this could indicate that a proportion of the KCl-extracted enzyme is insoluble on prolonged incubation in aqueous solution and cannot be maintained in solution by 0.3 - 0.6 M KCl. Indeed, DAG kinase activity is associated with the insoluble material recovered from dialysis of the KCl-extracted protein preparation against concentrations of KCl which maintain the KCl-dependent activity in solution, i.e. 0.3 M or greater (Fig 3.2). This insoluble activity does not represent a quantitative recovery of the enzyme activity lost from solution in a KCl-independent manner during dialysis. However, as discussed in Section 3.3, the DAG kinase activity present in the insoluble fraction may be underestimated. This possibility was not investigated further.

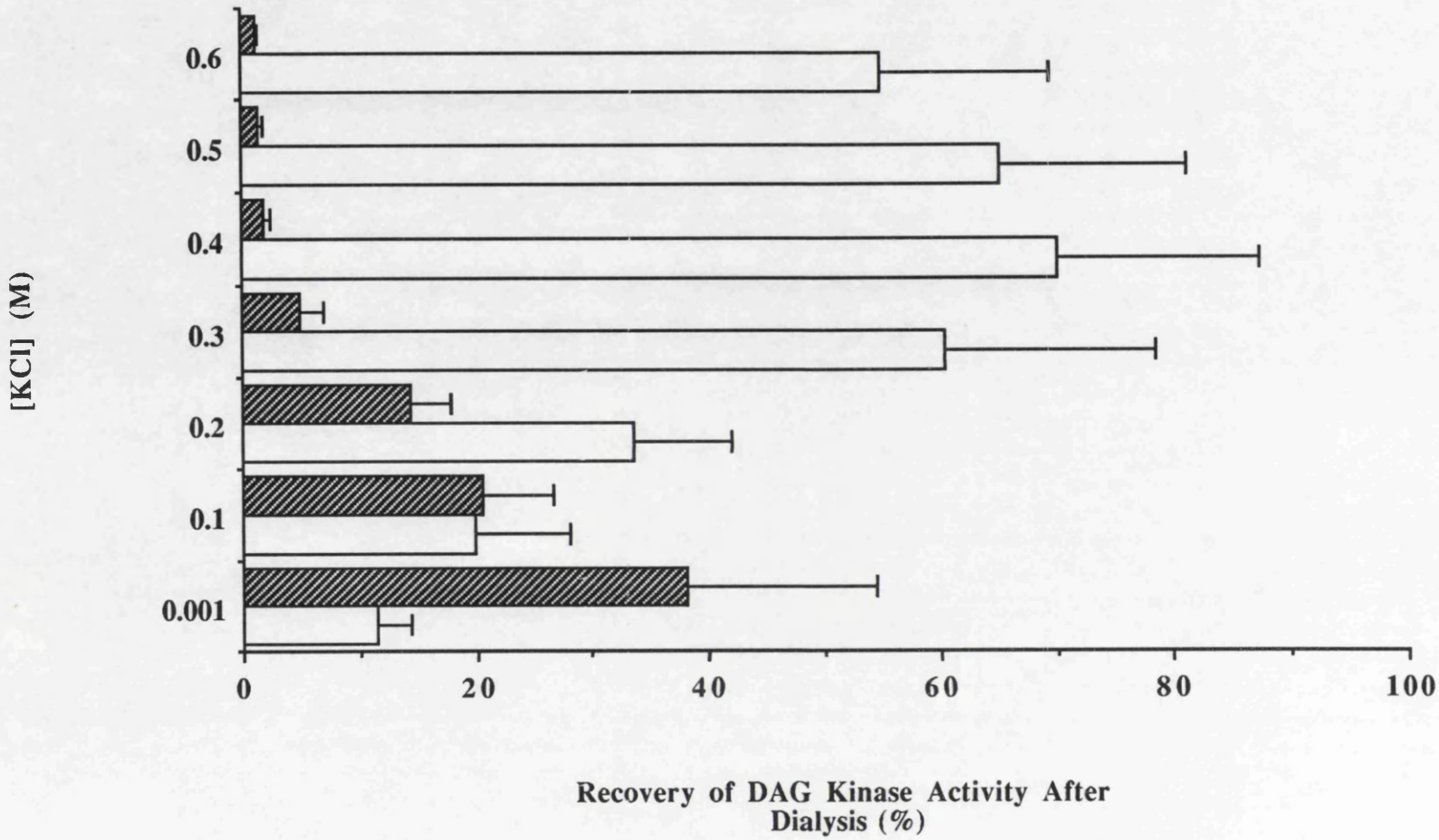

[KCl] (M)
0.6
0.5
0.4
0.3
0.2
0.1
0.001
0
20
40
60
80
100
Recovery of DAG Kinase Activity After Dialysis (%)

Fig 3.2. KCl Concentration Dependence of the KCl-Extracted DAG Kinase's Solubility.

KCl-extracted DAG kinase was prepared and centrifuged as described in Fig 3.6. 2.0 ml aliquots of the supernatant (2.0 mg protein) were dialysed against a range of KCl concentrations in dialysis buffer, as indicated. Dialysis was performed overnight against 2 x 500 ml volumes of buffer D supplemented with KCl as appropriate. The dialysates were clarified by centrifugation (100,000 xg, 1 h, 4°C). The supernatants were collected and the precipitates resuspended in 1.0 ml of dialysis buffer. DAG kinase activity and protein concentration were determined as described in Fig 3.1. DAG kinase activities are quoted as a percentage of the DAG kinase activity extracted from phospholipase C-treated membranes. This result is similar to an identical experiment. [*open bars*, soluble enzyme; *hatched bars*, insoluble enzyme]

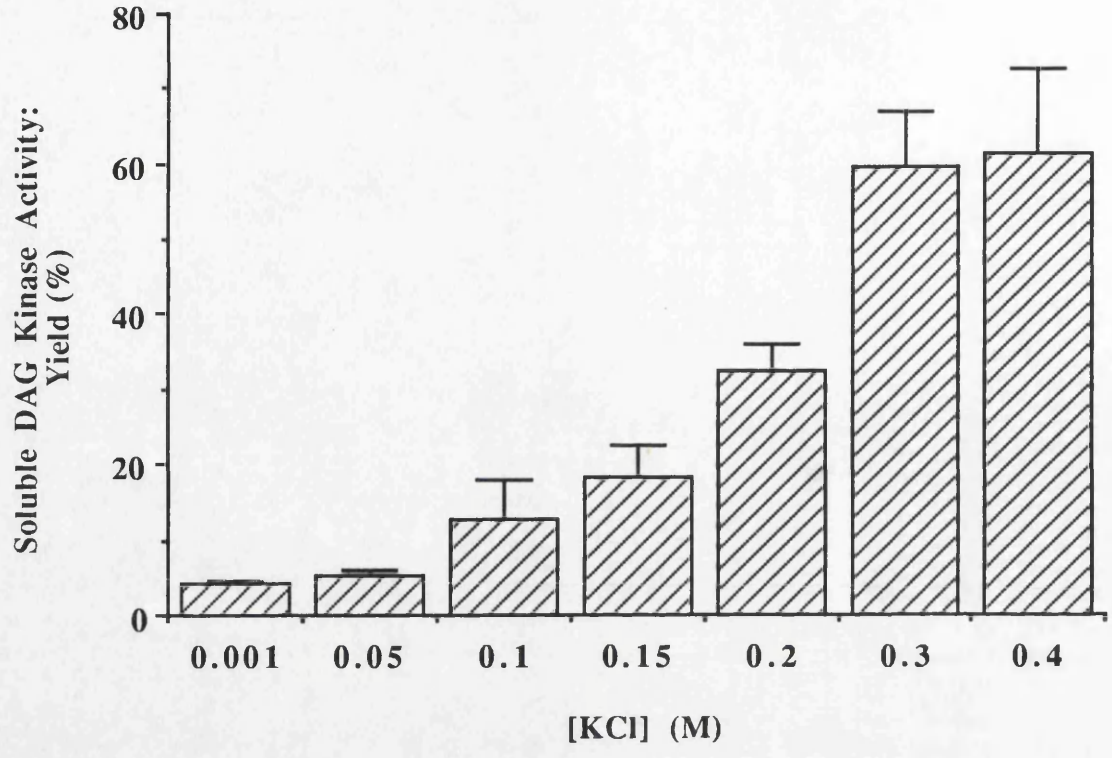
The non-ionic detergents Triton X-100, Nonidet P40 and Lubrol PX at final concentrations of both 0.1 and 0.01 % (w/v) did not resolubilise the KCl-extracted DAG kinase which precipitated on reductiuon of the KCl concentration below 300 mM (data not shown). This was also found to be the case for the zwitterionic detergent Zwittergent 3-14 at 0.1% (w/v) and the anionic detergent sodium deoxycholate at final concentrations of 0.5, 0.2 and 0.02 % (w/v) (data not shown). At these concentration none of the above detergents significantly increased the DAG kinase activity in solution nor apparently decreased the enzyme present in the insoluble material on reduction of the KCl concentration below 300 mM. This range of detergents under similar conditions was also unable to prevent the KCl-soluble enzyme from precipitating on reduction of the KCl concentration below 300 mM by dialysis (data not shown). Therefore, in conclusion, the membrane-associated DAG kinase solubilised from phospholipase C-treated rat brain membranes by incubation with 578 mM KCl must be maintained in the presence of 0.3 M or greater KCl for continued solubility of the enzyme and this requirement for 300 mM KCl cannot be replaced by detergent.

3.5. Purification of the KCl-Extracted DAG Kinase by Precipitation.

The ability to precipitate the KCl-extracted DAG kinase in an active state identified the possibility of exploiting the enzyme's solubility in increasing concentrations of KCl as a purification method. The enzyme could be precipitated by dialysis against KCl-free buffer and then selectively resolubilised from the insoluble material by increasing concentrations of KCl.

Extensive dialysis against 1.0 mM KCl (buffer D) resulted in the loss of 85.0 - 94.1 % of the KCl-extracted DAG kinase activity from solution (six determinations). Resuspending the insoluble material which formed in buffer of increasing concentrations of KCl resulted in a KCl-dependent increase in the soluble DAG kinase activity which was recovered (Fig 3.3A). Incubation of the insoluble material in 1.0 - 300 mM KCl resulted in an increase in the amount of

A


Soluble DAG Kinase Activity:
Yield (%)
80
60
40
20
0
0.001
0.05
0.1
0.15
0.2
0.3
0.4
[KCl] (M)

B

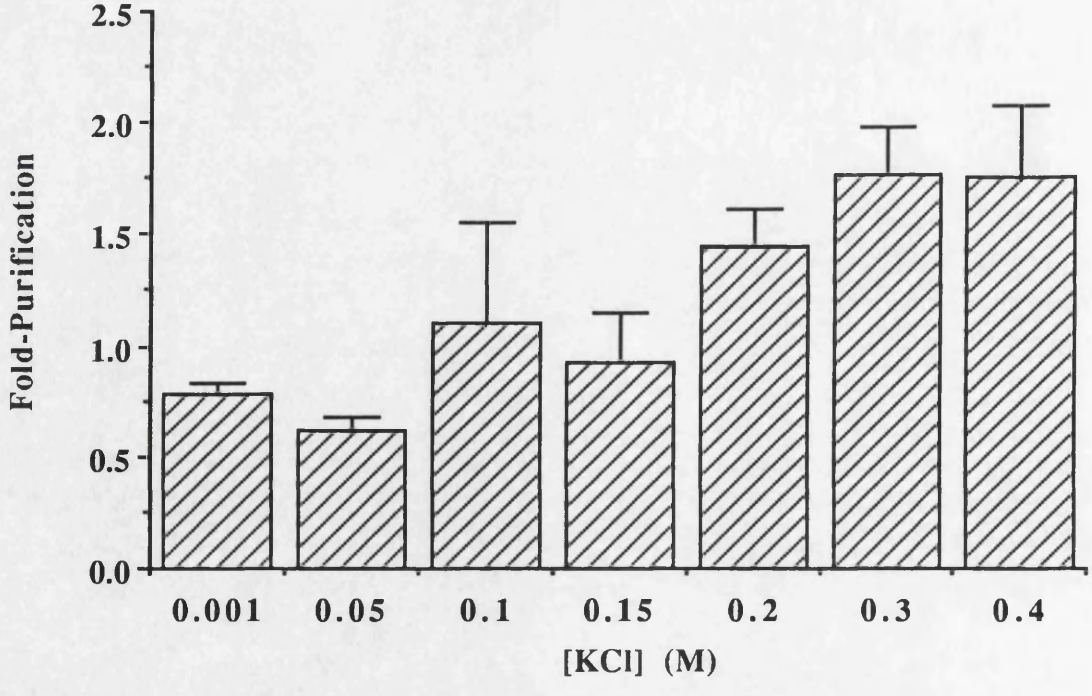

Fold-Purification
2.5
2.0
1.5
1.0
0.5
0.0
0.001
0.05
0.1
0.15
0.2
0.3
0.4
[KCl] (M)

Fig 3.3. Resolubilisation of Precipitated DAG Kinase: KCl Concentration Dependence.

A. Recovery of DAG kinase Activity From Insoluble Material Formed on Dialysis.

B. Fold-Purification of DAG kinase Activity Resolubilised From Hypotonic Precipitate.

KCl-extracted DAG kinase was prepared and centrifuged as described in Fig. 3.6. The KCl-extracted protein (14.4 mg, 41 ml) was dialysed against 2 x 5 l volumes of buffer D and the dialysate clarified by centrifugation (100,000 xg, 1 h, 4°C). The precipitate was resuspended in 9.0 ml of buffer D. 1.0 ml aliquots of the suspension were corrected to 5.0 ml KCl in buffer D to form a concentration range 1.0 mM - 0.4 M, as indicated. The insoluble material pelleted by centrifugation (100,000, 1 h, 4°C) after the suspensions had been incubated on ice for 1 h with frequent mixing. The supernatant was collected and the pellet resuspended in 1.0 ml of buffer D supplemented with KCl, as appropriate. DAG kinase activity was determined as described in Fig 3.1 and protein concentration measured by the Coomassie dye method (Section 2.2.3). Fold-purification was calculated as the increase in DAG kinase specific activity relative to the KCl-extracted protein preparation.

DAG kinase resolubilised. The activity resolubilised increased from 4.3 ± 0.2 % to 59.6 ± 6.7 % (relative to the KCl-extracted enzyme preparation) as the KCl concentration increased from 1.0 to 300 mM (Fig 3.3A). Incubation of the precipitated protein with greater than 300 mM KCl resulted in no further significant increase in DAG kinase activity recovered from the insoluble material (data not shown).

Purification of the KCl-extracted DAG kinase by this precipitation/resolubilisation procedure required resuspension of the precipitated protein in buffer containing 200 mM or greater KCl (Fig 3.3B). Incubation of the precipitated protein in solutions with lower than 200 mM KCl produced a resolubilised enzyme preparation with lower specific activity than the KCl-extracted protein. Thus, resolubilisation of the precipitated enzyme in buffer containing less than 200 mM KCl does not result in a net purification of the KCl-extracted DAG kinase (Fig 3.3B). However, elevation of the KCl concentration to 200 mM or greater did result in purification of the KCl-extracted enzyme (Fig 3.3B). The purification was greatest at 300 mM. Further increasing the KCl concentration from 300 mM to 600 mM did not result in any further increase in purification of the KCl-extracted activity (data not shown). In summary, dialysing the KCl-extracted protein preparation against 1.0 mM and resuspending the insoluble material which formed in 300 mM KCl resulted in a 1.76- to 2.54-fold purification of DAG kinase. The yield of enzyme activity recovered from this procedure was equivalent to between 48.5 and 59.6 % of that solubilised from the phospholipase C-treated membranes (n = 3).

It should be noted that 5.9 - 15.0 % of the KCl-extracted DAG kinase activity could not be removed from solution by extensive dialysis against 1.0 mM KCl. It would appear that the KCl-extracted enzyme preparation also contains a DAG kinase activity which remains soluble in the absence of 300 mM KCl, i.e. a KCl-independent DAG kinase. Exploitation of their different solubilities in reduced concentrations of KCl allows separation of the KCl-independent DAG kinase from

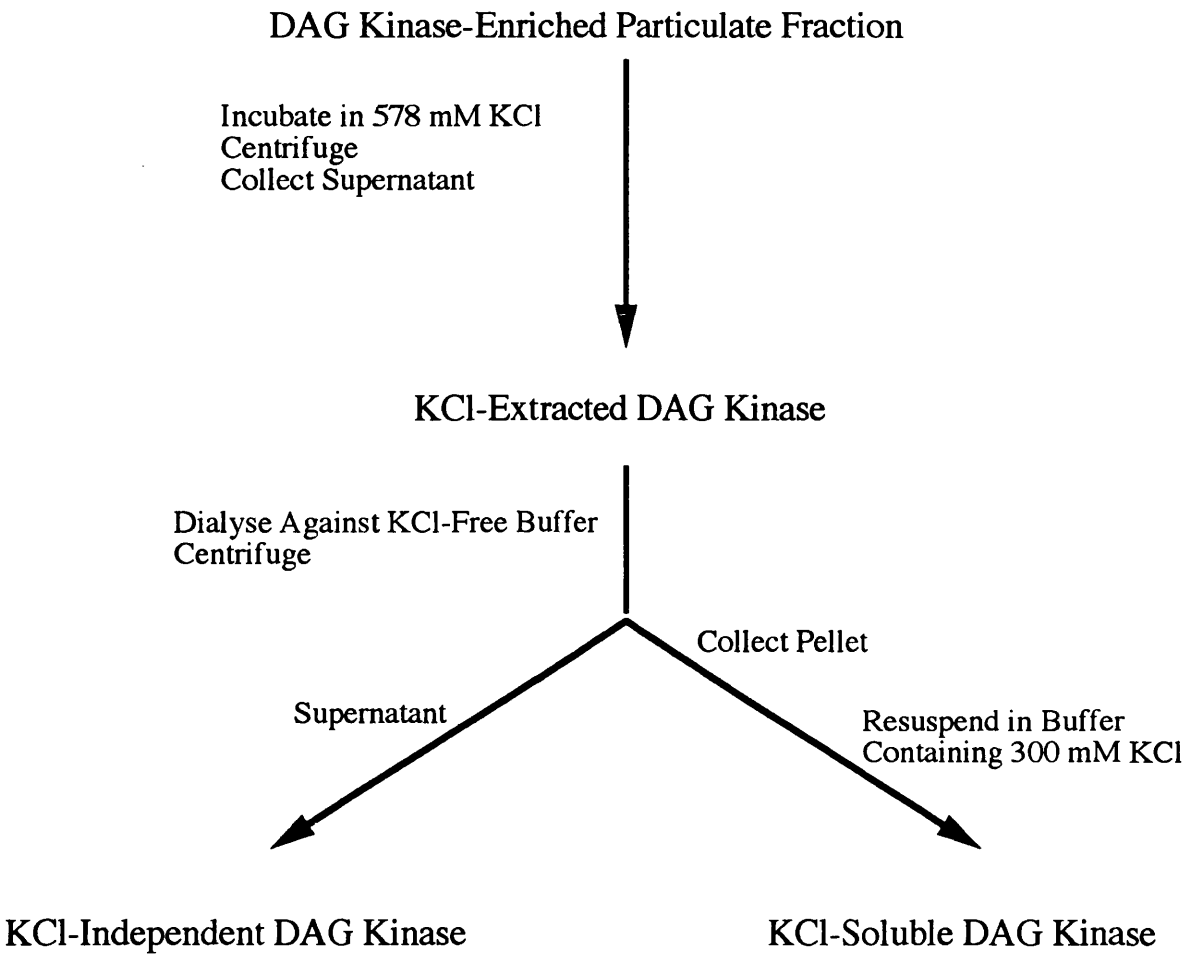


Fig 3.4. Separation of the KCl-Soluble and KCl-Independent DAG Kinases of Rat Brain Membranes: A Schematic Diagram.

the enzyme that does require KCl for solubility, i.e. the KCl-soluble DAG kinase. Therefore, in addition to providing a modest purification of the KCl-extracted DAG kinase the precipitation/resolubilisation procedure also allows fractionation of the KCl-extracted DAG kinase activity into KCl-independent and KCl-soluble enzymes.

3.6. KCl Maintains the KCl-Extracted DAG Kinase in Solution: Discussion.

It is clear that incubation of a phospholipase C-treated particulate fraction prepared from rat brain in buffer containing 578 mM KCl affects solubilisation of a considerable proportion of the membrane-associated DAG kinase. It is now also apparent that the solubilised DAG kinase activity is composed of two components; 1) a DAG kinase activity which remains freely soluble in the absence of greater than 1.0 mM KCl and 2) a DAG kinase activity which requires greater than 1.0 mM KCl to remain soluble with its KCl-dependent solubility being maximal in the presence of 300 mM or greater KCl. For the purposes of this thesis these enzymes have been refered to as the KCl-independent and the KCl-soluble DAG kinases respectively. It has also been possible to exploit these differential solubilities in solutions of reduced KCl concentration to separate the KCl-independent DAG kinase from the KCl-soluble enzyme (Fig 3.4). This has led to the development of a precipitation/resolubilisation procedure which apart from fractionating these enzymes also allows a modest purification of the KCl-soluble DAG kinase (Section 3.3).

The observation of a requirement for the continued presence of KCl (or salt in general) to maintain a DAG kinase in solution is novel and unique to the work reported in this thesis. Subsequent to preparing salt-solubilised fractions from rat brain, both Besterman *et al.* (1986b) and Kato and Takenawa (1990) purified membrane-associated DAG kinase by strategies which include dialysis against salt-free buffer. In both these reports the solubility of the membrane-associated DAG

kinases does not require the presence of greater than 1.0 mM concentrations of salt for continued solubility. Therefore, it can be concluded that both these reports are concerned with the KCl-independent DAG kinase and not the KCl-soluble enzyme which is described in Sections 3.3 & 3.4.

It may be possible that other workers have experienced a loss of a KCl-soluble DAG kinase. Prior to chromatography of the membrane-associated enzyme on AffiGel Blue, Kato and Takenawa (1990) determined the yield of DAG kinase activity solubilised from rat brain membranes by incubation with 2.0 M NaCl as only 39.3 %. Yet Coco Maroney and Macara (1989) reported that 78 % of the membrane-asssociated DAG kinase was solubilised from rat brain membranes by incubation with 0.5 M NaCl. This could indicate that Kato & Takenawa (1990) experienced a substantial loss of NaCl-extracted DAG kinase during dialysis against NaCl-free buffer in preparation for AffiGel blue chromatography? This would be analagous to the loss of KCl-extracted DAG kinase activity on dialysis against buffer containing 1 mM KCl (Section 3.1 & 3.3) as only 5.9 - 15 % of the solubilised enzyme remains in solution on dialysis, yet 55.3 - 87.1% of the membrane-associated enzyme is solubilised on incubation with 578 mM KCl. Regardless of this possibility, the data in Sections 3.3. & 3.4 is the first report of a membrane-associated DAG kinase from rat brain which retains a requirement for at least 300 mM KCl for continued solubility.

Further purification of the rat brain membrane-associated DAG kinase concentrated on the KCl-soluble enzyme alone. Characterisation of its KCl dependence for continued solubility allowed the development of a KCl dependent precipitation/resolubilisation purification procedure. Furthermore, the use of this precipitation step in the purification of DAG kinase was adopted in view of both its relative ease of use and also the defined environment of the resulting DAG kinase preparation. The KCl-soluble DAG kinase is thus present in 300mM KCl, the lowest KCl concentration that reproducibly conferred full solubility to the enzyme.

This removed the need for further desalting of the preparation prior to chromatography.

3.7. AffiGel Blue Chromatography.

AffiGel Blue, a dye-ligand matrix, belongs to the family of immobilised triazine dyes that have been effectively employed in the purification of lipoproteins, serum albumin and nucleotide-requiring enzymes (for review see Lowe and Pearson, 1984). With a possible application to the purification of DAG kinases as a whole, the chromatographic behavior of the KCl-soluble DAG kinase on AffiGel Blue was investigated.

KCl-soluble DAG kinase activity was found to reproducibly bind AffiGel Blue in the presence of the 300 mM KCl at pH 6.5. Once bound, the DAG kinase activity could be effectively eluted by increasing the KCl concentration of the chromatography buffer. The KCl-soluble DAG kinase eluted throughout a 0.3 - 2.0 M KCl gradient with the activity peaking between 0.9 - 1.2 M (data not shown). The inability of the linear KCl gradient to resolve DAG kinase from the other proteins eluting from the column suggested no benefit in its use compared to a step gradient. Therefore, elution by a 0.3 to 1.0 M KCl step-gradient was selected for routine purification of the KCl-soluble DAG kinase (Fig 3.5). As the step gradient resulted in the elution of DAG kinase a a sharp peak of activity it facilitated the recovery of the enzyme in a far smaller volume than would be achieved by a linear gradient. Further increasing the KCl concentration of the chromatography buffer from 1.0 to 2.0 M resulted in no further elution of DAG kinase activity (Fig 3.5). Thus, the 0.3 to 1.0 M KCl step gradient would appear to have efficiently removed all the bound DAG kinase activity from the AffiGel Blue column. Using this approach, the KCl-soluble DAG kinase was purified 1.29- to 1.94-fold across the column with a 50.6 - 94.2 % yield of activity (from four independent preparations, see Table 1).

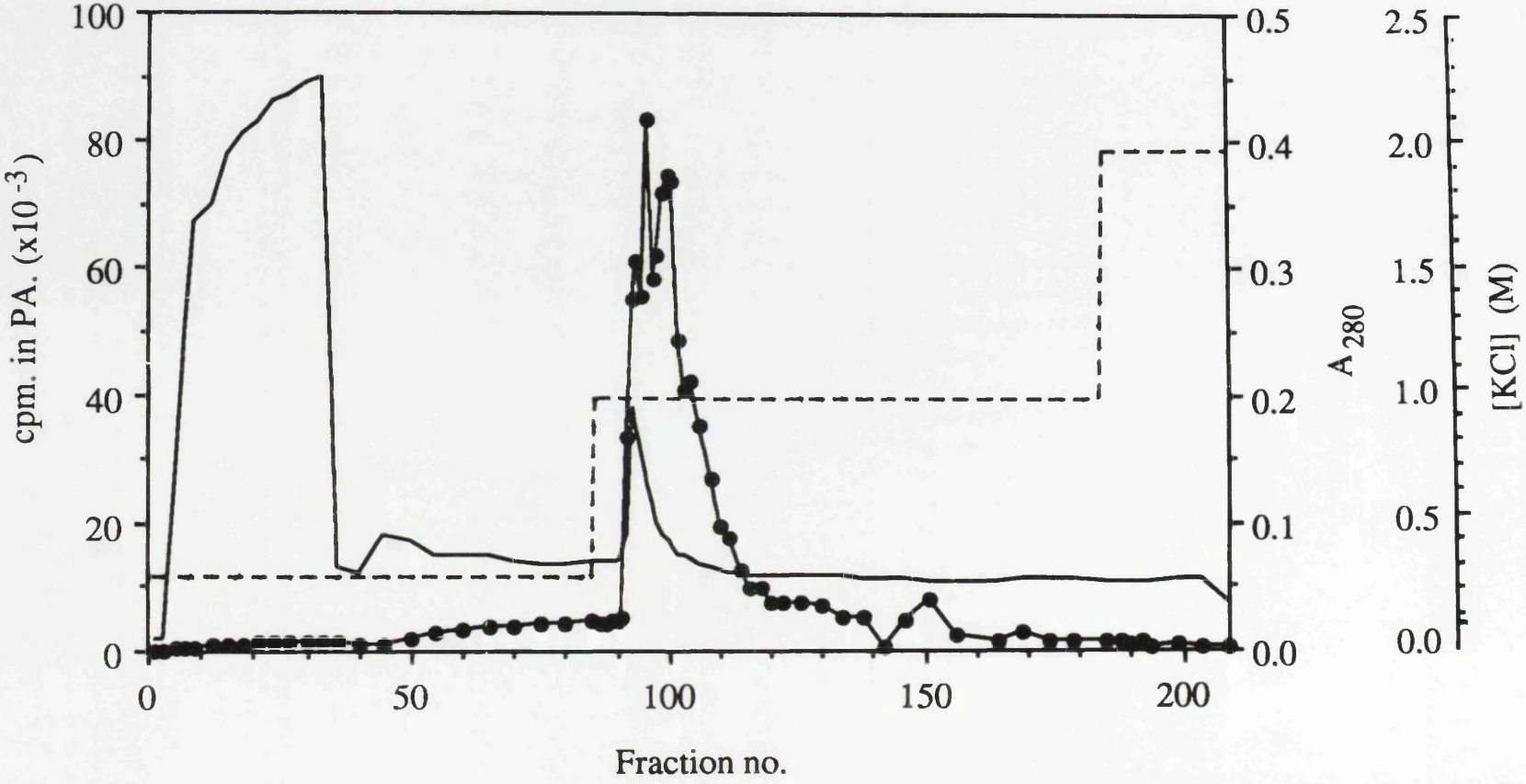

100
80
60
40
20
0
cpm. in PA. (x10 -3)
0
50
100
150
200
Fraction no.
0.5
0.4
0.3
0.2
0.1
0.0
A 280
2.5
2.0
1.5
1.0
0.5
0.0
[KCl] (M)

Fig 3.5. AffiGel Blue Chromatography of the KCl-Soluble DAG Kinase.

The KCl-soluble DAG kinase (79.0 mg protein, equivalent to 104 rats) was prepared as described in Section 2.6.1. The KCl-soluble protein preparation was applied to a 60 ml (2.6 x 11.3 cm) column of AffiGel Blue prepared and equilibrated in buffer F. The column was washed with buffer F to remove loosely bound proteins and developed with step gradients of 0.3 - 1.0 M KCl followed by 1.0 - 2.0 M KCl in buffer F (- - -). 6.4 ml fractions were collected and assayed for DAG kinase activity (-●-) as described in Section 2.4.2. The protein concentration of the column eluant was measured by absorption at 280 nm (*solid line*). Fractions containing DAG kinase activity (fractions 92-151) were pooled. (N.B. the pH of buffer F was found to remain at 6.5 during chromatography under the above conditions)

Despite the reported use of immobilised triazine dyes in the purification of many nucleotide-requiring enzymes (see Stellwagon *et al.*, 1990), purification of DAG kinases based on the exploitation of their requirement for ATP has mainly centred on the use of immobilised ATP in the form of ATP-agarose (Kanoh and Ohno, 1981; Kanoh *et al.*, 1983; Lin *et al.*, 1986; Sakane *et al.*, 1989; Kato & Takenawa, 1990; Schaap *et al.*, 1990; Yada *et al.*, 1990). Although Kato and Takenawa (1990) did employ AffiGel Blue in the initial fractionation of rat brain cytosolic protein during purification DAG kinase I (their notation) to homogeneity, they did not further exploit the nature of the interaction between the nucleotide-requiring enzyme and the dye-matrix. Elution of DAG kinase by washing the AffiGel Blue column with adenosine nucleotides, e.g. ATP, may possibly have resulted in greater fold-purification of the enzyme than was achieved by NaCl elution. Indeed, if the KCl-soluble DAG kinase binds AffiGel Blue via its nucleotide binding site, elution of the enzyme by ATP and other nucleotides could possibly result in increased purification of the enzyme across this column. To date, this possibility has not been investigated.

3.8. Hydrophobic Interaction Chromatography.

The need to include 300 mM KCl in all chromatography buffers prevented the use of ion-exchange at pH 6.5, whereas the binding of the KCl-extracted DAG kinases to hydrophobic interaction chromatography (HIC) media would be enhanced by its inclusion. Therefore, the feasibility of employing a HIC separation step in the purification protocol was investigated.

Phenyl-Sepharose CL-4B, octyl-Sepharose CL-4B, methyl-agarose, butyl-agarose and butyl-Sepharose 4B were screened for the ability to bind KCl-extracted DAG kinase activity in the presence of 1.0 M KCl at pH 6.5. Of these, the KCl-extracted activity was only observed to bind phenyl-Sepharose CL-4B and octyl-Sepharose CL-4B (data not shown). Reduction of the KCl concentration from 1.0 to 0.3 M did not elicit the elution of the KCl-extracted DAG kinases activity from

either of these matrices. Elution from either phenyl- or octyl-Sepharose CL-4B was found to require both reduction of the KCl concentration to 0.3 M and inclusion of 30 - 60 % (v/v) ethanediol in the chromatography buffer (data not shown).

The inclusion of 0.02 % (w/v) Triton X-100 in buffers employed during phenyl-Sepharose CL-4B chromatography resulted in an altered elution pattern of DAG kinase activity from the HIC matrix. Previously, DAG kinase was found to reproducibly elute from phenyl-Sepharose CL-4B with concentrations of between 30 and 50 % (v/v) ethanediol in the presence of 0.3 M KCl (data not shown). The incorporation of 0.02 % (v/v) Triton X-100 in the buffers employed during phenyl-Sepharose CL-4B chromatography resulted in the elution of DAG kinase from the HIC matrix on reduction of the KCl concentration from 1.0 to 0.3 M alone, without the inclusion of ethanediol. Therefore, an optimised procedure for the use of phenyl-Sepharose CL-4B in the presence of Triton X-100 was employed in the purification protocol. KCl-soluble DAG kinase was applied to the phenyl-Sepharose CL-4B in the presence of 1.0 M KCl, 0.02 % (w/v) Triton X-100 in buffer E and the DAG kinase was eluted in a single peak of activity by introducing ethanediol to the chromatography buffer at a final concentration of 40 % (v/v) whilst reducing the KCl concentration to 0.3 M (Fig 3.6). No further DAG kinase could be eluted from the phenyl-Sepharose CL-4B on raising the ethanediol concentration of the elution buffer to 50 % (v/v).

The fold-purification achieved by this optimised elution procedure was increased to some extent by the use of a butyl-Sepharose 4B pre-column in series with the the phenyl-Sepharose CL-4B (Fig 3.6). In the presence of 1.0 M KCl, the butyl-Sepharose 4B column bound up to 25 % of the protein present in the post-AffiGel blue enzyme preparation without binding any DAG kinase activity. On disconnection of the butyl-Sepharose 4B pre-column, DAG kinase could be eluted from the phenyl-Sepharose CL-4B column with higher specific activity than that observed without the use of the pre-column.

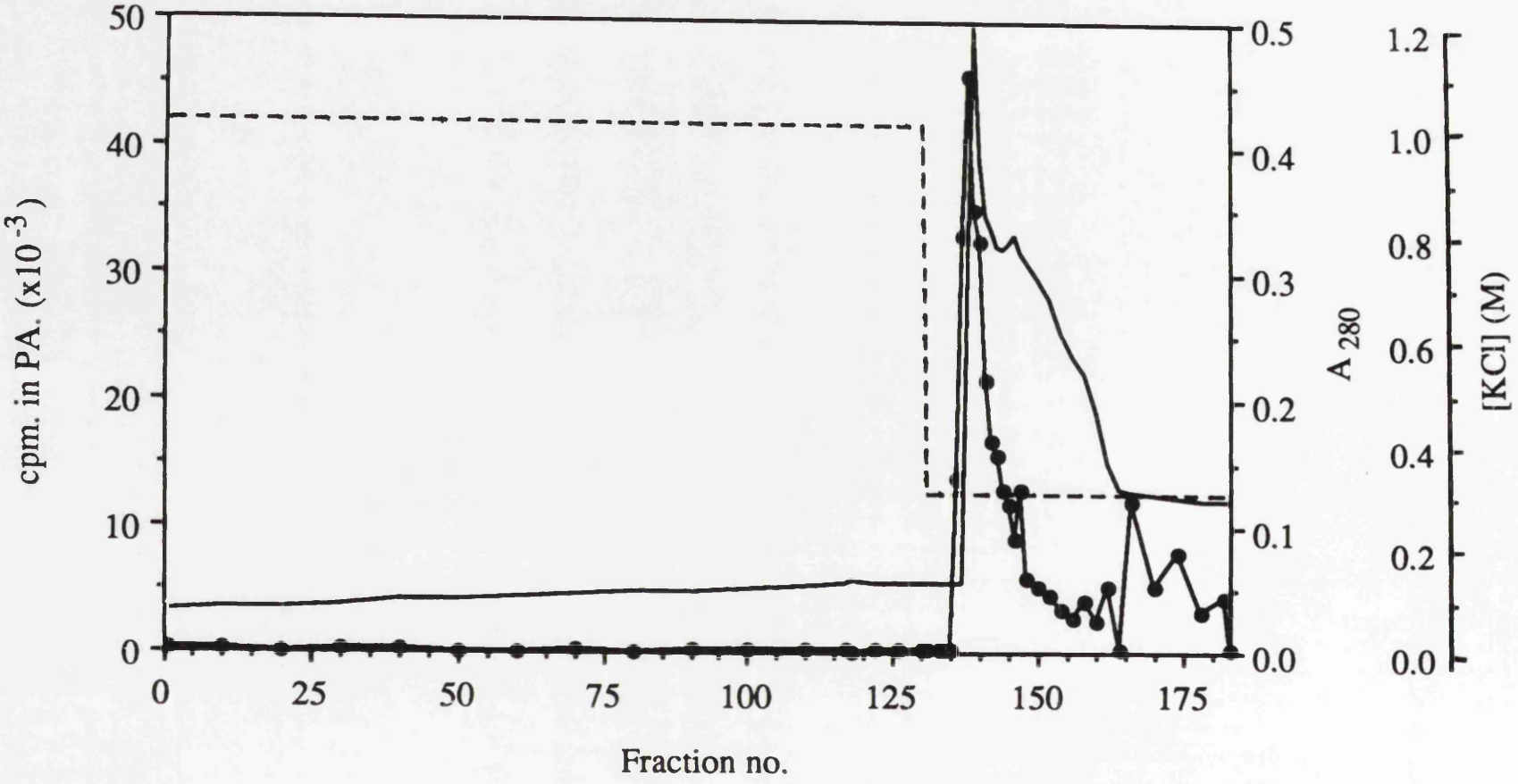

cpm. in PA. (x10^{-3})
50
40
30
20
10
0
0
25
50
75
100
125
150
175
Fraction no.
0.5
0.4
0.3
0.2
0.1
0.0
A_{280}
1.2
1.0
0.8
0.6
0.4
0.2
0.0
[KCl] (M)

Fig 3.6. Hydrophobic Interaction Chromatography of the KCl-Soluble DAG Kinase: Chromatography on a Butyl-Sepharose 4B/Phenyl-Sepharose CL-4B Column Combination Run in Series. A 7.5 ml (1.3 x 5.7 cm) column of butyl-Sepharose 4B was connected upstream of a 15 ml (1.6 x 7.5 cm) column of phenyl-Sepharose CL-4B, both columns had been prepared and equilibrated in buffer G as described in Section 2.6.2. The pooled DAG kinase preparation from AffiGel Blue chromatography (43.2 mg protein, 360 ml) was applied to the column combination and the columns washed with buffer G to remove loosely bound protein (fractions 117-131). The butyl-Sepharose 4B column was disconnected and the phenyl-Sepharose CL-4B developed with 20mM Tris/HCl pH 6.5, 300mM KCl, 10mM $MgCl_2$, 2mM EDTA, 2mM β-mercaptoethanol, 40% (v/v) ethanediol, 20% (v/v) glycerol, 0.2mM PMSF, 1μM E64, 1μM pepstatin A (N.B. The pH of this buffer was found to remain at 6.5 during chromatography). 4.0 ml fractions were collected and assayed for DAG kinase activity (-●-) by the deoxycholate/phosphatidylserine dispersion assay (Section 2.4.2). The protein concentration of the column eluant was monitored at 280 nm (*solid line*). Fractions containing DAG kinase activity (fractions 136-156) were pooled. [- - -, KCl concentration].

In summary, after characterisation of HIC, it proved extremely difficult to effect further significant purification of membrane-associated DAG kinase by this method. Recovery of DAG kinase after combined butyl-Sepharose 4B/phenyl-Sepharose CL-4B chromatography was measured to be only 29.6 % , with 0.7-fold purification of the enzyme over these columns. Therefore, at best, HIC chromatography could be employed in the purification protocol as a concentration step.

3.9. Gel Filtration Chromatography.

The ability to further purify the KCl-soluble DAG kinase by gel filtration chromatography of the post phenyl-Sepharose CL-4B preparation on Sephacryl S-300 SF was investigated.

The pooled post phenyl-Sepharose CL-4B DAG kinase preparation was concentrated by dialysis against polyethyleneglycol 6000 and subjected to gel filtration chromatography on a 1.6 x 90 cm column of Sephacryl S-300 SF (Fig 3.7). DAG kinase activity eluted from the Sephacryl S-300 SF column as a single peak of activity. The peak typically eluted in a volume of 94.0 to 97.0 ml of buffer (from four determinations). After determination of the column's v_o value from the elution of blue dextran 2000, the DAG kinase's elution volume was calculated to correspond to v_e/v_o values ranging between 1.41 and 1.46. Comparison of DAG kinase's elution volume to those of standard proteins of known molecular weight allowed calculation of the native molecular weight of DAG kinase (Fig 3.8). The native molecular weight of the KCl-soluble DAG kinase was calculated to be 160 ± 10 kDa (mean ± 1 SD, n = 4), with determined values ranging from 175 kDa to 150 kDa.

Sephacryl S-300 SF gel filtration chromatography typically resulted in 0.50- to 2.26-fold purification of the post AffiGel Blue preparation with only 13.2 to 38.5 % yield of activity (n = 4). However, SDS-PAGE analysis of the protein

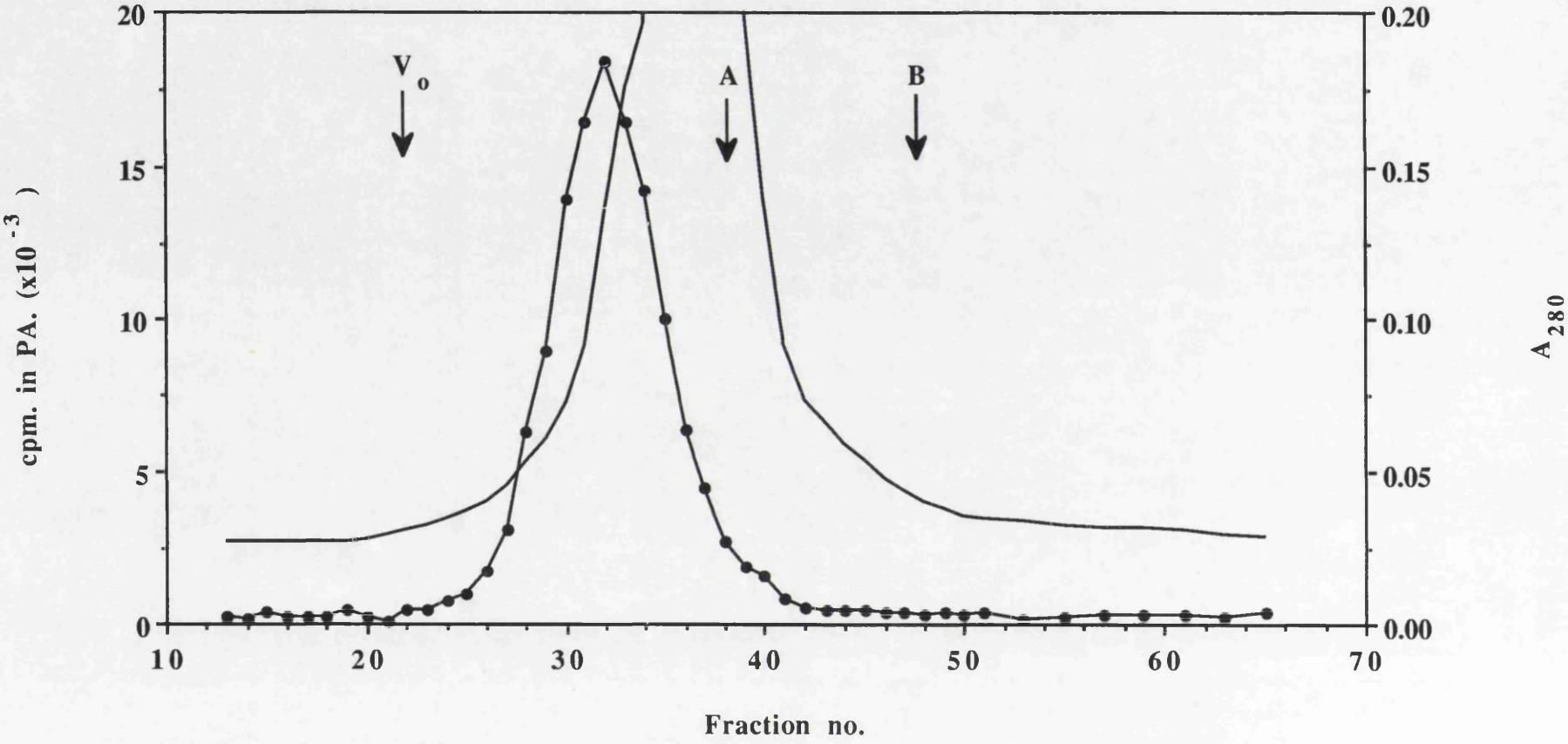

20
15
10
5
0
0.20
0.15
0.10
0.05
0.00
V_o
A
B
10
20
30
40
50
60
70
cpm. in PA. (x10^{-3})
A_{280}
Fraction no.

Fig 3.7. Sephacryl S-300 SF Gel Filtration Chromatography of the KCl-Soluble DAG Kinase.

The DAG kinase preparation (13.2 mg protein) pooled from hydrophobic interaction chromatography, as described in Fig 3.6, was concentrated to 8.0 ml by dialysis against 50 % (w/v) polyethylene glycol 6000 in buffer F. The dialysate was applied to a 180 ml (1.6 x 90 cm) column of Sephacryl S-300 SF equilibrated buffer H as described in Section 2.6.3. The column was developed with buffer H and the protein concentration of the eluant monitored at 280 nm (*solid line*). 3.0 ml fractions were collected and assayed for DAG kinase activity (-●-) by the deoxycholate/phosphatidylserine dispersion assay (Section 2.4.2). The fractions containing DAG kinase activity (fractions 26-39) were pooled.

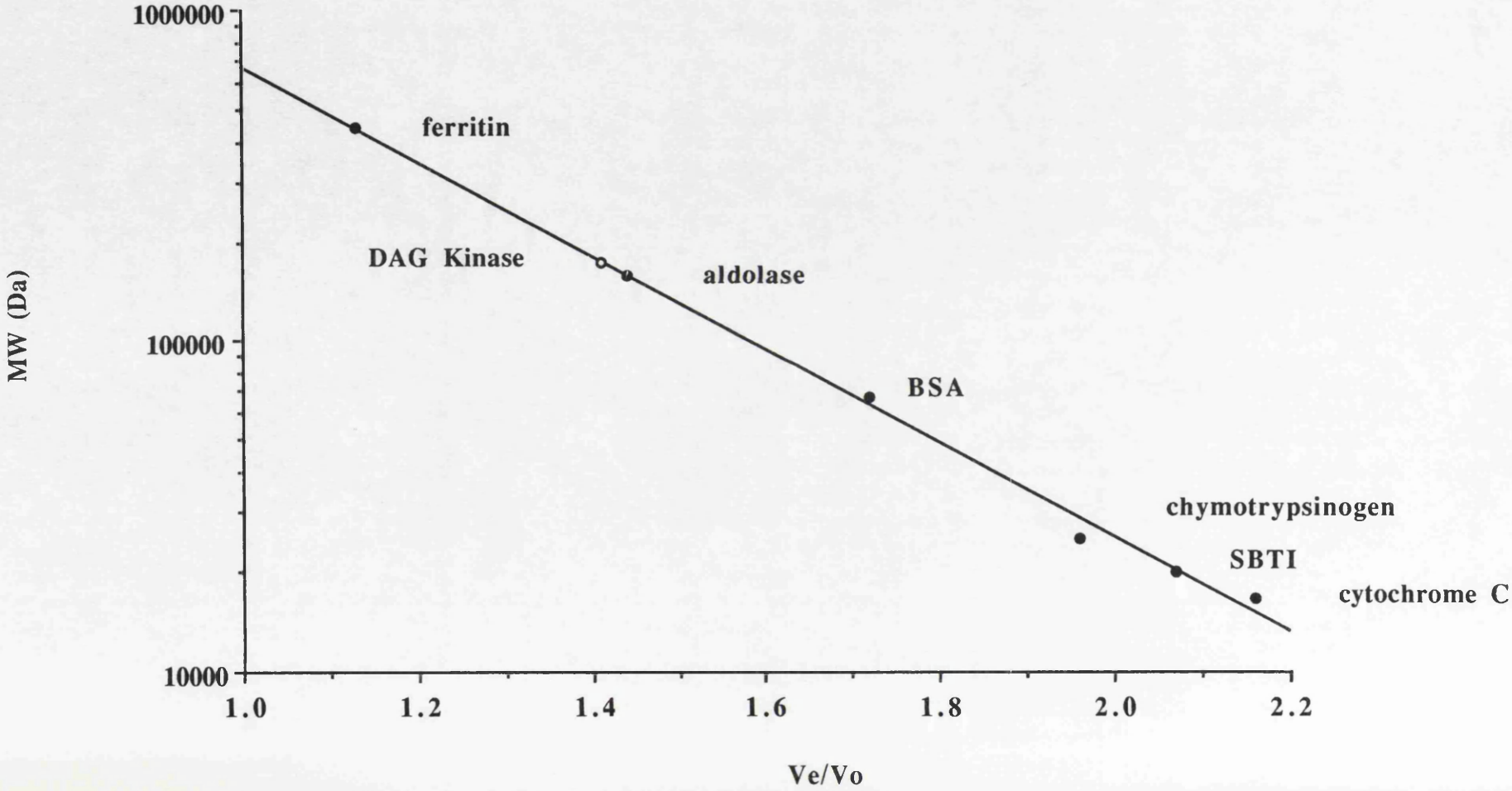

1000000
100000
10000
MW (Da)
ferritin
DAG Kinase
aldolase
BSA
chymotrypsinogen
SBTI
cytochrome C
1.0
1.2
1.4
1.6
1.8
2.0
2.2
Ve/Vo

Fig 3.8. Calibration Curve of the Sephacryl S-300 SF Gel Filtration Column.

The 180 ml (1.6 x 90 cm) column of Sephacryl S-300 SF employed in Fig 3.7 was equilibrated in buffer H. Protein standards (8 - 16 mg) or post HIC DAG kinase preparation were applied to the column in 8.0 ml and the column developed with buffer H at a flow rate of 8.0 ml/h. The void volume (v_o) of the column was determined from the elution of blue dextran 2000 monitored at 280 nm. The elution of ferritin (440 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), chymotrypsinogen (25 kDa), soybean trypsin inhibitor (20.1 kDa) and cytochrome (16.5 kDa) from the column were also monitored at 280nm and their elution volume (v_e) determined. The v_e of the KCl-soluble DAG kinase was determined for the elution of DAG kinase activity from the column (see Fig 3.7). The apparent molecular weight of the KCl-soluble DAG kinase was calculated from the calibration curve of logMW against v_e/v_o.

populations before and after Sephacryl S-300 SF chromatography revealed them to be quite different (Fig 3.11). Thus, the low fold-purifications across the gel filtration column may result from the enzyme's lability rather than the inability of Sephacryl S-300 SF to fractionate protein. Despite the poor yield and low level of purification across this column, it was retained in the purification protocol because of its additional ability to remove the ethanediol and Triton X-100 present in the post HIC DAG kinase preparation. DAG kinase activity had previously been observed to be inhibited by the presence of either Triton X-100 or ethanediol at concentrations greater than 0.01 % (v/v) and 5 % (v/v), respectively (data not shown). Therefore, it was advantageous that the KCl-soluble DAG kinase to be exchanged into ethanediol-free buffer of defined Triton X-100 concentration.

Fold-purification could be improved by reducing the volume of the post HIC DAG kinase preparation applied to the gel filtration column. In the present purification protocol the pooled protein preparation from the HIC step is loaded in a volume equivalent to 4.4 % of the column bed volume (8.0 ml). Further concentration of the DAG kinase preparation towards a volume equivalent to 1.0 % of the column bed volume could have increased the resolution of the individual proteins in the DAG kinase preparation. This was not undertaken due to concern about the recovery of protein and DAG kinase activity on concentration to volumes of less than 8.0 ml by dialysis against polyethyleneglycol 6000 (Section 2.8.2). The possibility of increasing the fold-purification across the Sephacryl S-300 SF column by further reducing the volume of protein applied to it is attractive. Examination of the protein preparation pooled from the Sephacryl S-300 SF column by SDS-PAGE reveals that it consists of polypeptides with widely variant molecular masses (ranging from 100 kDa to less than 29 kDa, see Fig 3.11). As the KCl-soluble DAG kinase elutes from Sephacryl S-300 SF with an apparent molecular mass of 160 kDa (see above) it would appear that increasing the resolution of the column by reduction of its load volume could increase the fold-purification across the column. However, examination of the post HIC and post

gel filtration DAG kinase preparations by SDS-PAGE reveals that the protein molecular mass distribution does not markedly alter on gel filtration, although the composition does. This confirms that size fractionation is occurring, although many smaller polypeptides elute with an apparent molecular mass of 160 kDa. Therefore, it is unlikely that reduction of the gel-filtration load volume will significantly increase the fold-purification achieved on Sephacryl S-300 SF.

The native molecular weight of the KCl-soluble DAG kinase was determined by gel filtration on Sephacryl S-300 SF to be 160 kDa (see above). This value is similar to the 150 kDa molecular weight reported for the membrane-associated DAG kinase previously purified from rat brain by Kato and Takenawa (1990). These workers estimated this value from SDS-PAGE analysis of the purified protein and did not determine a native molecular weight by gel filtration. However, as the purified protein resolved as a single band on SDS-PAGE it was possible to assume that the native molecular weight was 150 kDa, although, this does assume that the protein does not exist a multimeric complex consisting of similar subunits in the native state. This conclusion also assumes that the protein does not migrate anomalously with respect to molecular weight on SDS-PAGE. Despite the native molecular weight of the KCl-soluble DAG kinase being determined as 160 kDa, the subunit structure of the protein has still to be determined. Thus, the KCl-soluble DAG kinase may not resolve as a single polypeptide of molecular mass 160 kDa on SDS-PAGE. Additionally, within the error of the methods used, it cannot be concluded that the KCl-soluble DAG kinase is of identical molecular mass to the enzyme purified by Kato and Takenawa (1990).

High resolution gel filtration by HPLC could possibly be employed as a preparative step in the latter stages of the purification protocol where its increased resolution over low-pressure gel filtration may be beneficial. Preparations of the KCl-soluble DAG kinase from various stages of the purification protocol were subjected to gel-filtration chromatography on a TSK-G 3000SW (7.5 x 600 mm)

column using a Pharmacia HPLC apparatus. DAG kinase activity from all stages of the purification protocol proved to be highly labile. No measurable amount of activity eluted from the column, despite its correct functioning as assessed by elution of protein standards (data not shown). The reason for the lability of the KCl-soluble DAG kinase activity on this column compared its stability on Sephacryl S-300 SF is unknown and prevented further use of this method as either an analytical or preparative step.

3.10. Heparin-Agarose Chromatography.

The nature of interaction of proteins with heparin-agarose is still a matter of some contention. Chromatography on heparin-substituted agaroses consists of both an ion-exchange process between protein and the immobilised polyanion and also an undefined binding process that is independent of such a cation-exchange process. Nevertheless, immobilised heparin has been successfully employed in the purification of DNA-binding proteins, kinases, lipases and phospholipases. These include a receptor and G-protein regulated polyphosphoinositide-specific phospholipase C from turkey erythrocytes (Morris *et al.*, 1990), a phosphoinositide-specific phospholipase C from bovine liver membranes (Taylor *et al.*, 1990) and an inositol (1,3,4,5)-tetrakisphosphate 3-phosphatase from rat liver (Nogimori *et al.*, 1991). With reference to its application to DAG kinases, heparin-agarose has been employed in the purification of both the 150 kDa heat-stable DAG kinase of porcine thymus cytosol (Sakane *et al.*, 1989) and the cytosolic DAG kinases of human platelets (Yada *et al.*, 1990). Additionally, it has successfully resolved DAG kinase isoenzymes present in both rat brain and NIH 3T3 fibroblast cytosol (Strathopoulos *et al.*, 1990).

The DAG kinase activity present in the post gel filtration preparation was found to bind to heparin-agarose in the presence of 0.3 M KCl at pH 6.5 (Fig 3.10). The KCl-soluble DAG kinase was routinely eluted as a single peak of activity from heparin-agarose by a linear gradient of 0.3 -1.3 M KCl. This peak

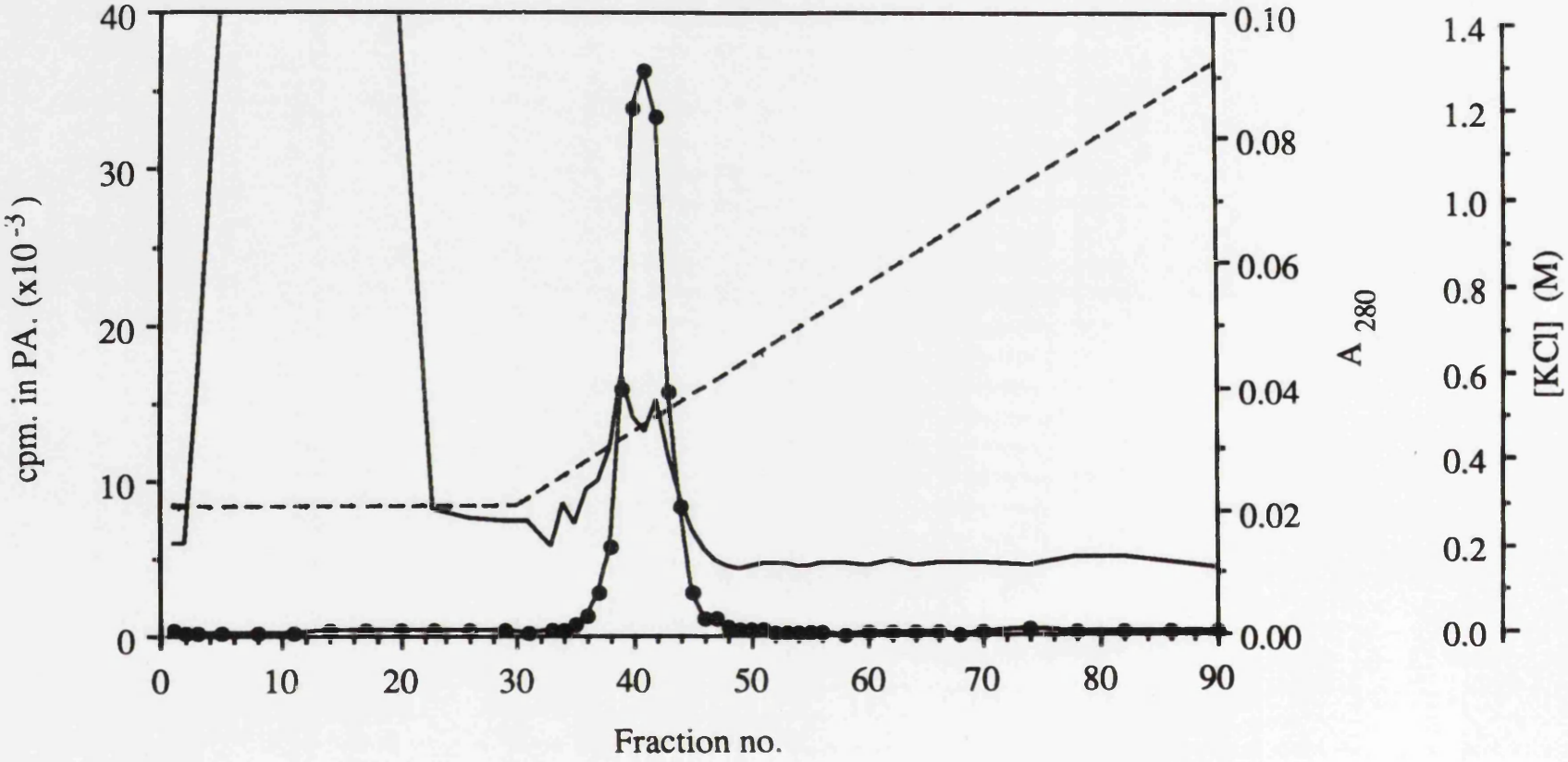

cpm. in PA. (x10^{-3})
40
30
20
10
0
0
10
20
30
40
50
60
70
80
90
Fraction no.
0.10
0.08
0.06
0.04
0.02
0.00
A 280
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
[KCl] (M)

Fig 3.9. Heparin-Agarose Chromatography of the KCl-Soluble DAG Kinase.

The DAG kinase preparation pooled from Sephacryl S-300 SF chromatography (12.4 mg protein, 48.0 ml) was applied to a 7.0 ml (1.0 x 8.9cm) column of heparin-agarose (type 1) prepared and equilibrated in buffer H. The column was washed with buffer H and developed with an 182 ml linear gradient of 0.3 - 1.3 M KCl prepared in the same buffer. The protein concentration and DAG kinase activity of the column eluant was determined as described in Fig 3.7. Fractions containing DAG kinase activity (fractions 36 - 46) were pooled.

(-●-, DAG kinase activity; - - -, KCl concentration; *solid line*, A_{280}).

was typically found to elute at a position in the gradient equivalent to 0.4 - 0.5 M KCl. Routinely, heparin-agarose chromatography of the post gel filtration enzyme preparation resulted in the 2.22- to 4.69-fold purification (n = 4). Recovery of the activity applied to the heparin-agarose column varied between 40.0 and 97.5 % (n = 4). Heparin-agarose was further employed in the purification of the KCl-soluble DAG kinase as its use had resulted in moderate fold-purification of the enzyme with reasonable recovery of activity.

Why the KCl-soluble DAG kinase should bind heparin-agarose in the presence of 0.3 M KCl at pH 6.5 is unknown. It is doubtful if the interaction is of simple ionic nature as the KCl-dependent DAG kinase does not bind the cation-exchange media SP-Sephadex C-50 under similar conditions (data not shown). Despite this, a complex ionic interaction may be occurring with the polyanionic heparin. Alternatively, the multiple sulphonyl moieties of the immobilised heparin molecule may allow the kinase to bind in a manner similar to its interaction with the polyanionic triphosphate moiety of ATP. If kinases interact with immobilised heparin via their nucleotide binding sites in a manner similar to affinity chromatography, the high degree of purification often observed with such proteins on heparin-agarose may be explained.

3.11. Hydroxyapatite Chromatography.

The possibility of further purification of the post heparin-agarose DAG kinase preparation by hydroxyapatite chromatography was assessed. Proteins adsorbed to a hydroxyapatite chromatography matrix can be specifically eluted on the basis of their isoelectric point. Gorbunoff (1990) summarised procedures that allow specific elution of basic, acidic and neutral proteins. Systematic disruption of the complex electrostatic interaction between the proteins' amino and carboxyl groups and the charged calcium and phosphate of the hydroxyapatite crystal surface facilitates separation of proteins based on their isoelectric point. Moreover, chromatography on hydroxyapatite has been successfully employed in several DAG

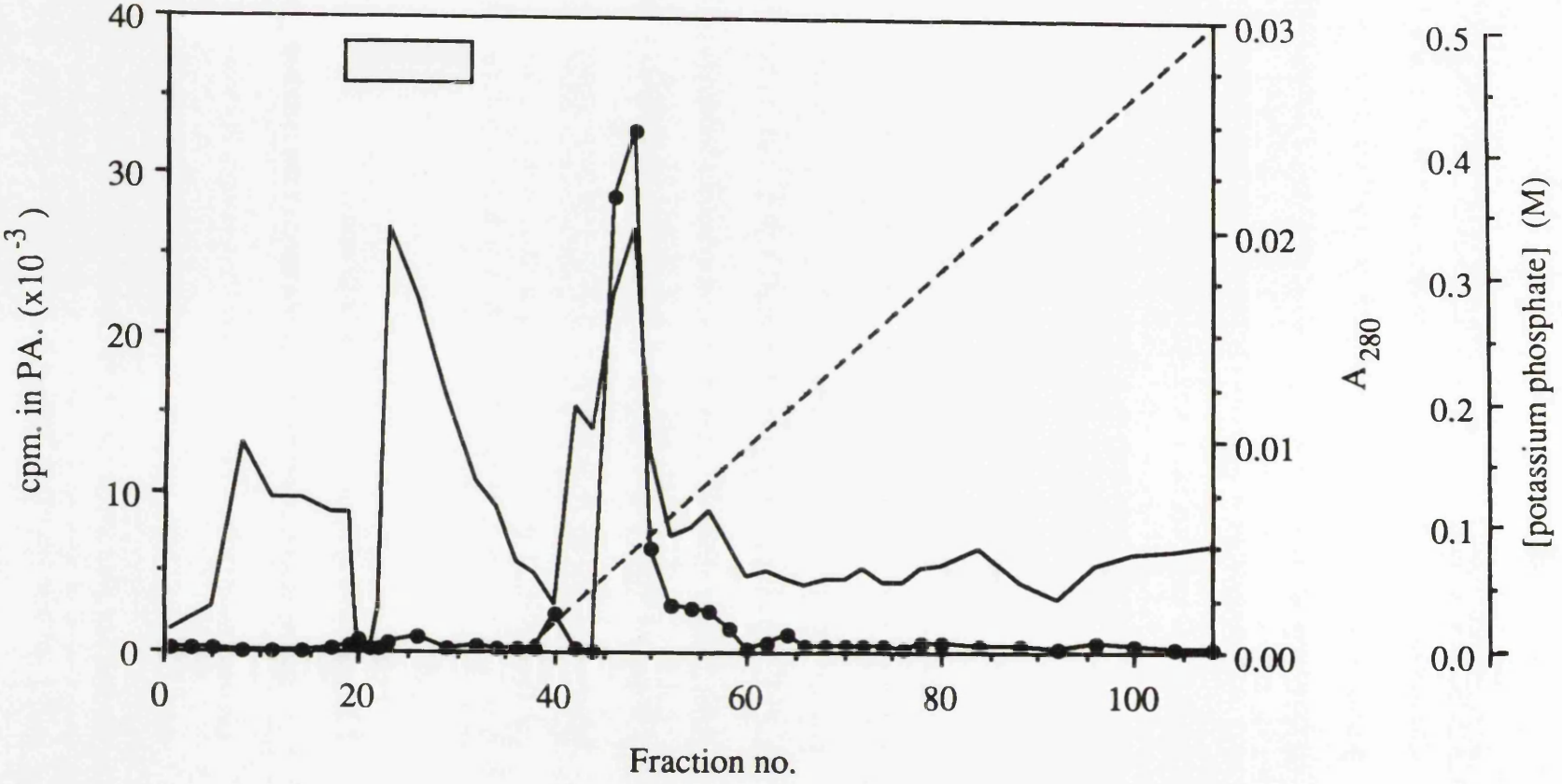

cpm. in PA. (x10^{-3})
40
30
20
10
0
0
20
40
60
80
100
Fraction no.
0.03
0.02
0.01
0.00
A_{280}
0.5
0.4
0.3
0.2
0.1
0.0
[potassium phosphate] (M)

Fig 3.10. Hydroxyapatite Chromatography of the KCl-soluble DAG Kinase.

A 5.0 ml (1.0 x 6.4 ml) column of hydroxyapatite (BioGel HTP)prepared and equilibrated buffer I as described in Section 2.6.5. The DAG kinase preparation pooled from heparin-agarose (2.7 mg protein, 35 ml) was applied to the column and the column was washed with buffer I followed by 5 column volumes of 1.0 M KCl, 10 mM $MgCl_2$ in buffer I (*open bar*). Finally, the column was washed with 12.0 ml of buffer I and developed with an 150 ml linear gradient of 0.0 to 0.5 M potassium phosphate, pH 6.5, in buffer I. The DAG kinase activity and the protein concentration of the column eluant were determined as described in Fig 3.7. 2.0 ml fractions were collected and fractions containing DAG kinase activity pooled (fractions 45 - 58; 2.1 mg protein, 33.0 ml). [-●-, DAG kinase activity; - - -, potassium phosphate concentration; *solid line*, A_{280}]

kinase purifications (Kanoh and Ono, 1984; Kato & Takenawa, 1990; Yada *et al.*, 1990).

Partially purified DAG kinase activity, pooled after heparin-agarose chromatography, was found to bind hydroxyapatite (BioGel HTP) in the presence of 0.3 M KCl at pH 6.5 (data not shown). Bound DAG kinase activity was retained on the column during washing with either 10 mM $MgCl_2$ or 1.0 M KCl in buffer D. It did, however, elute during washing with 0.3 M potassium phosphate, pH 6.5 (data not shown). From this information, a protocol for the elution of KCl-soluble DAG kinase was constructed (Fig 3.10). The protocol employed washing the column with a combined 10 mM $MgCl_2$, 1.0 M KCl step gradient in buffer D, to selectively elute basic and neutral proteins from the hydroxyapatite, followed by a linear gradient of 0 - 0.5 M potassium phosphate in buffer D to displace the DAG kinase. Reproducibly, the KCl-soluble DAG kinase eluted as a single peak of activity at a position in the gradient equivalent to 50 - 75 mM potassium phosphate. Typically, hydroxyapatite chromatography of KCl-soluble DAG kinase obtained from heparin-agarose resulted in 0.49- to 0.72-fold purification with 48.0 to 55.6 % recovery of activity across the column. The use of hydroxyapatite chromatography to further purify the post heparin-agarose preparation of the KCl-soluble DAG kinase is a matter for review. The inability of preparative scale columns to provide the purification previously recorded with pilot-scale experiments may require hydroxyapatite chromatography to be ommitted from the purification protocol in future.

3.12. Purification of the KCl-Soluble DAG Kinase: A Summary.

At the completion of the work towards this thesis, the following rationalised protocol was employed in the routine partial purification of the KCl-soluble DAG kinase. The order of the individual steps are as follows: phospholipase C-catalysed enrichment of rat brain particulate fraction with DAG kinase activity, KCl-extraction of the membrane-associated DAG kinase from the phospholipase C-treated

particulate fraction, fractionation of the KCl-soluble DAG kinase from the KCl-independent enzyme by KCl-dependent precipitation/resolubilisation, AffiGel Blue chromatography, combined butyl-Sepharose 4B and phenyl-Sepharose CL-4B hydrophobic interaction chromatography, Sephacryl S-300 SF gel filtration chromatography, heparin-agarose chromatography, and finally hydroxyapatite chromatography on BioGel HTP.

Partial purification of the KCl-soluble DAG kinase has been performed by the above protocol, as far as the heparin-agarose chromatography step, on four separate occaisions with similar results. The DAG kinase recovered from each step was pooled an analysed by SDS-PAGE (Fig 3.11). A typical purification summary is given in Table 1. The above purification protocol, employed as far as the heparin-agarose separation, results in 27.0- to 59.7-fold purification of the KCl-soluble DAG kinase relative to the total membrane-associated activity, with 0.6 to 1.3 % yield of activity. The resulting post heparin-agarose preparations were measured to have specific activities ranging from 44.1 to 116.0 nmol PtdOH formed/min/mg. Further purification of the post heparin-agarose DAG kinase by hydroxyapatite chromatography has been achieved (Fig 3.11). However, the post heparin-agarose enzyme was measured to be 0.55- to 0.81-fold purified by hydroxyapatite chromatography. This decrease in fold-purification is most likely to result both from the lability of the partially purified protein and the problems associated with accurate quantitation of the protein.

The fractions obtained from hydroxyapatite chromatography which contained DAG kinase activity were analysed by SDS-PAGE (Fig 3.12). It is impossible to identify any single polypeptide which co-migrates with DAG kinase activity. There is also an absence of any polypeptide with molecular mass of 160 kDa, the apparent native molecular mass of the KCl-soluble DAG kinase when determined by gel filtration chromatography (see Section 3.9).

In conclusion, the KCl-soluble DAG kinase is substantially purified, although not to homogeneity, by the above protocol. Several polypeptides obtained

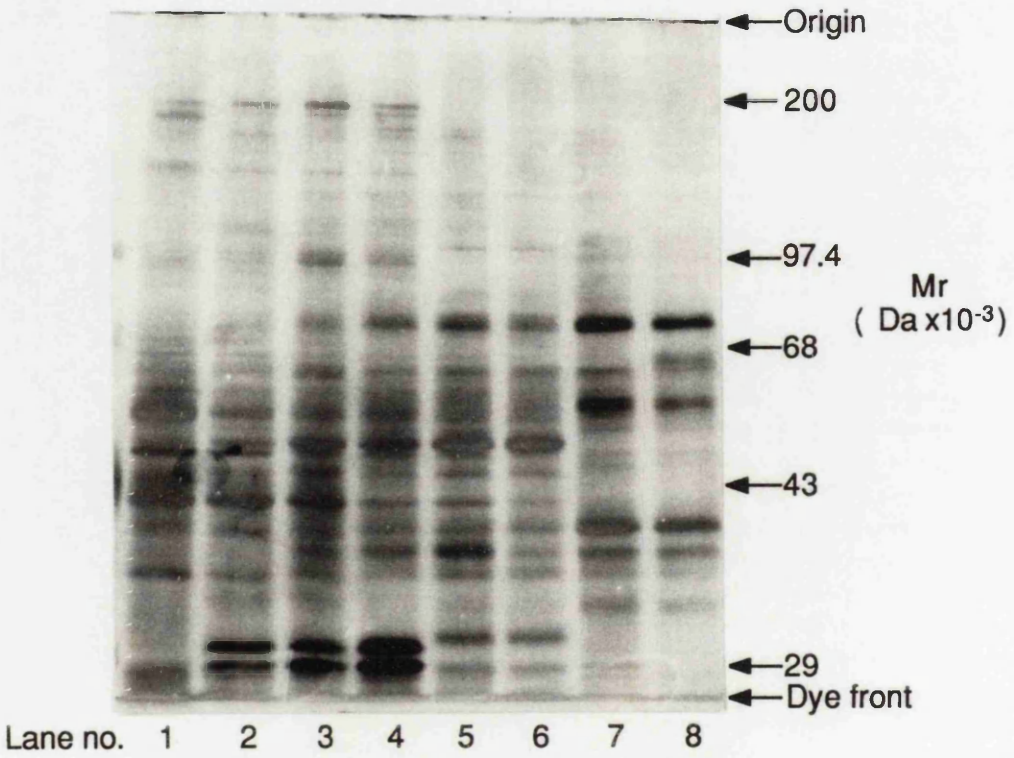

Origin
200
97.4
68
43
29
Dye front
Mr
(Da x10-3)
Lane no. 1 2 3 4 5 6 7 8

Fig 3.11. Purification of the KCl-soluble DAG kinase: SDS PAGE Analysis.

The KCl-soluble DAG kinase was purified by the method summarised in Section 4.9. 20 μg of the DAG kinase preparation pooled from each step of the purification was analysed by SDS-PAGE on a 10 % gel, as described in Section 2.3, and silver stained by the method described in Section 2.3.7.

Lane 1: DAG kinase-enriched particulate fraction.

Lane 2: KCl-extracted protein preparation.

Lane 3: KCl-soluble protein preparation.

Lane 4: post AffiGel blue.

Lane 5: post hydrophobic interaction chromatography.

Lane 6: post gel filtration.

Lane 7: post heparin-agarose.

Lane 8: post hydroxyapatite

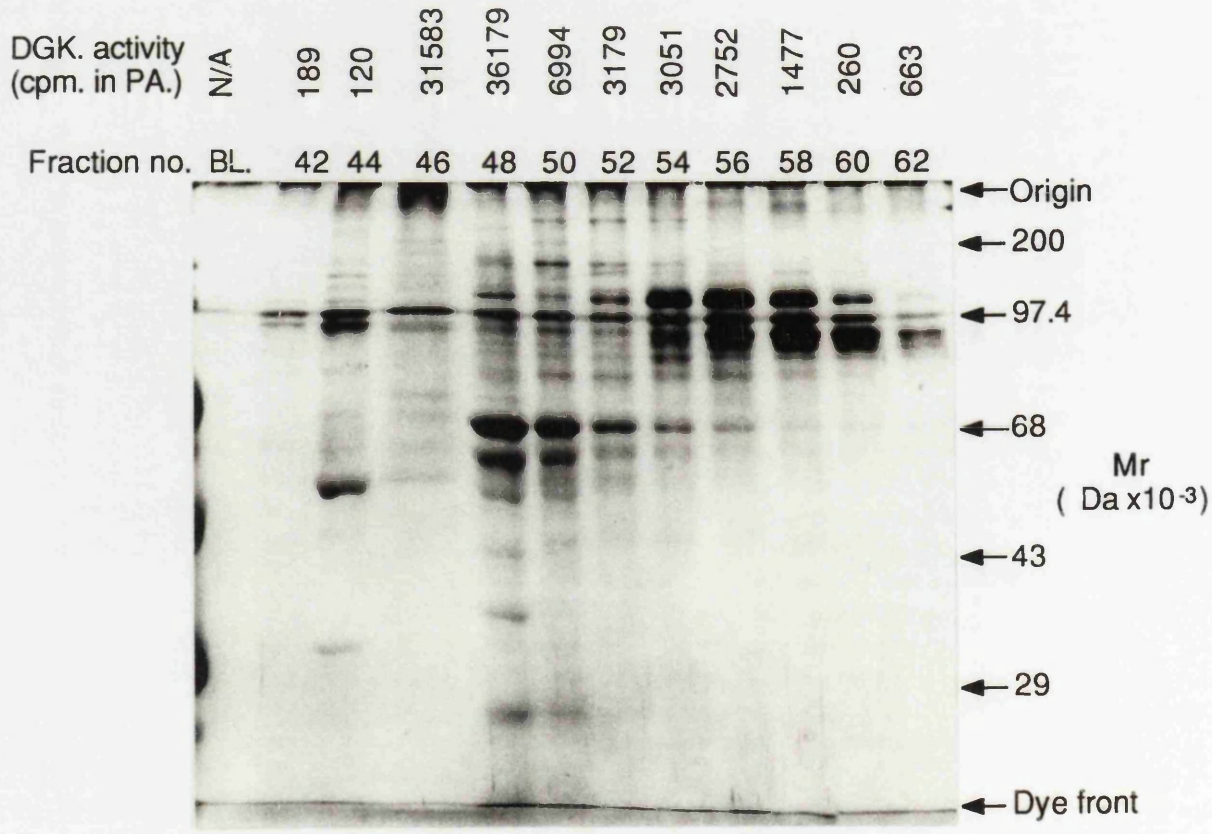

DGK. activity
(cpm. in PA.)
N/A
189
120
31583
36179
6994
3179
3051
2752
1477
260
663
Fraction no.
BL.
42
44
46
48
50
52
54
56
58
60
62
Origin
200
97.4
68
Mr
(Da x10-3)
43
29
Dye front

Fig 3.12. SDS-PAGE of the Fractions Co-Purifying With DAG Kinase Activity During Hydroxyapatite Chromatography.

100 μl of each fraction co-eluting with DAG kinase activity from the hydroxyapatite column discussed in Fig 3.10 was analysed by SDS-PAGE. A 10 % gel was run as described in Section 2.3 and silver stained by the method described in Section 2.3.7. The DAG kinase activity contained in a 10 μl aliquot of each fraction is marked on each lane as appropriate. DAG kinase activity was measured as described in Fig 3.7.

Table 1. Purification of the KCl-Soluble DAG Kinase of Rat Brain.

Step	Protein (mg)	DAG kinase activity (nmol/min)	Specific activity (nmol/min/mg)	Fold-purification	Yield (%)
Particulate fraction	5110.0	8850.00	1.73	1.00	100.00
KCl extract	283.00	1965.00	6.94	4.00	22.20
Resuspended extract	70.80	932.20	13.17	7.60	10.53
AffiGel Blue	34.40	878.10	25.53	14.74	9.92
HIC	14.60	259.70	17.78	10.27	2.93
Sephacryl S-300 SF	4.48	116.20	25.94	14.98	1.31
Heparin-agarose	0.96	99.15	103.40	59.70	1.12
Hydroxyapatite [1]					

This table summarises purification of the KCl-soluble DAG kinase from 100 rat brains.
10 - 20 µl of each fraction was assayed for DAG kinase activity by the *n*-octyl-β,D-glucopyranoside/PtdSer mixed micellar method (Section 2.3.3). 2.0 mM ATP, 2.0 mM *sn* -1-stearoyl-2-arachidonylglycerol were employed as substrates and the reaction mixture incubated for 10min.
[1] In this preparation, KCl-soluble DAG kinase was not further purified by hydroxyapatite chromatography, the rationalised purification protocol (as discussed in Section 3.12) was only employed as far as heparin-agarose chromatography. Typically, hydroxyapatite chromatography of the post heparin-agarose preparation provided an 18 - 32 % reduction in the protein present in the active fraction with only 45.0 - 55.5 % recovery of the DAG kinase activity appplied to the column as described in Fig 3.10.

after heparin-agarose and hydroxyapatite chromatography are observed to be substantially purified from the phospholipase C-treated particulate fraction of rat brain by the above protocol (Fig 3.11) and the KCl-soluble DAG kinase remains to be identified. It is, however, notable that none of these polypeptides are observed to co-purify with DAG kinase activity on hydroxyapatite chromatography (Fig 3.12).

3.13. Purification of the KCl-Soluble DAG Kinase to Homogeneity: A Discussion.

Despite considerable purification of the membrane-associated KCl-soluble DAG kinase, many polypeptides co-purified with the DAG kinase preparation recovered from heparin-agarose and hydroxyapatite chromatography (Fig 3.11). This indicates that further purification of the KCl-soluble DAG kinase is required. The KCl-soluble enzyme pooled after heparin-agarose chromatography possessed a specific activity of 103.4 nmol/min/mg (Table 1), a value 50-fold less than the V_{max} (5.2 μmol/min/mg) of the membrane-associated DAG kinase previously purified to homogeneity from rat brain (Kato and Takenawa, 1990). Caution must be exercised when comparing DAG kinase reaction velocitiies determined by different assay methodologies. The above specific activity of the post heparin-agarose was determined by an *n*-octyl-β,D-glucopyranoside mixed micellar assay, whereas Kato and Takenawa (1990) employed a sodium deoxycholate/*sn*-1,2-diacylglycerol dispersion method. It is now well documented that these methods support DAG kinase-catalysed phosphorylation of *sn*-1,2-diacylglycerol with widely different reaction velocities (Coco Maroney and Macara, 1989; Lemaitre *et al.*, 1990). Similarly, the specific activity of the post heparin-agarose DAG kinase described in Table 1 was determined to be 794 nmol/min/mg when measured by the deoxycholate/PtdSer dispersion method described in Section 2.4.2. This value is over 7-fold greater than the 103.4 nmol/min/mg determined with the *n*-octyl-β,D-glucopyranoside/PtdSer mixed micellar method (Section 2.4.3). This clearly

indicates the problems associated with comparing DAG kinase specific activities when different assay methodology has been employed. Nevertheless, the protein preparation obtained after heparin-agarose chromatography represents a DAG kinase preparation purified 59.7-fold relative to the phospholipase C-treated membranes (Table 1). Yet Kato and Takenawa (1990) reported their homogeneous preparation of rat brain membrane-associated DAG kinase was 7410-fold purified compared to brain particulate fraction. Similarly, Besterman *et al.* (1986b) reported the 500- to 750-fold partial purification of the membrane-associated DAG kinase from phospholipase C-treated rat brain homogenate. From these reports it is clear that substantial further purification of the post heparin-agarose KCl-soluble DAG kinase is required if a homogeneous protein preparation is to be obtained. In order that the KCl-soluble DAG kinase could be purified to homogeneity, many purification strategies in addition to those discussed above could be employed.

The purification protocol, as summarised in Section 3.12, does not include a true ion-exchange separation. However, the existing purification protocol does employ heparin-agarose chromatography (see Section 3.10). In this case, ionic interaction may be occurring between the DAG kinase and the multiple sulphonyl groups of the immobilised heparin in the presence of 300 mM KCl at pH 6.5. It is also possible that DAG kinase interacts with heparin by a mechanism still to be established, in a manner more similar to affinity than ion-exchange chromatography. This was found to be the case for the heat-stable 150 kDa DAG kinase of pig thymus (Sakane *et al.*, 1989). During the purification of the 150 kDa DAG kinase Sakane and co-workers (1989) found the enzyme able to bind both anion-exchange media (DE 52) and heparin-Sepharose at pH 7.4. As the enzyme bound to both an anion-exchange and a heparin-Sepharose column at the same pH, it may be possible to conclude that the interaction of the enzyme with the immobilised heparin is by a process other than ion-exchange.

It would be highly desirable to include an ion-exchange separation in the purification of the KCl-soluble DAG kinase as it could add a further dimension to

the purification of the enzyme. To date, the enzyme's requirement for 300 mM KCl has prevented the KCl-soluble DAG kinase from binding DEAE-cellulose (DE 52), QAE-Sephadex A-50 and SP-Sephadex C-50 at pH 6.5 (data not shown). Reduction of the KCl concentration below 300 mM would be required if an ion-exchange separation is to be successfully incorporated into the purification protocol. The exact nature of the KCl requirement for solubility remains to be established. It is possible that the solubility of a protein with exposed hydrophobic surfaces may become more favourable in a solution of increased strength. Therefore, as the solvation of such a protein becomes more favourable it is less likely to aggregate with other amphiphilic proteins or precipitate. This may be especially pertinent to the proteins solubilised from rat brain particulate fraction by incubation with 578 mM KCl. Such treatment solubilises non-integral membrane-associated proteins, i.e. proteins that are juxtaposed to the surface of the membrane. It would not be inconceivable that the protein surface normally associated with the phospholipid bilayer would possess some hydrophobic character. This is very likely to be the case for the KCl-soluble DAG kinase, whose function is to phosphorylate *sn*-1,2-diacylglycerol intercalated in the phospholipid bilayer. Although this may be a generalisation for all the proteins solubilised by KCl treatment, it does seem that a considerable proportion of these proteins possess sufficient hydrophobic character to cause their precipitation in KCl-free buffer.

Detergents have proven unable to replace the 300 mM KCl required to maintain the KCl-soluble DAG kinase in solution (Section 3.4.). It may be possible, however, to replace KCl with another salt that would maintain the KCl-soluble DAG kinase in solution at concentrations below 300 mM. If successful, this could renew the possibility of ion-exchange chromatography. Consideration of the Hofmeister lyotrophic series of anions and cations would suggest that the use of a salt with an increased 'salting out' nature compared to KCl may maintain DAG kinase in a fully soluble state at concentrations below 300 mM, e.g. $(NH_4)_2SO_4$, $(NH_4)_3PO_4$ or $((CH_3)_4N)_2SO_4$.

Additionally, if exposure of a hydrophobic surface is the cause of the protein's limited solubility in solution of low ionic strength, the addition of a co-solvent to the protein solution could terminate the requirement for KCl. The introduction of polyhydric alcohols to the aqueous solution results in 'preferential hydration' of the protein molecule, a result of increased surface tension at the protein:solution interface (Gekko and Timasheff, 1981a, b; Lee and Lee, 1987). This elicits stabilisation of the protein's shell of hydration, resulting in maintenance of the protein in the soluble state. Therefore, the introduction of co-solvents such as ethanediol, propanediol, polyethyleneglycol, or sucrose may maintain the KCl-soluble DAG kinase in solution in the absence of KCl. Thus, the KCl-soluble DAG kinase could be applied to ion-exchange media under conditions that would not prevent the protein from binding.

Alteration of the pH that ion-exchange chromatography is performed at from pH 6.5 would enhance the possibility of the KCl-soluble DAG kinase binding an ion-exchange column in the presence of 300 mM. As discussed in Section , the activity of the KCl-extracted DAG kinase was stable on exposure to a pH ranging from 4.5 to 9.0 for 1 h. This indicates that this approach would not be impeded by instability of the KCl-soluble DAG kinase' activity in buffer of pH much altered from neutrality. It may therefore be possible to successfully apply an ion-exchange separation in the presence of 300 mM KCl, but only if the chromatography was undertaken at a pH much removed from the protein's pI value. Moreover, the possibility of the KCl-soluble DAG kinase binding ion-exchange media under such pH conditions would be further increased on reduction of the KCl concentration at which chromatography was initiated. However, the success of this strategy again depends on the identification of a co-solvent that maintains the KCl-soluble DAG kinase's solubility in the presence of less than 300 mM KCl (see above). The stability of the KCl-soluble enzyme on prolonged exposure to co-solvents and pH altered from 6.5 has still to be established.

Cation-exchange chromatography on phosphocellulose P11 could be re-examined. The KCl-soluble DAG kinase had previously been found to bind phosphocellulose P11 in the presence of 300 mM KCl at pH 6.5. This is notable as the KCl-soluble enzyme was unable to bind either QAE-Sephadex A-50 or SP-Sephadex C-50 under these conditions. However, phosphocellulose P11 was not further employed in the purification protocol due to problems associated with its routine preparation and use in columns of 10 ml bed-volume or greater. These problems could be circumvented by employing a phospho-agarose cation-exchange medium. Alternatively, the phosphocellulose P11 cation-exchange separation could be introduced at a later stage of the purification. During the latter stages of the purification a small bed-volume column of phosphocellulose P11 would be sufficient, thus avoiding large bed-volume columns which had previously proven difficult to operate.

As reported in Section 3.7, AffiGel Blue binds the KCl-soluble DAG kinase in the presence of 300mM KCl at pH 6.5. Once bound the DAG kinase activity was eluted from the dye-matrix by increasing concentrations of KCl rather than by the use of ATP or other nucleotides. AffiGel Blue was initially considered for use in the purification of the KCl-soluble DAG kinase on the basis of dye-ligand matrices' ability to bind nucleotide-requiring enzymes. Thus, if the KCl-soluble DAG kinase binds via its nucleotide binding site, it would seem feasible to elute the bound enzyme by free nucleotides, especially ATP. Moreover, due to the selective nature of elution by free nucleotide compared to elution with increasing concentrations of KCl, greater purification of the kinase across AffiGel blue would result. However, ATP, ADP, AMP and adenosine were unable to displace the KCl-soluble DAG kinase from AffiGel Blue when employed at 2.0 mM (S. Gardner, personal communication). It would seem that the KCl-soluble enzyme does not bind the dye-matrix via a nucleotide binding site. Therefore, the resolution of the KCl-soluble DAG kinase gained on AffiGel Blue chromatography cannot be improved by the use of free nucleotide to displace the enzyme. This conclusion

may be premature as the enzyme could elute on developing the column with concentrations of nucleotide and nucleoside greater than 2.0 mM.

Subsequent to the completion of the work conducted towards this thesis, a series of dye-matrices were examined for the ability to bind the KCl-soluble DAG kinase. In addition to the previously characterised AffiGel Blue (see Section 3.7), Matrex Orange A, Matrex Red A and Matrex Green A were screened for the ability to bind the enzyme in the presence of 300 mM KCl at pH 6.5. The KCl-soluble DAG kinase bound to all these dye-matrices in the presence of 300 mM KCl, although binding to Matrex Orange A was only partial. Washing the dye-matrices with 600 mM KCl elicited elution of DAG kinase activity from Matrex Orange A alone. Further increasing the KCl concentration to 1.0 M initiated the elution of activity from Matrex Green A and AffiGel Blue. Finally, increasing the KCl concentration to 2.0 M resulted in elution of the DAG kinase from Matrex Red A, while also accomplishing further elution of activity from Matrex Green A and AffiGel Blue. (S. Gardener, personal communication). This ordered elution would indicate increasing affinity of the KCl-soluble DAG kinase for dye-matrices in the order: Matrex Orange A<AffiGel Blue<Matrex Green A<Matrex Red A (group 1, group 3, group 5 and group 3, respectively; as discussed by Stellwagen, 1990). The arrangement of dye-affinity matrices into groups is based on the individual dye-matrix's ability to bind protein, with group 1 binding least and group 5 most. Therefore, the KCl-soluble DAG kinase binds the above dye-matrices with a ranked order of increasing affinity that is a departure from that predicted by Stellwagen (1990). If a second dye-affinity column were to be incorporated into the purification protocol, the above departure would indicate that Matrex Red A is probably the best candidate for providing further purification. The increased affinity of the KCl-soluble DAG kinase for Matrex Red A allowing more selective elution of the enzyme than would be achieved with Matrex Green A. Furthermore, it would seem possible that the KCl-soluble DAG kinase could bind Matrex Red A in the presence of KCl concentrations that would affect its elution from AffiGel

Blue. Thus, the KCl-soluble DAG kinase activity eluting from an AffiGel Blue column could be selectively collected on a Matrex Red A column. This illustrates the possibility of employing AffiGel Blue and Matrex Red A columns in tandem, whereas incorporation of a second dye-affinity column during the latter stages of the purification is equally possible. Altogether it would appear that the incorporation of a second dye-affinity column could further purify the KCl-soluble DAG kinase towards homogeneity. Indeed, the consecutive application of two different dye-matrix columns has been reported in the successful purification of lysophosphatidylcholine transacylase from bovine heart muscle (Sanjanwala *et al.*, 1989).

If a second dye-affinity column were to be incorporated into the purification, the possibility of more selective elution of the bound DAG kinase by free nucleotide compared to increasing concentrations of KCl should be investigated. As reported above, the KCl-soluble DAG kinase binds Matrex Red A with greater affinity than predicted by the classification of dye-matrices discussed by Stellwagen (1990). It is possible that this may indicate the KCl-soluble DAG kinase binds Matrex Red A by a process different from that which it associates with the other dye-matrices. Although it would appear that the KCl-soluble DAG kinase does not interact with AffiGel Blue via its nucleotide binding site (see above), it may do so when binding Matrex Red A. Therefore, if Matrex Red A chromatography were to be included in the purification, it would be worth investigating the elution of the KCl-soluble DAG kinase by free nucleotide.

Chromatofocusing can not be applied to the purification of KCl-soluble DAG kinase for much the same reasons that ion-exchange chromatography cannot be employed. In essence, chromatofocusing is an adaptation of ion-exchange chromatography. Rather than eluting a bound protein with increasing concentrations of salt, a decreasing pH gradient is developed across the anion-exchange column and proteins elute as their net positive charge is lost. Thus, chromatofocusing involves the application of proteins to a strong anion-exchange

medium a pH above their pI values in order that it may bind. However, this binding process will be prevented by the 300 mM KCl required to maintain solubility of the KCl-soluble DAG kinase. As is the case for ion-exchange chromatography, development of conditions that would maintain the KCl-soluble DAG kinase in solution in salt-free buffer are required before this chromatography method can be considered further.

Affinity chromatography of DAG kinase has mainly revolved around the ATP substrate of the enzyme, e.g. rat liver cytosol (Kanoh & Ohno, 1981), pig brain cytosol (Kanoh *et al.*, 1983), bovine brain cytosol (Lin *et al.*, 1986), pig thymus cytosol (Sakane *et al.*, 1989), human platelet cytosol (Yada *et al.*, 1990), human white blood cell cytosol (Schaap *et al.*, 1989), and rat brain cytosol and membranes (Kato and Takenawa, 1990). In all these cases the DAG kinase bound the ATP-agarose and requires more than 300 mM NaCl to displace the bound enzyme. The KCl-soluble DAG kinase, however, does not bind ATP-agarose in the presence of 300 mM KCl at pH 6.5 (data not shown).

Affinity chromatography on immobilised *sn*-1,2-diacylglycerol or phospholipid would appear to be attractive alternatives. Uchida and Filburn (1984) successfully employed an affinity matrix of *sn*-1,2-diacylglycerol and PtdSer immobilised in a polyacrylamide gel to purify protein kinase C from rabbit renal cortex. It may be significant that Besterman *et al.* (1986b) have employed polyacrylamide-immobilised *sn*-1,2-diacylglycerol to purify the membrane-associated DAG kinase of rat brain to near homogeneity. However, work previously conducted in this laboratory has found rat brain DAG kinase unable to bind such polyacrylamide immobilised *sn*-1,2-diacylglycerol (S. Palmer & M.J.O. Wakelam, personal communication). Alternatively, the covalent attachment of *sn*-1,2-diacylglycerol to a chromatography support may provide a suitable affinity chromatography medium. PtdCho covalently immobilised via its acyl chains to an agarose support has been employed successfully in the purification of *Crotalus adamanteus* venom phospholipase A_2 (Rock & Snyder, 1975). Unfortunately it is

not known what effect the presence of 300 mM KCl would have on the interaction of the KCl-soluble DAG kinase with immobilised *sn*-1,2-diacylglycerol affinity media. The affinity of KCl-soluble DAG kinase for the immobilised *sn*-1,2-diacylglycerol may be sufficiently great to overcome any displacing effect that results from the presence of 300 mM KCl. As a purification strategy, the use of immobilised *sn*-1,2-diacylglycerol could possibly result in substantial purification of the KCl-soluble DAG kinase. The greater relative selectivity of immobilised *sn*-1,2-diacylglycerol for DAG kinases, compared to agarose-immobilised nucleotides, must indicate the former as most likely to lead to the final purification of the KCl-soluble enzyme.

Preparative-scale non-denaturing electrophoresis of the final KCl-soluble DAG kinase preparation obtained from the established purification protocol is unlikely to resolve the enzyme to homogeneity. The final preparation contains many polypeptides (Fig 3.11) and it is unlikely that they will separate sufficiently on non-denaturing electrophoresis. This will also prevent the final identification of the KCl-soluble DAG kinase. Those polypeptides that do not co-migrate with DAG kinase activity can, however, be eliminated from the identification process. Thus, the KCl-soluble DAG kinase could be identified as one of a small number of proteins. If this were the case, non-denaturing electrophoresis would at least allow further partial purification of the enzyme. Unfortunately, DAG kinase activity has never been recovered from non-denaturing electrophoresis of the KCl-soluble enzyme (data not shown). This inability to recover activity from a polyacrylamide gel has led to doubt about the KCl-soluble DAG kinase entering the gel and thus migrating through the gel during electrophoresis. Therefore, the use of preparative non-denaturing PAGE has not been considered further.

As yet, the KCl-soluble DAG kinase has still to be identified on SDS-PAGE analysis of the post hydroxyapatite protein preparation (see Section 3.11). This has prevented the purification of the KCl-soluble DAG kinase by preparative SDS-PAGE.

Purification of the KCl-soluble DAG kinase of rat brain could proceed by several possible strategies. Employing affinity chromatography on immobilised *sn*-1,2-diacylglycerol would seem to offer the best possibility of continued purification. Nevertheless, this conclusion remains speculative until the ability of immobilised *sn*-1,2-diacylglycerol to bind the KCl-soluble enzyme in the presence of 300 mM KCl is established.

3.14. Identification of the KCl-Soluble DAG Kinase Polypeptide: A Discussion.

In the absence of a homogeneous preparation of the KCl-soluble DAG kinase, its identification within a mixture of other polypeptides could allow protein sequencing. Identification would allow direct electroblotting of the polypeptide from an SDS-PAGE gel to a PVDF membrane. Solid-phase N-terminal sequencing could then be undertaken. This does require that the polypeptide to be sequenced is sufficiently resolved from the other proteins on SDS-PAGE. If this is not the case further partial purification would be required prior to SDS-PAGE and electroblotting to the PVDF support.

As reported in Section 3.11, it is not possible to identify the KCl-soluble DAG kinase from the mixture of proteins pooled from hydroxyapatite chromatography. SDS-PAGE analysis of the fractions collected failed to identify any polypeptide(s) that co-migrate with DAG kinase activity on hydroxyapatite (Fig 3.12). Furthermore, no 160 kDa polypeptides were observed on SDS-PAGE of the post hydroxyapatite enzyme preparation (Figs 3.11, 3.12), despite the apparent native molecular mass of the KCl-soluble DAG kinase being determined as approximately 160 kDa (Section 3.9). Identification of the KCl-soluble DAG kinase will thus require that further analytical methods be employed.

Identification of the KCl-soluble DAG kinase's catalytic polypeptide may be possible upon covalent modification with a photo-affinity ATP analogue. If the photo-affinity analogue were radiolabelled, the modified polypeptide could then be

subjected to SDS-PAGE and identified by autoradiography. The most likely candidate analogues are [α-^{32}P]8-azido ATP and [α-^{32}P]3'-*O* -(4-benzoyl)benzoyl ATP. Both these photo-activated analogues competitively occupy ATP binding sites, irreversibly inhibiting the enzyme on exposure to UV light . Indeed, [α-^{32}P]3'-*O* -(4-benzoyl)benzoyl ATP has been successfully employed in identifying the P_{2Y}-purinergic receptor of turkey erythrocyte plasma membranes by the above approach (Boyer *et al.*, 1990). However, the application of such photo-affinity analogues to the identification of the KCl-soluble DAG kinase requires the prior characterisation of their suitability as substrates. They must be able to competitively inhibit the binding of ATP to the catalytic site of the enzyme. The possibility could still exist that several ATP-binding proteins have co-purified with the KCl-soluble DAG kinase on AffiGel Blue and heparin-agarose chromatography. As it likely that such proteins would also be labelled with ATP photo-affinity analogues, the modification of the the KCl-soluble DAG kinase by their use must be carefully characterised. Conditions would need to be developed to optimise the labelling of the KCl-soluble DAG kinase over the other ATP-binding proteins that may possibly be present.

The use of analytical non-denaturing PAGE to both resolve and identify the KCl-soluble DAG kinase cannot be considered as a likely method. As discussed in 3.13, activity has yet to be recovered after non-denaturing PAGE of the KCl-soluble DAG kinase. The inability to recover DAG kinase activity from non-denaturing gels may be the result of any one of several causes. It is not clear whether the KCl-soluble DAG kinase entered the gel at the initiation of electrophoresis. Equally, it was not clear if the proteins applied to the non-denaturing gel migrated through the gel during electrophoresis. If the proteins did enter the gel and migrate on electrophoresis, problems may be associated with efficiently eluting the KCl-soluble DAG kinase from the non-denaturing gel prior to assay. Finally, it may also be possible that the KCl-soluble DAG kinase is unstable and denatures to an inactive form during non-denaturing electrophoresis.

In conclusion, the KCl-soluble DAG kinase is most likely to be identified by covalent labelling with photo-affinity ATP analogues. This may not automatically allow N-terminal sequencing of the protein or the peptides derived from it as the protein may not be clearly resolved from the others present in the post hydroxyapatite preparation on SDS-PAGE. It would, however, allow further purification of the enzyme by a more informed protocol. Additionally, if the protein is identified or selectively labelled within a mixture of other proteins, purification could proceed by methods that are not associated with the preservation of enzyme activity, e.g. reverse phase chromatography.

3.15. Comparison of the KCl-Soluble Enzyme to Membrane-Associated DAG Kinases Previously Purified From Rat Brain.

The DAG kinase purified in the work towards this thesis is different from the enzymes purified from rat brain by Besterman *et al.* (1986b) and Kato and Takenawa (1990). The membrane-associated DAG kinase reported within this thesis possesses an absolute requirement for the presence of salt for continued solubility, specifically 300 mM or greater KCl (see Section 3.4). Neither Besterman *et al.*(1986b) nor Kato and Takenawa (1990) reported DAG kinase activities which required the presence of salt for continued solubility. Indeed, both enzymes were substantially purified by protocols which required dialysis of the enzyme against salt-free buffer followed by clarification of the dialysate. Therefore, this thesis represents the first report of a membrane-associated DAG kinase which requires the presence of elevated concentrations of salt alone to remain soluble.

Additionally, a membrane-associated DAG kinase activity which did not require the presence of KCl for continued solubility was also observed in this KCl-extracted protein preparation employed in this thesis (see Sections 3.4, 3.5). It is notable that the KCl-independent DAG kinase displayed different chromatographic behavior from the KCl-soluble enzyme on hydroxyapatite (data not shown).

However, the KCl-independent enzyme eluted from hydroxyapatite under conditions similar to those employed in the purification of the 150 kDa DAG kinase. Both the KCl-independent DAG kinase reported within this thesis and the 150 kDa enzyme purified to homogeneity by Kato and Takenawa (1990) are eluted from hydroxyapatite by increasing concentrations of salt, whereas the KCl-soluble DAG kinase remains bound and requires in excess of 50 mM potassium phosphate for elution (Section 3.11). This strongly indicates that the KCl-independent enzyme discussed in Section 3.14 is likely to be the 150 kDa activity purified to homogeneity by Kato and Takenawa (1990) while the KCl-soluble enzyme is novel and not previously reported.

The differences between the KCl-soluble DAG kinase and the enzyme purified by Kato and Takenawa (1990) are further accentuated if their respective purification protocols are further examined. The 150 kDa DAG kinase was purified from a 2.0 M NaCl extract by sequential chromatography on Q-Sepharose Fast Flow (anion-exchange), phenyl-Superose, hydroxyapatite, and ATP-agarose (Kato and Takenawa, 1990). All these chromatographic methods have been applied to the purification of the KCl-soluble enzyme and have been found to be unsuccessful. The differences in the functional group, the degree of their substitution in QAE-Sephadex A-50 and Q-Sepharose Fast Flow and also the difference in pH employed (pH 6.5 and 7.4 respectively) prevent any comparison of the two enzymes' behavior on anion-exchange media. It is also difficult to compare the behavior of the two enzymes on phenyl-Superose. The 150 kDa elutes from phenyl-Superose as the NaCl concentration is dropped from 100 to 0 mM, whereas the KCl-Soluble enzyme cannot be purified by this method as it would precipitate on the phenyl-Superose column under these conditions of reduced salt concentration. It sholuld be noted that the KCl-soluble DAG kinase can be purified by phenyl-Superose chromatography. In the presence of 300 mM KCl and 0.02 % (w/v) Triton X-100 the KCl-soluble enzyme elutes from phenyl-Superose between 40 and 50 % (v/v) ethanediol. Apart from the unsuitability of the phenyl-Superose chromatography

method of Kato and Takenawa (1990) to the KCl-soluble DAG kinase, it is impossible to further compare their behavior on phenyl-Superose. As discussed above, the KCl-soluble DAG kinase and the 150 kDa enzyme previously purified from rat brain behave quite differently on hydroxyapatite. Finally, the 150 kDa DAG kinase was substantially purified on ATP-agarose and eluted with between 350 and 380 mM NaCl (Kato and Takenawa, 1990). The KCl-soluble DAG kinase, however, was found not to bind ATP-agarose in the presence of 300 mM KCl at pH 6.5 (S.D. Gardner, personal communication). Therefore the purification protocol of Kato and Takenawa (1990) is inapplicable to the KCl-soluble DAG kinase reported within this thesis.

Rather than using the separation methods which were successful with the 150 kDa DAG kinase, the KCl-soluble enzyme was purified by protocol which employed AffiGel blue, gel filtration, and heparin-agarose chromatography. AffiGel blue, heparin-agarose and gel filtration chromatography are procedures which have been employed in purification of DAG kinases distinct from the 150 kDa membrane-associated enzyme of rat brain. For example, AffiGel blue was employed as the initial chromatographic separation in the purification of the 110 kDa cytoolic DAG kinase of rat brain (Kato and Takenawa, 1990). Additionally, heparin-agarose and gel filtration chromatography have been successfully applied to purification of the porcine cytosolic DAG kinases (Sakane *et al.*, 1989). Therefore, the application of these separation procedures to the purification of a membrane-associated DAG kinase is novel and not previously reported.

As the KCl-soluble DAG kinase is only partially purified 59.7-fold by the protocol reported here, its V_{max} in the pure state cannot be compared to that of the enzyme purified to homogeneity by Kato and Takenawa (1990). The impurity of the final preparation of the KCl-soluble activity prevents determination of its molecular mass by SDS-PAGE. Although it possesses an apparent molecular mass of 160 kDa on gel filtration, it cannot be assumed to be of similar mass to the 150 kDa enzyme of Kato and Takenawa (1990). Similarly, this value cannot be

compared to the mass of the four polypeptides purified by *sn*-1,2-diacylglycerol/PtdSer affinity chromatography (Besterman *et al.*, 1986b).

In summary, apart from the KCl requirement for solubility, the KCl-soluble DAG kinase is purified by a protocol quite different from those previously employed for purification of membrane-associated DAG kinases. In the absence of a homogeneous preparation of the KCl-soluble enzyme, comparison of its chromatographic behavior to that of the 150 kDa DAG kinase previously purified from rat brain suggests that they are two different enzymes. Furthermore, this is the first report of a membrane-associated DAG kinase which is solubilised by hypertonic concentrations of salt and requires the continued presence of salt, specifically 300 mM or greater KCl, for solubility.

Chapter 4

Characterisation of a Triton X-100 Based *sn*-1,2-Diradylglycerol Mass Measurement Assay and Kinetic Characterisation of the KCl-Soluble DAG Kinase.

4.1. Characterisation of the Triton X-100 Method and Its Application to the Measurement of *sn*-1,2-Diradylglycerol Mass in Cell Lipid Extracts.

In order that the *sn*-1-acyl-2-archidonylglycerol selectivity of the KCl-soluble DAG kinase of rat brain can be assessed, it is necessary to determine the kinetic constants, K_m and V_{max}, of the enzyme when different *sn*-1,2-diacylglycerol substrates are employed, e.g. *sn*-1,2-dilauroylglycerol, *sn*-1,2-dioleoylglycerol and *sn*-1-stearoyl-2-arachidonylglycerol. This permits the identification of catalytic selectivity of the enzyme towards a particular substrate by the enzyme exhibiting a reduced K_m or elevated V_{max} towards any one substrate compared to other *sn*-1,2-diacylglycerol species. Therefore, the concentrations of *sn*-1,2-diacylglycerol substrates within the assay tube must be determined accurately if true values for the above kinetic constants, especially K_m, are to be determined. The *sn*-1,2-diacylglycerol concentrations employed in this study of the concentration dependence of the KCl-soluble DAG kinase's reaction velocity were normalised to that of *sn*-1-stearoyl-2-arachidonylglycerol by means of a quantitative assay that employs the DAG kinase of *Eschericia coli.*

Such a quantitative assay relies on the solubilisation of *sn*-1,2-diacylglycerol in a detergent mixed micellar system with subsequent phosphorylation of the *sn*-1,2-diacylglycerol in a reaction catalysed by the DAG kinase (*dgkA* gene product) of *E. coli.* The inclusion of [γ-^{32}P]ATP of known specific activity in the DAG kinase reaction mixture allows quantitation of the [^{32}P]phosphatidic acid produced. Comparison to a standard curve constructed from *sn*-1,2-diacylglycerol of known concentration and treated in an identical manner further allows quantitation of the *sn*-1,2-diacylglycerol mass originally present. Furthermore, assay conditions were developed to provide complete phosphorylation of *sn*-1,2-diacylglycerol to phosphatidic acid, eliminating possible erroneous quantitation that might arise from altered efficiency of conversion between the standard and experimental *sn*-1,2-diacylglycerol samples. Originally,

an *n*-octyl-β,D-glucopyranoside/cardiolipin mixed micellar method was reported to quantitatively measure *sn*-1,2-dioleoylglycerol over the range 25 pmol to 25 nmol with greater than 95 % efficiency (Preiss *et al.*, 1986, 1987). Complete conversion of *sn*-1,2-diacylglycerol to phosphatidic acid by this method was achieved by employing membranes from *E. coli* strain N4830/pJW10, a strain that overproduces 100-fold the membrane-associated DAG kinase of *E. coli*, to provide an initial reaction velocity of 43 nmol/min within the assay, and incubation of the reaction mixture for 30 min. Enzyme inactivation during this prolonged incubation period is reduced by inclusion of cardiolipin, DTT and DETAPAC in the reaction mixture and incubation at 30°C rather than 37°C (Walsh & Bell, 1986a).

The lipid/detergent mixed micellar system that could be provided by such use of *n*-octyl-β,D-glucopyranoside also allowed solubilisation of the lipid extracts from stimulated cells and quantitation of the *sn*-1,2-diacylglycerol within the extract (Preiss *et al.*, 1986). These workers reported that conversion of *sn*-1,2-diacylglycerol to phosphatidic acid was not impared by the addition of cell lipid extract prepared from 0.5×10^9 to 2.0×10^9 platelets (Preiss *et al.*, 1986). Therefore, the observed complete conversion of *sn*-1,2-diacylglycerol to phosphatidic acid in the presence of cell lipid confirmed that the *E. coli* DAG kinase assay could indeed be employed in the quantitation of *sn*-1,2-diacylglycerol mass contained within the cellular lipid extract prepared from cells. Workers in this, and other laboratories have, however, experienced difficulty in the adaptation of the assay methodology reported by Preiss *et al.* (1986). It has been the experience of the workers within this laboratory to observe incomplete conversion of the *sn*-1,2-diacylglycerol standard curve to phosphatidic acid. Typically, 57 ± 9.4 % of the *sn*-1-stearoyl-2-arachidonylglycerol over the range 100 pmol to 2 nmol is phosphorylated in the absence of cell lipid extract (Fig 4.1). Such inefficient conversion was not related to impurity of the *n*-octyl-β,D-glucopyranoside as recrystalised detergent was employed, as suggested by Preiss *et al.* (1987). Additionally, although reported as a possible source of inefficient

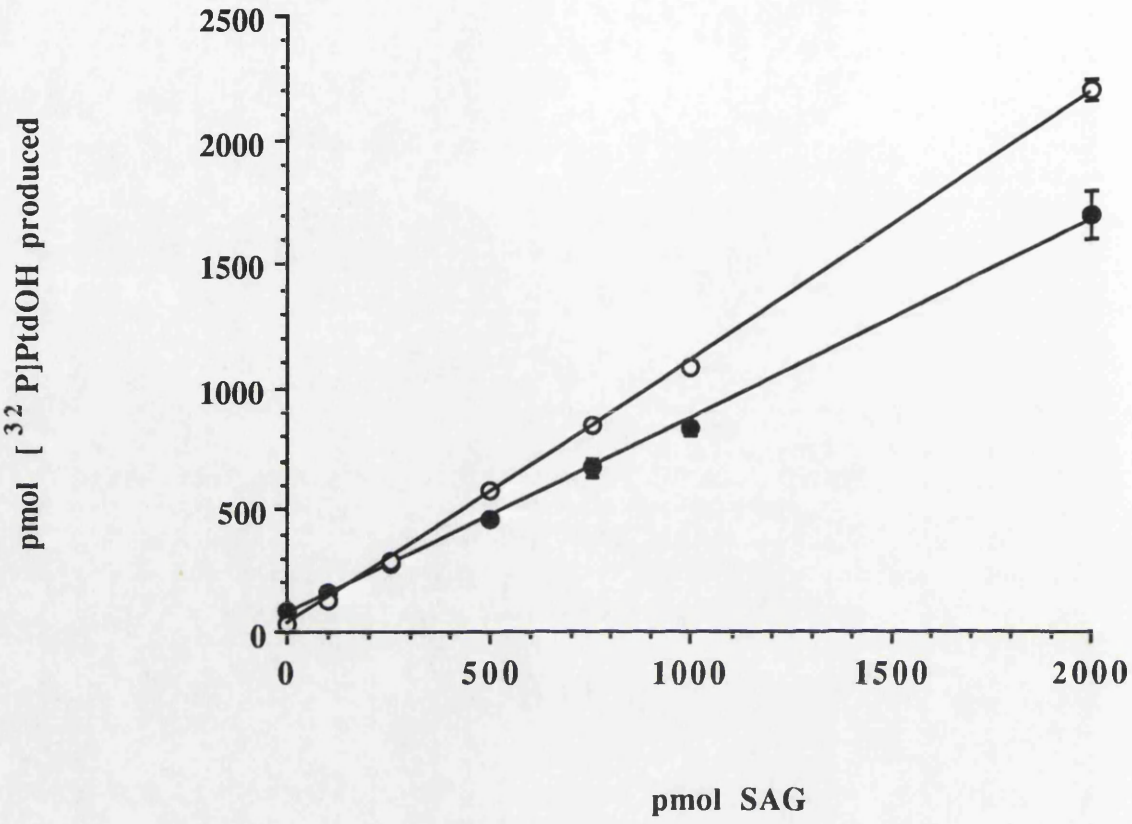

pmol [32 P]PtdOH produced
2500
2000
1500
1000
500
0
0
500
1000
1500
2000
pmol SAG

Fig 4.1. Conversion of *sn*-1-Stearoyl-2-Arachidonylglycerol to Phosphatidic Acid by *n*-Octyl-β,D-Glucopyranoside and Triton X-100 Micellar Methods.

Conversion of 100 pmol - 2 nmol *sn*-1-stearoyl -2-arachidonylglycerol to [^{32}P]phosphatidic acid by the *n*-octyl-β,D-glucopyranoside (●) and Triton X-100 (○) micellar systems was monitored in the absence of endogenous cell lipid. Quantitation by the Triton X-100 method was performed as described in Section 2.7 and the *n*-octyl-β,D-glucopyranoside assay performed as described in Section 2.4.1.

conversion by Preiss and co-workers (1986, 1987), the integrity of the [γ-^{32}P]ATP was established by PEI-cellulose t.l.c. (data not shown) and this possibility eliminated.

The possibility that, under the conditions reported by Preiss and co-workers (1986, 1987), *n*-octyl-β,D-glucopyranoside was unable to fully solubilise the *sn*-1,2-diacylglycerol substrate and, thus, prevented complete DAG kinase-catalysed phosphorylation was investigated. This was pertinent as the lipid extract prepared from Swiss 3T3 fibroblasts was visibly insoluble in the detergent conditions of the existing assay protocol (data not shown). Methodology adapted from Thompson *et al.* (1990), employing Triton X-100, was examined for its ability to provide increased conversion of *sn*-1,2-diacylglycerol to phosphatidic acid. This would indicate if 0.3 % (w/v) Triton X-100 (c.m.c. = 0.019 % (w/v)/4.8 mM) provides conditions within the assay mixture that are more favourable towards complete conversion of *sn*-1,2-diacylglycerol to phosphatidic acid than provided by the 52 mM *n*-octyl-β,D-glucopyranoside (c.m.c = 25 mM) employed by Preiss *et al.* (1986).

More efficient phosphorylation of *sn*-1-stearoyl-2-archidonylglycerol over the range 100 pmol - 2 nmol was observed when 4.8 mM [0.3 % (w/v)] Triton X-100, rather than 52 mM *n*-octyl-β,D-glucopyranoside, was employed to establish mixed micellar enviroment within the assay mixtutre (Fig 4.1). Routinely, quantitative phosphorylation of *sn*-1-stearoyl-2-arachidonylglycerol is observed over the range 100 pmol - 2 nmol (113 ± 1.9 %, Fig 4.1), indicating the suitability of Triton X-100 as the detergent to be employed in such a *sn*-1,2-diacylglycerol mass determination assay. Greater than 100 % conversion of the *sn*-1,2-diacylglycerol present within the standard curve is observed, this is most likely attributable to inaccurate quantitation of the *sn*-1-stearoyl-2-arachidonylglycerol stock solution employed in constructing the standard curve and also inaccurate determination of the specific activity of the [γ-^{32}P]ATP present in the DAG kinase incubation mixture. However, the degree of phosphorylation, although greater than

100 %, is similar at all points in the standard curve and confirms that the *sn*-1,2-diacylglycerol present is uniformly quantitated throughout the range 100 pmol - 2 nmol.

The Triton X-100 mixed micellar assay system proved suitable for the quantitation of *sn*-1,2-diacylglycerol mass within cell lipid extracts. The efficiency of conversion of 330 pmol *sn*-1-stearoyl-2-arachidonylglycerol added to increasing amounts of cell lipid extract prepared from confluent, quiescent Swiss 3T3 fibroblasts monolayers was determined for both the Triton X-100/PtdSer and *n*-octyl-β,D-glucopyranoside/cardiolipin methods (Table 2). Maximally, in the presence of endogenous cell lipid equivalent to that prepared from 2.25 x 10^5 fibroblasts, the *n*-octyl-β,D-glucopyranoside/cardiolipin method of Preiss *et al.* (1986) resulted in 80.4 % conversion of *sn*-1-stearoyl-2-arachidonylglycerol to phosphatidic acid (Table 2). As the concentration of cell lipid within the incubation mixture increased to that equivalent to 2.25 x 10^6 fibroblasts, the measured efficiency of conversion fell to 0.4 % (Table 2). In comparison, the conversion of *sn*-1-stearoyl-2-arachidonylglycerol was complete in the Triton X-100/PtdSer assay method at lipid concentrations equivalent to 2.25 x 10^5 - 4.50 x 10^5 fibroblasts, falling to 38.8 % in the presence of lipid equivelent to 2.25 x 10^6 fibroblasts (Table 2). Additionally, increased precision of *sn*-1-stearoyl-2-archidoylglycerol mass determination in the presence of cell lipid was observed with the Triton X-100/PtdSer method (Table 2).

In the presence of cell lipid equivalent to 2.25 x 10^6 Swiss 3T3 fibroblasts, quantitative recovery of [^{32}P]phosphatidic acid over the range 100 pmol - 1 nmol was recorded in a sham Triton X-100/PtdSer assay (data not shown). This indicated that the decreased efficiency of *sn*-1,2-diacylglycerol measurement in the presence of lipid equivalent to that produced from greater than 4.50 x 10^5 fibroblasts was not a product of limited phosphatidic acid recovery in the presence of elevated lipid concentrations but, rather, the product of inhibited DAG kinase

Table 2. Efficiency of *sn*-1,2-Diradylglycerol Mass Quantitation by the *n*-Octyl-β,D-Glucopyranoside and Triton X-100 Micellar Methods in the Presence of Increasing Endogenous Cell Lipid.

	Efficiency of phosphorylation (%)	
Cell No.	*n*-octyl-β,D-glucopyranoside	Triton X-100
2.25×10^5	80.4 ± 52.1	101.2 ± 2.1
4.50×10^5	60.4 ± 9.6	97.3 ± 16.8
7.50×10^5	4.45 ± 10.1	80.8 ± 7.6
2.25×10^6	0.4 ± 14.0	38.8 ± 8.5

Conversion of 330 pmol *sn*-1-stearoyl-2-arachidonylglycerol in the presence of four concentrations of endogenous cell lipid, prepared from confluent monolayers of Swiss 3T3 fibroblasts, was measured by the methods described in Fig 4.1.

reaction in the presence of such elevated lipid concentrations. Therefore, unlike the *n*-octyl-β,D-glucopyranoside method, the Triton X-100/PtdSer method is capable of supporting DAG kinase-catalysed phosphorylation of the *sn*-1,2-diacylglycerol present in cell lipid extracts with 100 % efficiency and, thus, complete quantitation of the *sn*-1,2-diacylglycerol present in cell lipid extracts. However, the capacity of the method for cell lipid is finite and the amount from which an *sn*-1,2-diacylglycerol mass determination is made must be carefully titrated to avoid lipid concentrations that are inhibitory to the DAG kinase reaction.

Without further characterisation, the Triton X-100/PtdSer method (Thompson *et al.*, 1990; Paterson *et al.*, 1991) would appear to quantitate the mass of *sn*-1,2-diacylglycerol over the range 100 pmol - 2 nmol, both, in the absence and presence of limited amounts of lipid derived from cell lipid extracts with a degree of

precision not experienced with the *n*-octyl-β,D-glucopyranoside detergent method (Preiss *et al.*, 1986). However, such a conclusion assumes the assay converts differing molecular species of *sn*-1,2-diacylglycerol with an efficiency equal to *sn*-1-stearoyl-2-arachidonylglycerol. This has not been investigated directly but, Walsh and co-workers (1990) observed the V_{max} of the *E. coli* DAG kinase to remain unaltered when incubated with *sn*-1,2-dihexanoylglycerol, *sn*-1,2-dioctanoylglycerol and *sn*-1,2-dioleoylglycerol in an *n*-octyl-β,D-glucopyranoside/phosphatidylglycerol mixed micellar system. The apparent K_m for each substrate was observed to fall with increasing acyl-chain length (*sn*-1,2-dihexanoylglycerol = 2.4 mol%; *sn*-1,2-dioctanoylglycerol = 1.9 mol%; *sn*-1,2-dioleoylglycerol = 0.37 mol%). Although V_{max} and K_m were not determined, MacDonald *et al.* (1988b) reported the *E. coli* DAG kinase to catalyse the phosphorylation of *sn*-1-stearoyl-2-linoleoylglycerol, *sn*-1,2-dioleoylglycerol and *sn*-stearoyl-2-oleoylglycerol in an *n*-octyl-β,D-glucopyranoside mixed micellar system with greater reaction velocity than measured with *sn*-1-stearoyl-2-arachidonylglycerol. This information has been gathered for the reaction velocity of DAG kinase on solubilisation of the *sn*-1,2-diacylglycerol substrate in *n*-octyl-β,D-glucopyranoside but, it does indicate that *sn*-1-stearoyl-2-arachidonylglycerol is a less favourable *sn*-1,2-diacylglycerol substrate than other molecular species, e.g. *sn*-1-stearoyl-2-oleoyolglycerol. Morever, how such initial reaction velocity information extrapolates to a situation of complete phosphorylation of *sn*-1,2-diacylglycerol, as encountered in the *sn*-1,2-diacylglycerol mass quantitation assay, is unknown. Although information has not been gathered for the substrate selectivity of the *E.* coli DAG kinase in a Triton X-100 assay system, the above data may indicate, however, that in a non-ionic detergent mixed micellar assay system the accuracy of quantitation of long-chain *sn*-1,2-diacylglycerols is assured if *sn*-1-stearoyl-2-arachidonylglycerol is observed to be converted to phosphatidic acid with 100 % efficiency under identical assay conditions.

It should be noted in the discussion of the application of enzymatic determination of *sn*-1,2-diacylglycerol that the *E. coli* DAG kinase can catalyse the phosphorylation of *sn*-1-*O*-alkyl-2-acylglycerol and *sn*-1-*O*-alkenyl-2-acylglycerol in *n*-octyl-β,D-glucopyranoside-based reaction mixtures with similar reaction velocities to those determined for *sn*-1,2-diacylglycerol substrates (Ford & Gross, 1990). Similarly, *sn*-1-*O*-alkyl-2-acylglycerol and *sn*-1-*O*-alkenyl-2-acylglycerol together with *sn*-1,2-diacylglycerol are phosphorylated in the Triton X-100/PtdSer method (Thompson *et al.*, 1990). As a result, all three *sn*-1,2-diradylglycerol classes, i.e. *sn*-1,2-diacylglycerol, *sn*-1-*O*-alkyl-2-acylglycerol and *sn*-1-*O*-alkenyl-2-acylglycerol, are phosphorylated in these enzymatic methods without discrimination. In this context, without prior knowledge of the *sn*-1,2-diradylglycerol subclass population, the assay must be considered as quantitating the *sn*-1,2-diradylglycerol content of cell lipid extracts. *sn*-1,2-Diacylglycerol mass can, however, be determined specifically if the *sn*-1-*O*-alkyl-2-acylglycerol and *sn*-1-*O*-alkenyl-2-acylglycerol mass within the total *sn*-1,2-diradylglycerol population is determined by enzymatic analysis employing phospholipase A_1 in combination with the DAG kinase enzymatic *sn*-1,2-diacylglycerol mass determination method (Thompson *et al.*, 1990; Tyagi *et al.*, 1989).

4.2. Kinetic Characterisation of the KCl-Soluble DAG Kinase: Introduction.

The aim of this project was to purify the DAG kinase involved in phosphorylating the *sn*-1,2-diacylgycerol produced on receptor-stimulated PtdIns(4,5)P_2 hydrolysis. This specific DAG kinase isoenzyme would appear to be identified by its substrate selectivity (Section 1.8.1). It catalyses the phosphorylation of *sn*-1-acyl-2-arachdonylglycerols with greater reaction velocities than when non-arachidonyl *sn*-1,2-diacylglycerols are employed as substrate. This highlights a property of the enzyme that can be employed in assessing a DAG kinase isoenzyme's contribution to the metabolism of *sn*-1,2-diacylglycerol

generated from PtdIns(4,5)P_2 hydrolysis. In addition, the *sn*-1-acyl-2-arachidonylglycerol selectivity of the PtdIns(4,5)P_2-linked DAG kinase was employed to facilitate the isolation of this isoenzyme from the non-selective enzymes encountered in tissue homogenates (Section 1.9).

The kinetic properties of the KCl-soluble DAG kinase shall now be investigated. This enzyme has been partially purified by the strategy proposed in Section 1.9. However, it remains to be determined if this enzyme is the DAG kinase involved in phosphorylating the PtdIns(4,5)P_2-derived *sn*-1,2-diacylglycerol. This chapter will compare some of the catalytic properties of the KCl-soluble enzyme of rat brain to those of the PtdIns(4,5)P_2-linked DAG kinase of Swiss 3T3 fibroblasts. This will establish if the enzymes share any common propetics and, thus, verify if the KCl-soluble DAG kinase is involved in the metabolism of PtdIns(4,5)P_2-derived *sn*-1,2-diacylglycerol. The activation of both these enzymes by PtdSer will also be investigated. When assayed in an *n*-octyl-β,D-glucopyranoside mixed micellar solution, the *sn*-1-acyl-2-arachidonylglycerol-selective enzyme of Swiss 3T3 fibroblasts is activated by PtdSer. Inclusion of 6 mol% PtdSer results in an elevation of the enzyme's V_{max} and also a reduction of its apparent K_m for *sn*-1-stearoyl-2-arachidonylglycerol (MacDonald *et al.*, 1988b). Therefore, activation of the KCl-soluble DAG kinase by PtdSer will be compared to that of the Swiss 3T3 fibroblast enzyme. Comparison of the *sn*-1,2-diacylglycerol substrate selectivity of the KCl-soluble and Swiss 3T3 DAG kinases and also the effects of PtdSer on their activity will provide some indication of the rat brain enzyme's ability to function as the DAG kinase involved in the phosphorylation of PtdIns(4,5)P_2-derived *sn*-1,2-diacylglycerol.

This chapter will also examine the ATP, Mg^{2+}, and Ca^{2+} concentration dependence of the KCl-soluble DAG kinase's reaction velocity.

The kinetic characterisation of the KCl-soluble DAG kinase has been performed with post gel filtration or post heparin-agarose preparations of the enzyme. These have been prepared by the existing purification protocol which is

summarised in Section 3.12. In both cases, the DAG kinase is present as single species, which migrates on gel filtration and heparin-agarose chromatography as a single peak of activity.

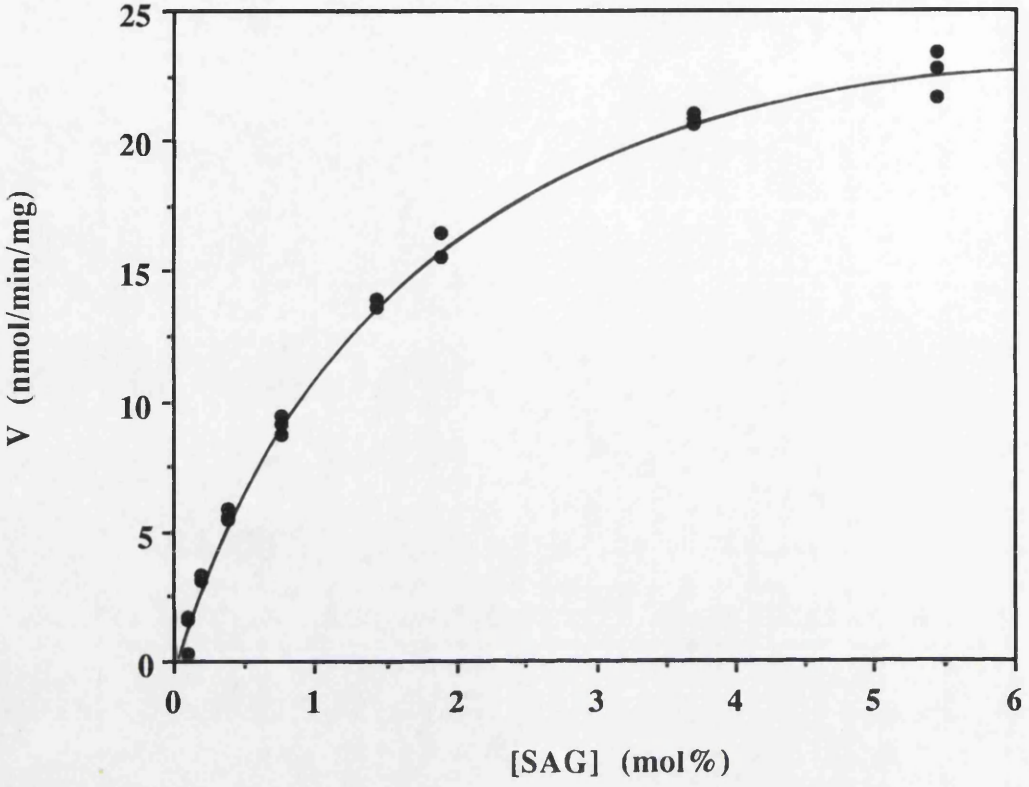
4.3. *sn*-1,2-Diacylglycerol Concentration Dependence.

4.3.1. Determination of Apparent K_m and V_{max} Values with *sn*-1-Stearoyl-2-Arachidonylglycerol as Substrate.

The KCl-soluble DAG kinase's rate of reaction increased as a hyperbolic function of *sn* -1-stearoyl-2-arachidonylglycerol concentration (Fig 4.2A). Over the range of *sn*-1-stearoyl-2-aqrachidonylglycerol concentrations employed, a maximal rate of reaction was not observed. The concentration of *sn* -1-stearoyl-2-arachidonylglycerol was expressed as a mol fraction of the *n*-octyl-β,D-glucopyranoside/PtdSer/DAG mixed micellar phase in the reaction mixture and the data fitted to the Michaelis-Menten kinetic model. Under these conditions, the post gel filtration enzyme was found to have a V_{max} of 29.9 nmol/min/mg and an apparent K_m of 1.7 mol%. These fitted values of V_{max} and apparent K_m correspond to those determined from Eadie-Hofstee analysis of the experimental data (Fig 4.2B).

The apparent K_m of the KCl-soluble DAG kinase for *sn*-1-stearoyl-2-arachidonylglycerol is similar to that of the membrane-associated enzyme of Swiss 3T3 fibroblasts (MacDonald *et al.*, 1988b). Using a similar *n* -octyl-β,D-glucopyranoside mixed micellar assay system, MacDonald and co-workers (1988b) determined the apparent K_m of the Swiss 3T3 enzyme, an *sn*-1-acyl-2-arachidonylglycerol-selective enzyme, to be 1.2 mol%, when measured in the presence of 6 mol% PtdSer. This compares to the 1.7 mol% determined for the rat brain KCl-soluble DAG kinase when measured in the presence of 7.8 mol% PtdSer. Under the detergent and phospholipid conditions employed, the apparent K_m of 1.7 mol% is comparable to 890 μM when expressed as a bulk concentration. This value is nearly 10-fold greater than the apparent K_m of 90 μM determined for

A


25
20
15
10
5
0
V (nmol/min/mg)
0
1
2
3
4
5
6
[SAG] (mol%)

B

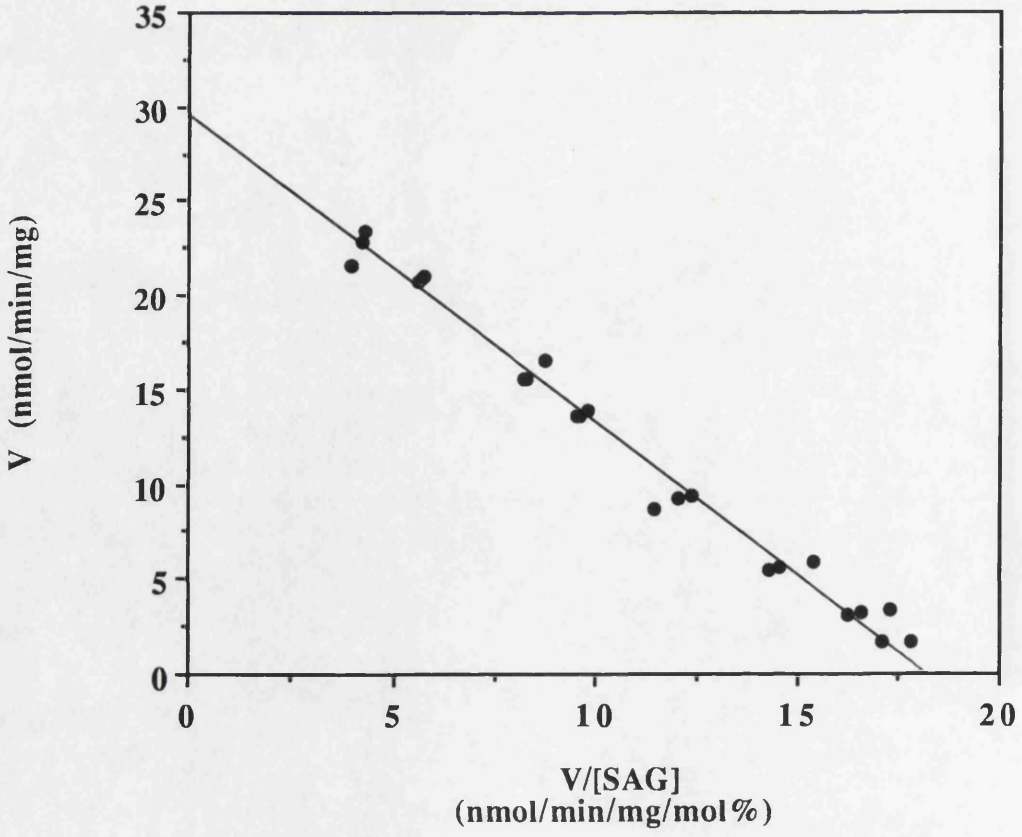

35
30
25
20
15
10
5
0
V (nmol/min/mg)
0
5
10
15
20
V/[SAG]
(nmol/min/mg/mol%)

Fig 4.2. DAG Kinase Reaction Velocity: Concentration Dependence on *sn*-1-Stearoyl-2-Arachidonylglycerol.

A. *sn*-1-stearoyl-2-arachidonylglycerol concentration dependence of the KCl-soluble DAG kinase's reaction velocity.

B. Eadie-Hofstee plot of data shown in A.

The DAG kinase rate of reaction was determined by the *n*-octyl-β,D-glucopyranoside/PtdSer assay method (Section 2.4.3) in the presence of the indicated concentrations of *sn*-1-stearoyl-2-arachidonylglycerol and 1.0 mM ATP. The reaction velocity was determined with 20 μl of the post gel filtration enzyme preparation diluted to 50 μl with buffer E. After 15 min, the reaction was terminated and the [^{32}P]phosphatidic acid quantitated as described in Section 2.4.3. The protein concentration of the DAG kinase preparation was determined by the Coomassie dye method (Section 2.2.3).

Table 3. Reported K_m and V_{max} Values of Selected DAG Kinases.

Tissue	Location (cytosol/membrane)	Mass (kDa)	V_{max} (μmol/min/mg)	K_m for ATP (μM)	K_m for DAG	Reference
pig brain	cytosol	80	8.0	300	60 μM [2]	Kanoh *et al.* (1983) [A]
rat brain	cytosol	110	11.5	125	70 μM [3]	Kato & Takenawa (1990) [A]
rat brain	membrane	150	5.2	250	90 μM [3]	Kato & Takenawa (1990) [A]
rat liver	cytosol	121	1.49	170	3.0 μM [1]	Kanoh & Ohno (1981) [A]
Swiss 3T3 fibroblast	membrane	N.D.	1.28×10^{-3}	N.D.	1.2 mol% [3]	MacDonald *et al.* (1988b) [B]
rat brain KCl-soluble	membrane	160	29.9×10^{-3} *	330 μM	1.7 mol % [3]	This thesis [B]

The above enzymes were assayed by the following methods: [A], dispersed deoxycholate/*sn*-1,2-diacylglycerol; [B], *n*-octyl-β,D-glucopyranoside/PtdSer mixed micelle.

The *sn*-1,2-diacylglycerol substrates employed were as follows: [1], *sn*-1,2-diacylglycerol prepared from egg PtdCho; [2], *sn*-1,2-dioleoylglycerol; [3], *sn*-1-stearoyl-2-arachidonylglycerol.

* The V_{max} value given for the KCl-soluble DAG kinase was determined with the post gel filtration preparation of the enzyme.

N.D., not done.

the 150 kDa DAG kinase of rat brain with the same *sn*-1,2-diacylglycerol substrate (see Table 3). However, as the *sn*-1,2-diacylglycerol concentration dependence of the 150 kDa DAG kinase's reaction velocity was examined with a deoxycholate dispersion assay, no true comparison can be made between this K_m and the value determined for the KCl-soluble enzyme by a mixed micellar system (see Section 5.5). Similarly, due to the differences in DAG kinase reaction velocities obtained by different methods of assay, no comparison can be made between the V_{max} values obtained for the previously purified 150 kDa DAG kinase and the KCl-soluble DAG kinase. Although the K_m values may be comparable between the KCl-soluble DAG kinase and the membrane-associated enzyme of Swiss 3T3 fibroblasts, the V_{max} values obtained for these activities cannot be compared. Despite reaction velocity being measured under near-identical conditions, the Swiss 3T3 DAG kinase is prepared solely by fractionation of the total membrane component of a cell lysate, whereas the post gel filtration preparation of KCl-soluble enzyme has been purified 7-fold from the particulate fraction of rat brain (see Table 1). Therefore, their disparate levels of purity prevents a meaningful comparison of their V_{max} values.

4.3.2. Determination of V_{max} and Apparent K_m Values with *sn*-1,2-Dilauroylglycerol and *sn*-1,2-Dioleoylglycerol as Substrates. Is the Kinase Selective for its Substrate?

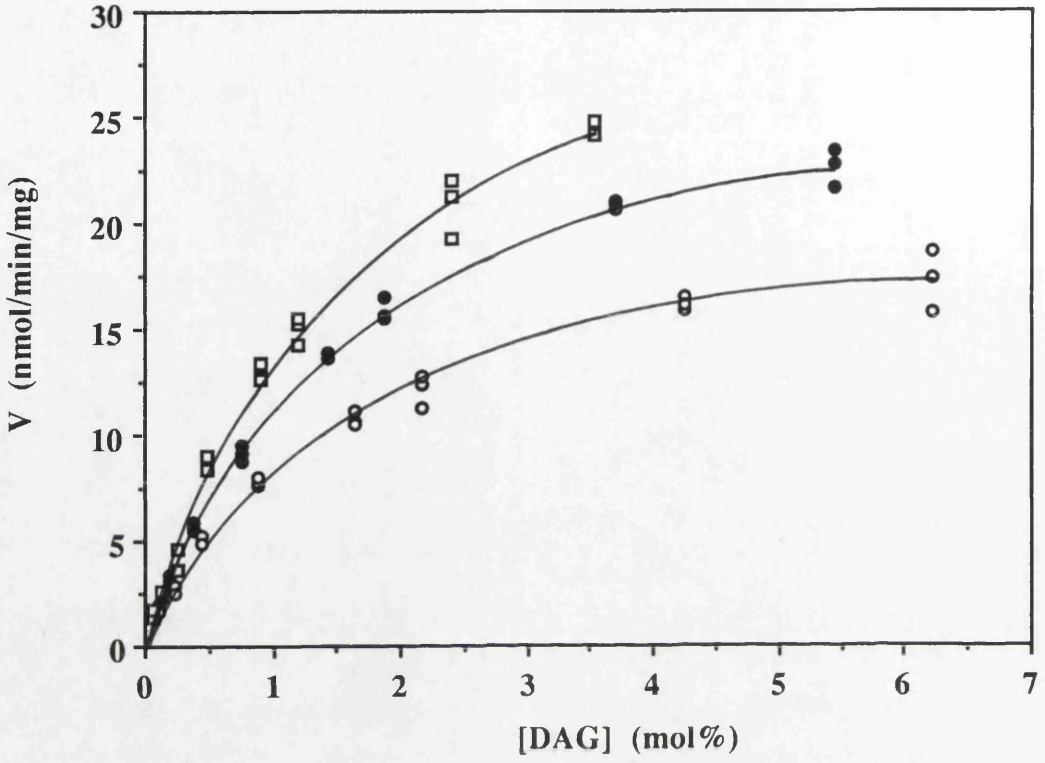
As discussed in Section 1.8.1, the DAG kinase which phosphorylates the *sn*-1,2-diacylglycerol produced Swiss 3T3 fibroblasts on PDGF stimulation is membrane-associated and selective for *sn*-1,2-diacylglycerols possessing arachidonate in the 2-acyl position (MacDonald *et al.*, 1988a, b). Further DAG kinases which are selective for *sn*-1-acyl-2-arachidonylglycerol substrates have been identified in rat brain microvessels (Hee-Chong *et al.*, 1985), rabbit aorta (Severson & Hee-Chong, 1986), and baboon brain (Lemaitre *et al.*, 1990). To assess if the KCl-soluble DAG kinase is selective towards different *sn*-1,2-

diacylglycerol substrates, especially *sn*-1-acyl-2-arachidonylglycerol, the enzyme's V_{max} and apparent K_m were determined with *sn*-1,2-dioleoylglycerol and *sn*-1,2-dilauroylglycerol and the values obtained compared to those calculated with *sn*-1-stearoyl-2-arachidonylglycerol.

The reaction velocity of the post gel filtration KCl-soluble DAG kinase increased with increasing concentrations of both *sn*-1,2-dioleoylglycerol and *sn*-1,2-dilauroylglycerol, the rates increasing as a hyperbolic function of *sn*-1,2-diacylglycerol concentration in both cases (Fig 4.3A). Expressing the concentrations of *sn*-1,2-dioleoylglycerol and *sn*-1,2-dilauroylglycerol as a mol fraction of the *n*-octyl-β,D-glucopyranoside/PtdSer/*sn*-1,2-diacylglycerol mixed micellar phase in the assay mixture, the data was fitted to the Michaelis-Menten kinetic model. The enzyme possesses an apparent K_m of 1.6 mol% for both *sn*-1,2-dioleoylglycerol and *sn*-1,2-dilauroylglycerol. The fitted V_{max} values were calculated as 34.8 nmol/min/mg and 21.7 nmol/min/mg for *sn*-1,2-dioleoylglycerol and *sn*-1,2-dilauroylglycerol, respectively. These values agree with those determined by Eadie-Hofstee analysis of the same data (Fig 4.3B).

Comparison of these values to those obtained with *sn*-1-stearoyl-2-arachidonylglycerol (apparent K_m = 1.7 mol% and V_{max} = 29.9 nmol/min/mg) suggests that the KCl-soluble DAG kinase does not exhibit any selectivity towards *sn*-1-acyl-2-arachidonylglycerols. The enzyme displayed similar apparent K_m values for all three *sn*-1,2-diacylglycerol substrates with only small alteration in the V_{max} between substrates. The specificity constant V_{max}/K_m was calculated as 17.6, 21.8, and 13.6 nmol/min/mg/mol% for *sn* -1-stearoyl-2-arachidonylglycerol, *sn*-1,2-dioleoylglycerol and *sn*-1,2-dilauroylglycerol, respectively. Although these values would suggest that the KCl-soluble enzyme displays some specificity for *sn*-1,2-dioleoylglycerol, this was not observed consistently. Routinely, similar V_{max} and apparent K_m values were determined for the KCl-soluble DAG kinase with the above three *sn*-1,2-diacylglycerol substrates. Thus, the KCl-soluble enzyme of rat brain is not *sn*-1-acyl-2-arachidonylglycerol-selective as it neither possesses a

A


30
25
20
15
10
5
0
V (nmol/min/mg)
0
1
2
3
4
5
6
7
[DAG] (mol%)

B

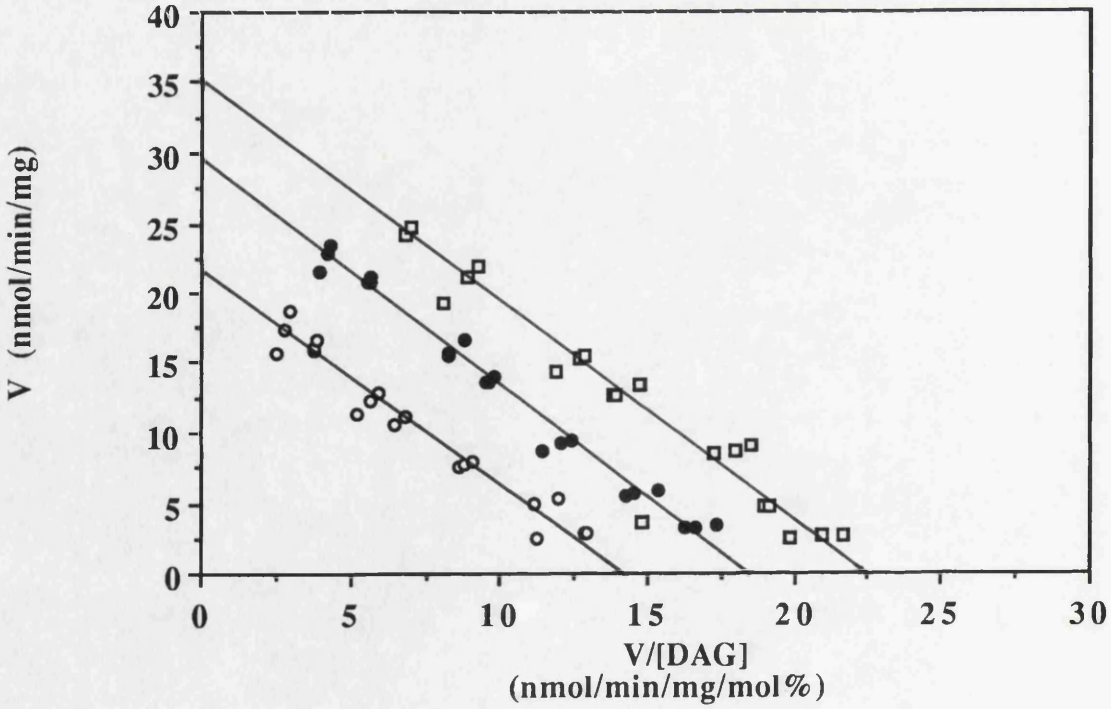

40
35
30
25
20
15
10
5
0
V (nmol/min/mg)
0
5
10
15
20
25
30
V/[DAG]
(nmol/min/mg/mol%)

Fig 4.3. DAG Kinase Reaction Velocity: Concentration Dependence on *sn*-1,2-Dialaurylglycerol, *sn*-1,2-Dioleoylglycerol and *sn*-1-Stearoyl-2-arachidonylglycerol.

A Concentration dependence on *sn*-1,2-dilauroylglycerol, *sn*-1,2-dioleoylglycerol and *sn*-1-stearoyl-2-arachidonylglycerol.

B. Eadie-Hofstee plot of data shown in A.

The DAG kinase reaction velocity was determined by the *n*-octyl-β,D-glucopyranoside/PtdSer assay method (Section 2.4.3) in the presence of the indicated concentrations of *sn*-1,2-diacylglycerols, as appropriate, and 1.0 mM ATP. 20 μl of the post gel filtration preparation of the KCl-soluble DAG kinase (1.95 μg protein) was diluted to 50 μl with buffer E and employed to determine the reaction velocity. After 15 min, the reaction was terminated and the [^{32}P]phosphatidic acid quantitated as described in Section 2.4.3. The protein concentration of the DAG kinase preparation was determined by the Coomassie dye method (Section 2.2.3). [□, *sn*-1,2-dioleoylglycerol; ●, *sn*-1-stearoyl-2-arachidonylglycerol; ○, *sn*-1,2-dilaurylglycerol]

reduced K_m for *sn*-1-stearoyl-2-arachidonylglycerol compared to *sn*-1,2-dioleoylglycerol or *sn*-1,2-dilaurylglycerol, nor does it catalyse the phosphorylation of the *sn*-1-acyl-2-arachidonylglycerol with increased V_{max}. This is different from the *sn*-1-acyl-2-arachidonylglycerol-selective DAG kinase of Swiss 3T3 fibroblasts (MacDonald *et al.*, 1988b). This enzyme catalyses the phosphorylation of *sn*-1-stearoyl-2-arachidonylglycerol with a rate 2- to 8-fold higher than other *sn*-1,2-diacylglycerols, including *sn*-1,2-dioleoylglycerol. This is despite the fibroblast enzyme being incubated with *sn*-1,2-diacylglycerols concentrations capable of supporting maximal reaction velocities. Similarly, the *sn*-1-acyl-2-arachidonylglycerol-selective DAG kinase of baboon brain catalyses the phosphorylation of *sn*-1-acyl-2-arachidonylglycerols with a reaction rate 3-fold higher than those measured with other *sn*-1,2-diacylglycerols (Lemaitre *et al.*, 1990). Therefore, the KCl-soluble DAG kinase is not comparable to any previously characterised *sn*-1-acyl-2-arachidonylglycerol-selective enzyme and is unlikely to be the rat homologue of the selective enzymes identified in Swiss 3T3 fibroblasts and baboon brain. It may be possible that the KCl-soluble DAG kinase loses its physiological selectivity for *sn*-1,2-diacylglycerol species when assayed in the *n*-octyl-β,D-glucopyranoside/PtdSer mixed micellar system. Possibly the KCl-soluble enzyme could catalyse the phosphorylation of *sn*-1-acyl-2-arachidonylglycerol with elevated velocity compared to non-arachidonyl *sn*-1,2-diacylglycerols in the environment of the cell membrane, but loses this ability when incubated with substrate in a detergent mixed micelle. This is discussed further in Section 5.5. In addition, it must be acknowledged that the above investigation of *sn*-1,2-diacylglycerol selectivity of the KCl-soluble DAG kinase is made in the absence of a positive control. As discussed above, several groups have demonstrated *sn*-1-acyl-2-arachidonylglycerol-selective DAG kinases using the *n*-octyl-β,D-glucopyranoside/PtdSer assay method. However, a positive control in the form a selective enzyme was not included in the study reported here. Such a control could be provided by the DAG kinase associated with the membranes of

Swiss 3T3 fibroblasts (MacDonald *et al.*, 1988b). In the absence of such a control it is not apparent if the assay method would have allowed identification of an arachidonyl-selective enzyme.

Finally, it should be noted that the conclusions made about the substrate selectivity of the KCl-soluble DAG kinase assume that the *sn*-1,2-diacylglycerol concentrations were accurately determined. The enzymatic method described in Section 2.7 and in Paterson *et al.* (1991), which utilises the DAG kinase of *E. coli*, was employed to measure the concentrations of the *sn*-1,2-diacylglycerol stock solutions. This allowed the concentrations of *sn*-1,2-dioleoylglycerol and *sn*-1,2-dilaurylglycerol to be normalised to that of *sn*-1-stearoyl-2-arachidonylglycerol. However, this does assume that the *E. coli* DAG kinase assay quantitates *sn*-1,2-diacylglycerols of different acyl-chain composition with equal identical efficiency. This is discussed further in Section 2.7.2.

4.4. ATP Concentration Dependence: Determination of V_{max} and Apparent K_m Values.

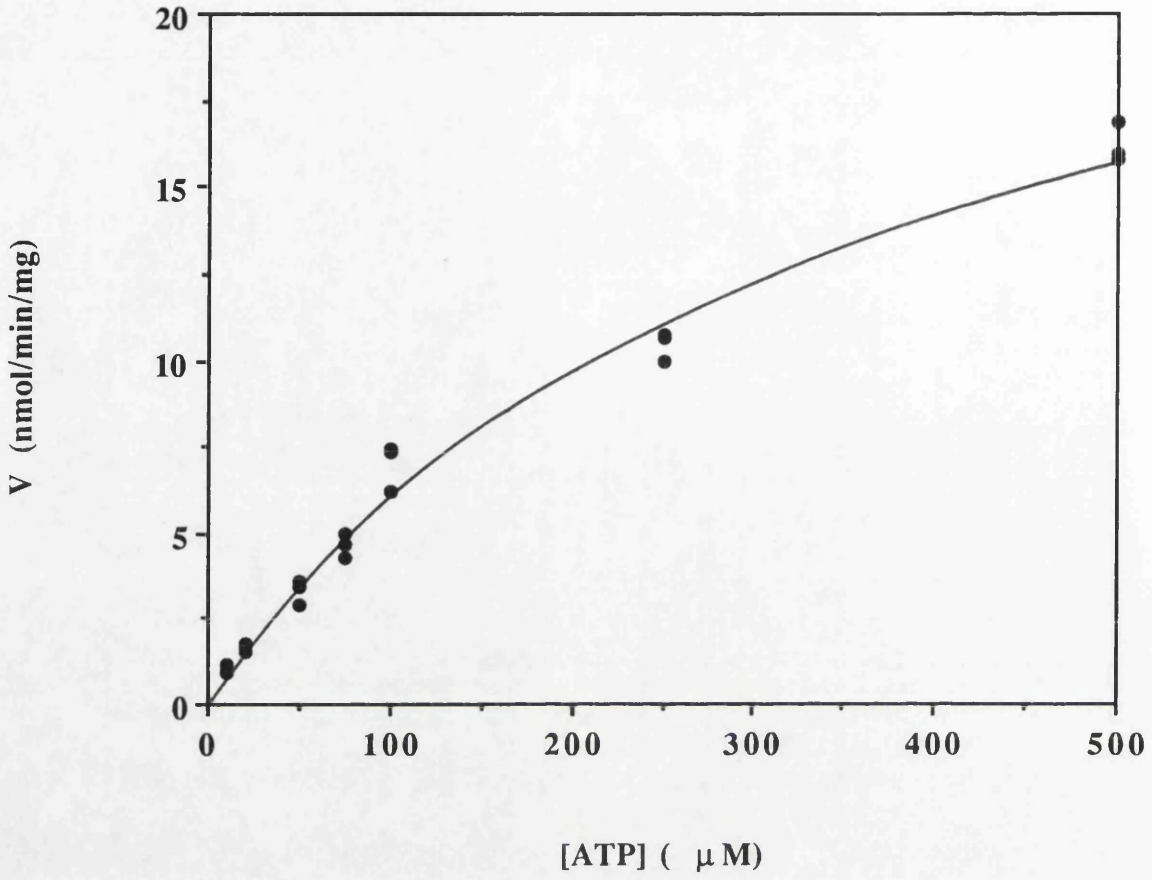
The reaction velocity of the KCl-soluble DAG kinase was measured over a range of ATP concentration (10 - 500 μM), under conditions of near-saturating substrate concentrations with respect to *sn* -1-stearoyl-2-arachidonylglycerol (Fig 4.4A).

Employing the *n*-octyl-β,D-glucopyranoside/PtdSer mixed micellar methodwith 3.7 mol% (2.0 mM) *sn*-1-stearoyl-2-arachidonylglycerol, the reaction velocity of the KCl-soluble DAG kinase increased as a hyperbolic function of ATP concentration (Fig 4.4A). Although maximal rates of reaction were not obtained over the above range of ATP concentrations, near maximal rates have been obtained with greater than 1.0 mM ATP (data not shown). The data was fitted to a Michaelis-Menten kinetic model and the V_{max} and apparent K_m of the KCl-soluble DAG kinase for ATP determined as 26.4 nmol/min/mg and 330 μM respectively. These values were found to correspond to those values determined graphically by

an Eadie-Hofstee plot (Fig 4.4B). It is noticeable in the Eadie-Hofstee plot that the experimental values deviate from the fitted line. However, a Hill plot of the experimenta data was linear, with no deviation of the experimental values from the fitted line (Fig 4.4C). The Hill coefficient was determined as 0.93 and was taken to indicate that the enzyme was not allosterically regulated by increasing concentrations of ATP. The deviation of the experimental values from the fitted Eadie-Hofstee plot at low reaction velocities is most likely due to problems associated with accurate quantitation of the ATP concentrations within the assay incubation mixture. Eadie-Hofstee transformation of the experimental data served to emphasise this error and led to the deviation, whereas this did not occur with the Hill transformation.

The apparent K_m of the KCl-soluble DAG kinase for ATP (330 μM) is similar to the K_m values determined for several other DAG kinases. These values range from 125 to 300 μM (Table 3) and are all somewhat lower than the intracellular concentrations of ATP. This is illustrated in the example of rat brain where the steady-state concentration of ATP is 2.5 mM (Lehninger, 1982), yet the cytosolic and membrane-associated DAG kinases purified from this tissue by Kato and Takenawa (1990) have K_m values of 125 μM and 250 μM, respectively, and the KCl-soluble enzyme has a K_m for ATP of 330 μM. This would seem to indicate that all three rat brain activities discussed here, would operate at near maximal rates of reaction with respect to ATP concentration. This would indicate cellular DAG kinase activity is not modulated by intracellular ATP concentrations, but by another mechanism, e.g. by *sn*-1,2-diacylglycerol concentration or the enzyme's phosphorylation state. If cellular DAG kinase activity were to be regulated by intracellular ATP concentrations, the K_m of the enzyme would require to be altered by a process independent of ATP concentration, e.g. alteration of the enzymes' K_m for ATP in response to phosphorylation/dephosphorylation of the protein or Ca^{2+}-binding to a regulatory domain, as is observed with the porcine 80 kDa DAG kinase (Sakane *et al.*, 1991) Until methods that would allow

A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10
5
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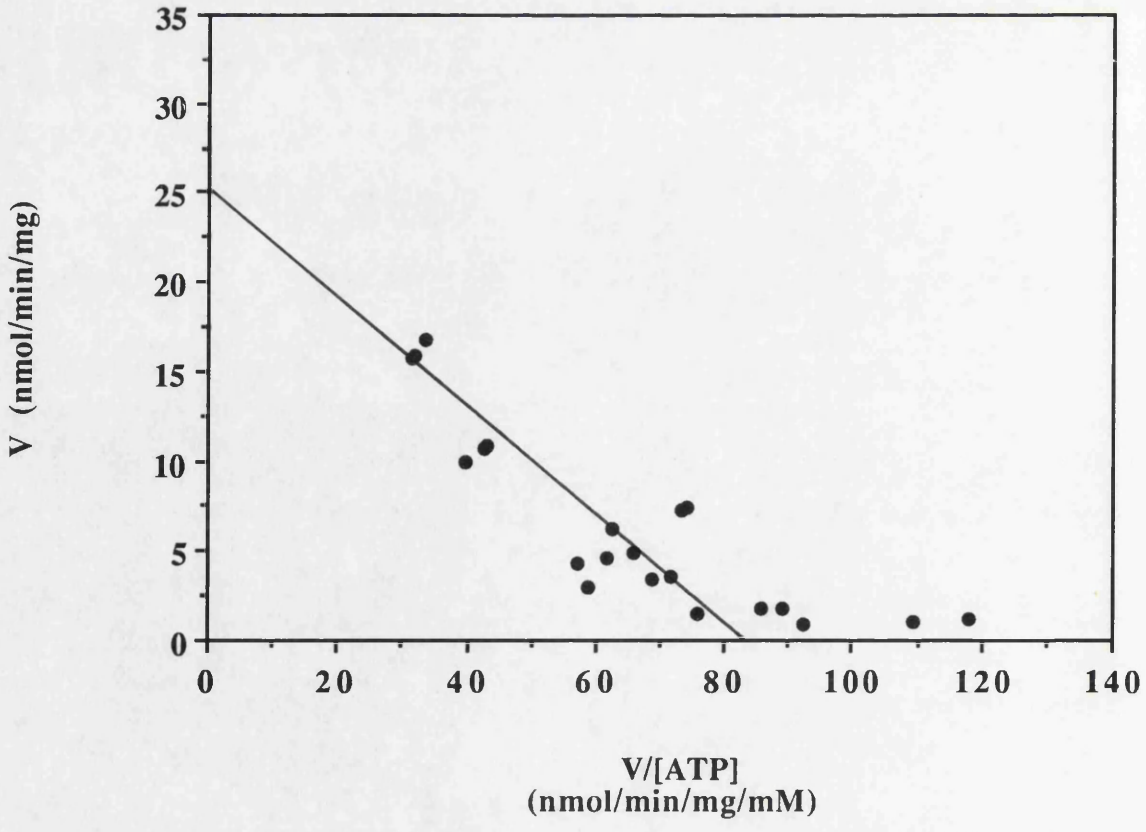
100
200
300
400
500
[ATP] (μM)

B


35
30
25
20
15
10
5
0
V (nmol/min/mg)
0
20
40
60
80
100
120
140
V/[ATP]
(nmol/min/mg/mM)

C

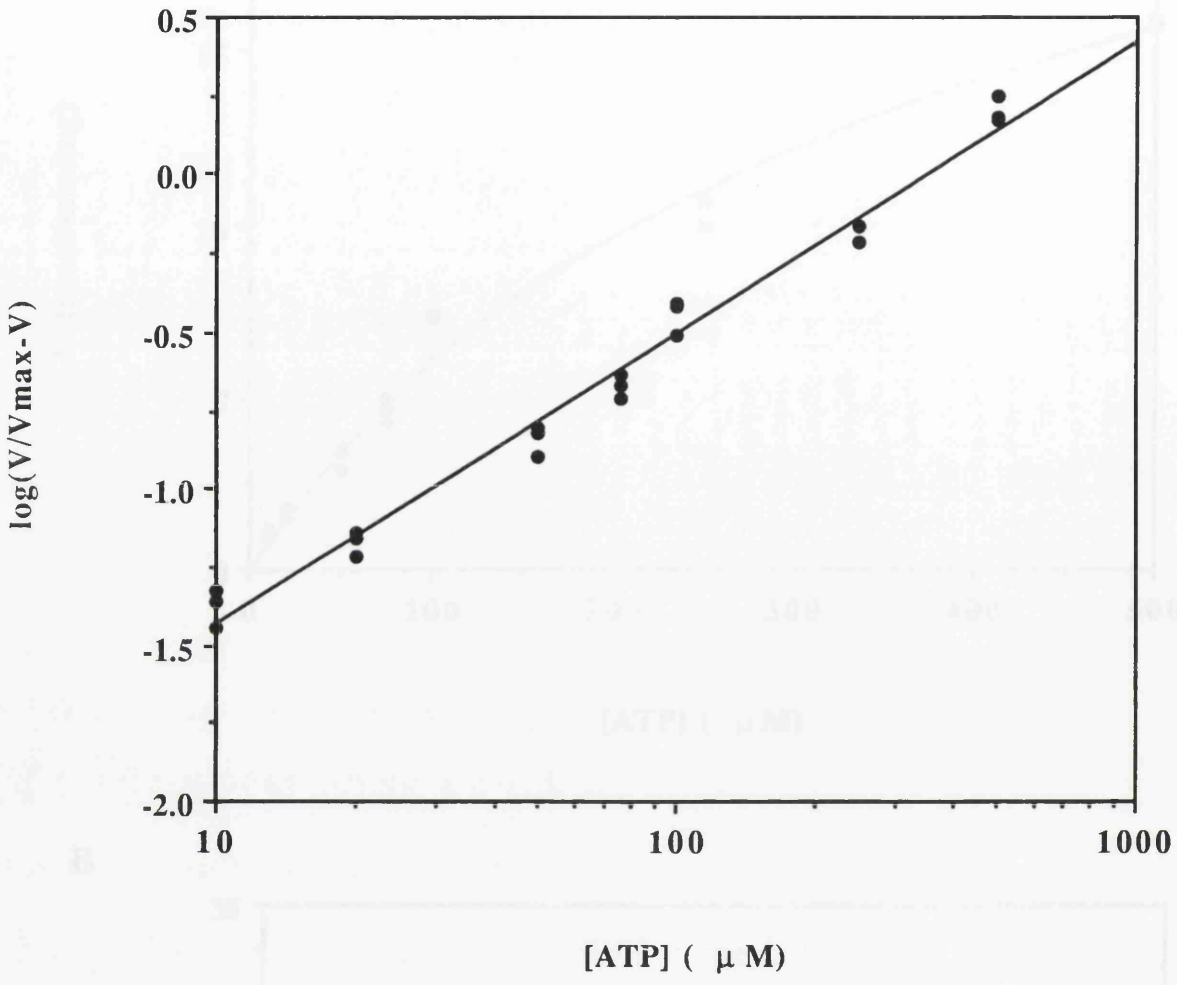


Fig 4.4. DAG Kinase Reaction Velocity: Concentration Dependence on ATP.

A. ATP concentration dependence of the KCl-soluble DAG kinase's reaction velocity.

B. Eadie-Hofstree plot of data shown in A.

C. Hill plot of the data shown in A.

DAG kinase reaction rate was determined as described in Fig 4.2. 20 μl of the post gel filtration preparation of the KCl-soluble DAG kinase (1.95 μg protein) was diluted to 50 μl with buffer E and incubated with 2.0 mM (3.7 mol%) *sn* -1-stearoyl-2-arachidonylglycerol and the indicated concentrations of ATP. The protein concentration of the KCl-soluble DAG kinase preparation was determined as decribed in Fig 4.2.

the study of such changes in DAG kinase's kinetic parameters on cell activation are developed, this question remains open.

Further characterisation of the nucleotide requirements of KCl-soluble DAG kinase and, indeed, the concentration dependence of the enzyme on nucleotides as substrates or competitive inhibitors was not investigated. It should, however, be noted that the reaction rate of the DAG kinase partially purified from rat liver cytosol was reduced by 72.1% by the addition of 2.0 mM ADP (K_m = 170 μM) when present with 2.0 mM ATP substrate (Kanoh & Ohno, 1981). Subsequently, ADP was found to inhibit that rat liver enzyme with a K_i of 50 μM (Kanoh & Ohno, 1981). Moreover, both the cytosolic and membrane-associated DAG kinases of rat brain were inhibited 77 % and 66 %, respectively, by 500 μM ADP when assayed with 1.0 mM ATP (Kato & Takenawa, 1990). Thus, from this *in vitro* evidence, it would appear that the cellular DAG kinase activity may be regulated by alteration of the cell's energy charge. Is the physiological reaction velocity of DAG kinase tightly coupled to the energy charge of the cell? As discussed earlier, it would seem that under cellular ATP concentrations, the DAG kinases operate at maximal rates with respect to ATP. However, intracellular ADP concentrations in rat brain, for example, have been reported at 730 μM (Lehninger, 1982). Thus, due to ADP's action as a competitive inhibitor of rat brain and liver DAG kinases (see above), intracellular concentrations of ADP would be expected to reduce the physiological reaction velocity of these enzymes from the values obtained in the presence of ATP alone. Therefore, acceleration of cell metabolism and alteration of the energy charge of the cell may result in modulation of the cellular DAG kinase reaction velocity. Nevertheless, it is more likely that the reaction velocity of PtdOH production within the cell is dependent on the rate of *sn*-1,2-diacylglycerol production within the cell and not inhibition of the DAG kinase activity responsible for its removal. However, inhibition of DAG kinase by cellular concentrations of ADP could dictate the longevity of *sn*-1,2-diacylglycerol within the cell and, thus, the signal modulated by this second messenger. This

observation is tentative and very preliminary in nature as investigation of DAG kinase rates of reaction in the presence of ATP and ADP concentrations equivalent to those experienced in a resting cell and also those experienced during physiological fluctuations of cellular energy charge have still to be undertaken.

4.5. Dependence on Phosphatidylserine for Activity?

The DAG kinase activity in Swiss 3T3 fibroblasts identified by MacDonald *et al.* (1988a) as the cellular activity responsible for the phosphorylation of the *sn*-1,2-diacylglycerol produced in response to PDGF was membrane-associated and apparently selective for *sn*-1-acyl-2-arachidonylglycerol. These workers also observed the DAG kinase reaction rate of Swiss 3T3 cell lysates to be increased by inclusion of PtdSer in the reaction mixture, with the effect being maximal at 6 mol% (MacDonald *et al.*, 1988a). Similar activation was observed with the DAG kinase activity of membranes fractionated from Swiss 3T3 cell lysates (MacDonald *et al.*, 1988b). This activation was found to result both from an increase in the enzyme's V_{max} and a lowering of its apparent K_m for *sn*-1-stearoyl-2-arachidonylglycerol. PtdSer has also been reported to enhance the Ca^{2+}-dependent activation of the porcine cytosolic 80 kDa DAG kinase when the enzyme has been assayed in an *n*-octyl-β,D-glucopyranoside mixed micellar assay system (Sakane *et al.*, 1991a). Although a small increase in the porcine DAG kinase's reaction rate could be measured in the presence of 10 μM Ca^{2+} alone, the presence of 10 μM Ca^{2+} and 20 mol% PtdSer resulted in a 10-fold reduced K_m for ATP and approximately 4-fold higher V_{max} without any significant alteration in the apparent K_m for *sn*-1,2-diacylglycerol (Sakane *et al.*, 1991). However, this phospholipid-dependent activation of the 80 kDa DAG kinase, in the presence of 10 μM Ca^{2+}, was only observed at concentrations above 10 mol% PtdSer and greater than 20 mol% PtdSer was required to provide maximal Ca^{2+}-stimulated activity (Sakane *et al.*, 1991a). These concentrations of PtdSer are somewhat higher than the 6 mol% found to produce maximal activation of the membrane-associated DAG kinase of Swiss 3T3

fibroblast membranes (MacDonald *et al.*, 1988b). This may reflect the difference in the two observed phenomena: a Ca^{2+}/phospholipid dependency of the porcine cytosolic enzyme and a PtdSer dependency, alone, of the Swiss 3T3 membrane-associated activity. Activation of DAG kinase reaction velocity by PtdSer is not limited to these two enzymes; although less well characterised, PtdSer has been reported to increase the reaction rate of the 80 kDa enzyme of pig brain (Kanoh *et al.*, 1983) and platelets (Yada *et al.*, 1990).

To assess if the KCl-soluble DAG kinase was activated by PtdSer, the reaction velocity of the enzyme was measured in the *n*-octyl-β,D-glucopyranoside mixed micellar assay system with increasing concentrations of PtdSer. Increasing the PtdSer concentration of the detergent mixed micelle solution from 0 to 14 mol% resulted in no significant increase in the reaction velocity of the KCl-soluble enzyme (Fig 4.5). The reaction velocity of the enzyme was measured with near-saturating concentrations of ATP and *sn*-1,stearoyl-2-arachidonylglycerol, therefore it can be concluded that no significant alteration in the DAG kinase's V_{max} was observed. However, the effect of PtdSer on the enzyme's apparent K_m for either substrate was not assessed. Furthermore, direct comparison of the KCl-soluble DAG kinase's V_{max} and K_m values in the presence and absence of PtdSer was not made. In the absence of such information and from the information presented in Fig 4.5, it would appear that PtdSer does not activate the KCl-soluble DAG kinase by elevating the enzyme's V_{max}. Thus, the KCl-soluble DAG kinase is unlike the membrane-associated enzyme of Swiss 3T3 fibroblasts as it is neither *sn*-1-acyl-2-arachidonylglycerol-selective in an *n*-octyl-β,D-glucopyranoside mixed micelle, nor is it activated by PtdSer.

4.6. Magnesium Concentration Dependence.

The effect of magnesium on the reaction rate of the KCl-soluble DAG kinase was assessed. The rate of PtdOH formation was determined over the range 6.0×10^{-7} M to 23.4×10^{-3} M Mg^{2+} (Fig 4.6A; personal communication, S.

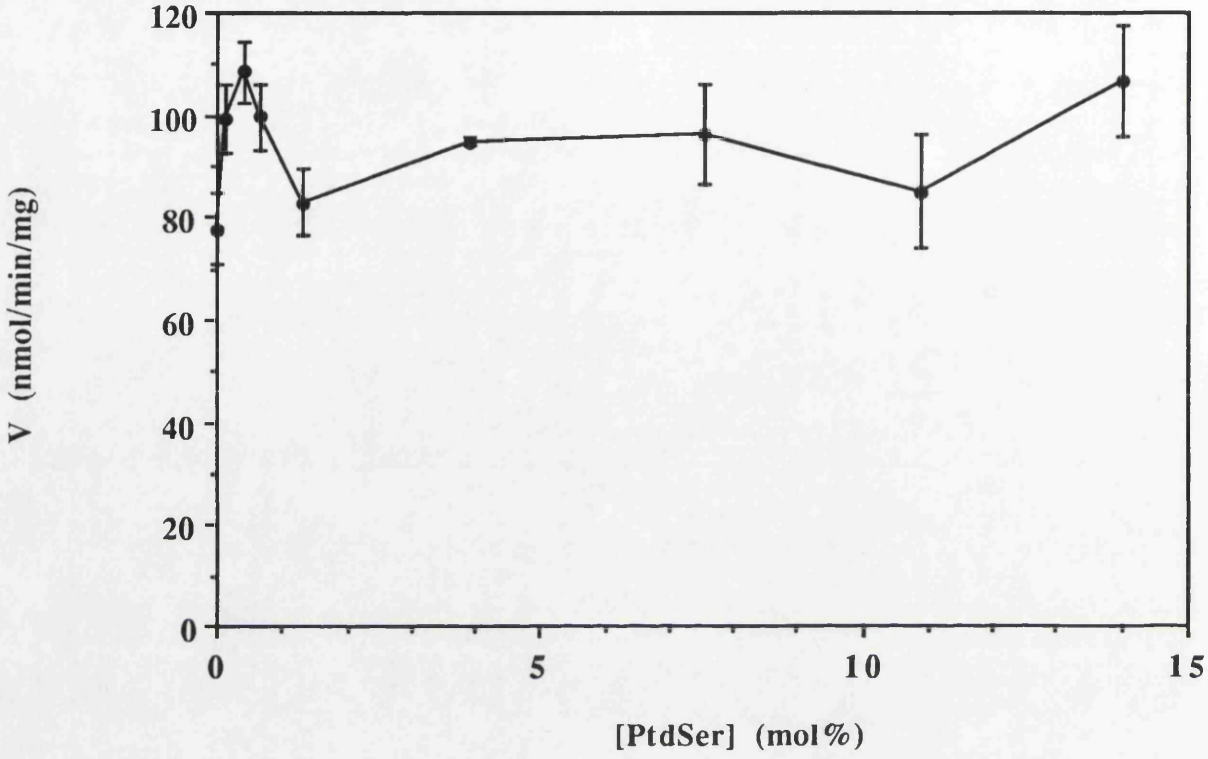

120
100
80
60
40
20
0
V (nmol/min/mg)
0
5
10
15
[PtdSer] (mol%)

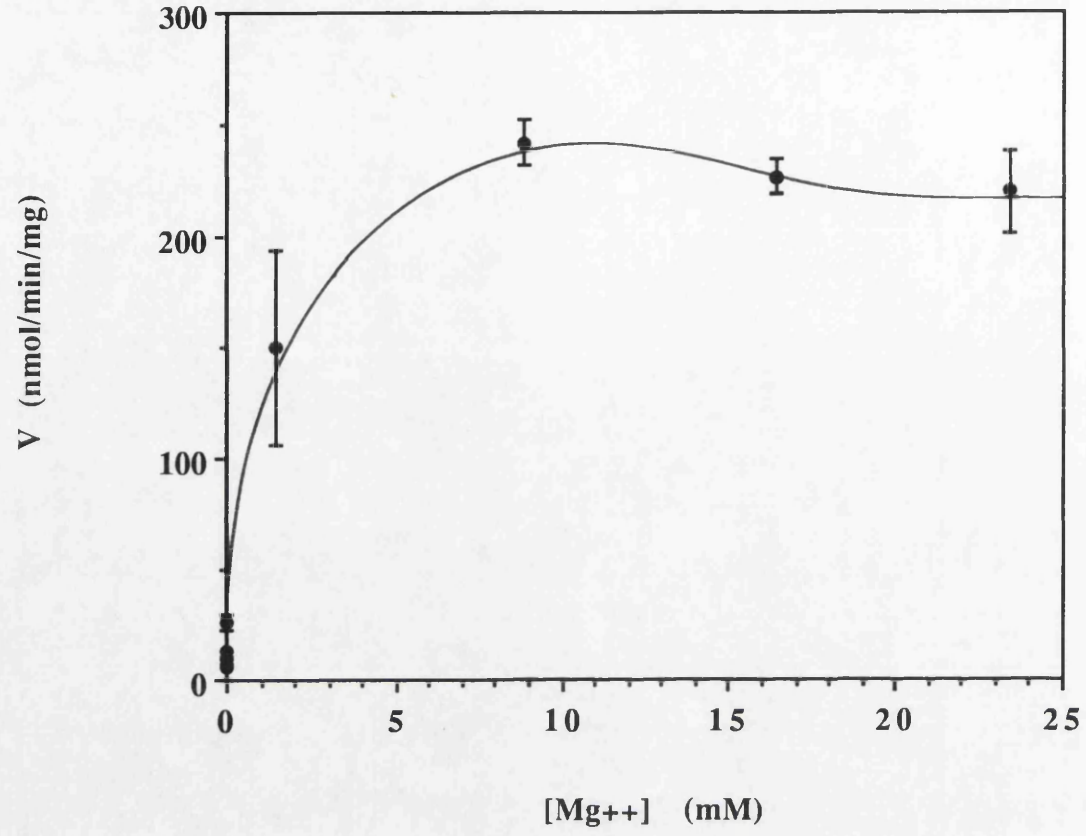
Fig 4.5. Effect of PtdSer on the Reaction Velocity of the KCl-soluble DAG Kinase.

10 μl of the post heparin-agarose preparation of the KCl-soluble DAG kinase (0.8 μg protein) was diluted to 50 μl with buffer E and assayed for DAG kinase activity in the presence of the indicated concentrations of PtdSer, 2.0 mM ATP and 2.0 mM (3.7 mol%, at 7.5 mol% phosphatidylserine) *sn* -1-stearoyl-2-arachidonylglyceol by the method described in Fig 4.2. The protein concentration of the DAG kinase preparation was determined by the Coomassie dye method (Section 2.2.3).

Gardner). The enzyme's rate of reaction was found to increase as a hyperbolic function of Mg^{2+} concentration when measured in the *n*-octyl-β,D-glucopyranoside/PtdSer mixed micellar assay system in the presence of 1.0 mM ATP and 3.7 mol% (2.0 mM) *sn*-1-stearoyl-2-arachidonylglycerol (Fig 4.6A). Although the rate of reaction was not determined at concentrations less than 600 nM, it would appear that the KCl-soluble DAG kinase is dependent on the presence of magnesium for activity, regardless of whether the magnesium concentration is plotted as the sum of free Mg^{2+} and $MgATP^{2-}$ together, or as free Mg^{2+} alone (Fig 4.6B). Maximal rates of reaction were obtained with 7.0 mM free Mg^{2+}, with no further significant increase in the measured rate of reaction at greater concentrations of Mg^{2+} (Fig 4.6B).

The Mg^{2+}-dependence of the KCl-soluble DAG kinase is comparable to that of the porcine 80 kDa enzyme (Kanoh *et al.*, 1983) and the 150 kDa membrane-associated DAG kinase of rat brain.(Kato & Takenawa, 1990). The reaction velocities of both of these purified DAG kinases increase as a hyperbolic function of Mg^{2+} concentration when measured by a deoxycholate/*sn*-1,2-diaylglycerol dispersion assay method, reaching maximal values at 5.0 mM. However, when the deoxycholate in the reaction mixture is replaced by 0.5 mM PtdSer or 0.5 mM PtdCho, the porcine 80 kDa enzyme requires 8.0 and 24.0 mM Mg^{2+}, respectively, for maximal reaction velocities. Not only does the porcine enzyme require greater concentrations of magnesium, in the presence of PtdSer and PtdCho, than the KCl-soluble DAG kinase for maximal reaction velocities, its rate of reaction increased as a sigmoidal rather than hyperbolic function of increasing Mg^{2+} concentration in the presence of these phospholipids (Kanoh *et al.*, 1983). The 110 kDa cytosolic DAG kinase of rat brain was also found to display a sigmoidal dependence on Mg^{2+} for activity, reaching maximal rates at 20.0 mM when assayed in a deoxycholate/*sn*-1,2-diacylglycerol dispersion assay system (Kato & Takenawa, 1990). Nevertheless, such sigmoidal rate dependencies on magnesium haven't, however, been observed with the KCl-soluble DAG kinase.

A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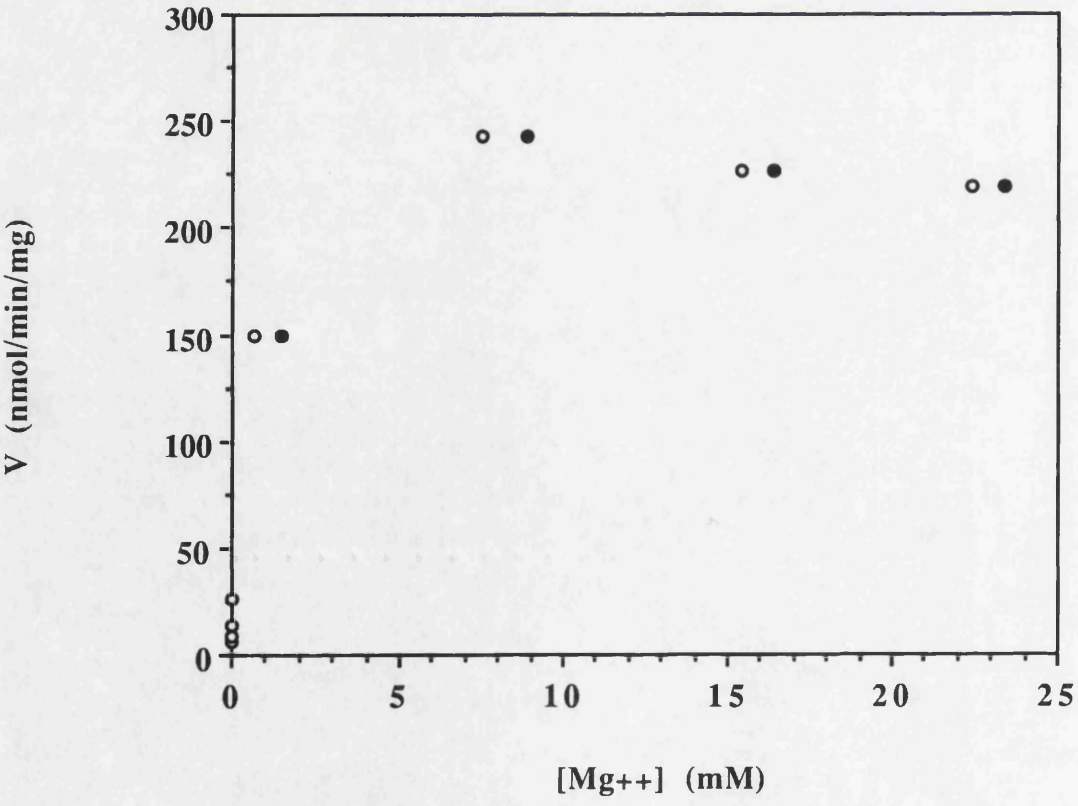
5
10
15
20
25
[Mg++] (mM)

B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50
0
V (nmol/min/mg)
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5
10
15
20
25
[Mg++] (mM)

Fig 4.6. Mg^{2+} Concentration Dependence of DAG kinase Activity.

A. Concentration dependence on free Mg^{2+}.

B. Concentration dependence on free Mg^{2+} compared to the sum of free Mg^{2+} and $MgATP^{2-}$.

20 µl of the post-hydroxyapatite preparation of the KCl-soluble DAG kinase (1.2 µg protein) was diluted to 50 µl with buffer E and assayed for DAG kinase activity. DAG kinase reaction velocity was determined in the presence of 1.0 mM ATP, 2.0 mM (3.7 mol%) *sn*-1-stearoyl-2-arachidonylglyceol and the indicated concentrations of Mg^{2+} by the method described in Fig 4.2. The desired Mg^{2+} concentrations were established by increasing concentrations of Mg^{2+} in the presence of 2.0 mM EDTA and 1.0 mM EGTA in the reaction mixture. The total Mg^{2+} concentration required was calculated with the program React 2, a kind gift from Dr. G. Smith. The protein concentration of the DAG kinase preparation was determined by the Coomassie dye method (Section 2.2.3). [O, free Mg^{2+}; ●, free Mg^{2+} with $MgATP^{2-}$]

Whilst this investigation of the Mg^{2+}-dependency of the KCl-soluble DAG kinase is preliminary in nature, it would seen reasonable to conclude that inclusion of at least 7.0 mM $MgCl_2$ in the reaction mixture allows the enzyme reaction to proceed under conditions where the Mg^{2+} concentration is not limiting. Thus, under the reaction conditions of the DAG kinase assay described in Section 2.4.3, enzyme activity is determined under conditions where reaction velocity is not limited by the Mg^{2+} concentstion.

4.7. Dependence on Calcium for Activity?

Cloning of the purified porcine 80 kDa DAG kinase revealed that the primary structure of this protein possessed two sequences with high levels of homology to the E-F hand motifs typical of calcium binding proteins (Sakane *et al.*, 1990), e.g. calmodulin; Ca^{2+}-activated neutral protease, calpain; troponin C; and calcineurin B (Moncrief *et al.*, 1990). Sakane and co-workers found the activity of the 80 kDa DAG kinase to be enhanced *in vitro* by 10^{-7} - 10^{-4} M Ca^{2+} in the presence of deoxycholate or sphingosine. Furthermore, the porcine 80 kDa DAG kinase bound Ca^{2+} with high affinity (K_d = 0.3 μM) and a stoichiometry of two mol of Ca^{2+} per mol of enzyme (Sakane *et al.*, 1991a). Binding was observed to be cooperative (Hill coefficient = 1.4), with both Ca^{2+} ions required to be bound for Ca^{2+} activation of the enzyme. Activation of the porcine DAG kinase by 10^{-5} M Ca^{2+}, in an *n*-octyl-β,D-glucopyranoside mixed micellar assay, was much greater when in the presence of PtdSer. When measured by this method, activation was observed as a 4-fold elevation of the DAG kinase's V_{max} and a 10-fold reduction in its K_m for ATP , with no alteration in its apparent K_m for *sn*-1,2-diacylglycerol (Sakane *et al.*, 1991a).

Activation by Ca^{2+} is not restricted to the porcine 80 kDa DAG kinase, the reaction velocity of the 110 kDa cytosolic DAG kinase of rat brain is enhanced approximately 5-fold over the range 10^{-5} - 10^{-3} M Ca^{2+} (Kato & Takenawa, 1990). Although requiring greater concentrations of (10^{-4} - 10^{-3} M), the 150 kDa

membrane-associated DAG kinase is also elevated by Ca^{2+} (Kato & Takenawa, 1990). It should be noted, however, that the measured reaction rates of the porcine 80 kDa DAG kinase and both rat brain enzymes are apparently inhibited by greater than 10^{-3} M Ca^{2+}. Therefore, the effect of Ca^{2+} on the reaction rate of the KCl-soluble DAG kinase was investigated. In particular, the effect of increasing the Ca^{2+} concentration through the physiological concentration range of the cation was investigated.

The reaction rate of the partially purified KCl-soluble DAG kinase was measured in the *n*-octyl-β,D-glucopyranoside/PtdSer mixed micellar assay system in the presence of 10^{-8} - 10^{-5} M free Ca^{2+} (Fig 4.7). Under these conditions, the enzyme was unresponsive to alterations of the free Ca^{2+} concentration within the assay mixture. Alteration of the free Ca^{2+} from 10^{-8} to 10^{-5} M in a Ca^{2+}/EGTA buffer system resulted in no significant alteration of the reaction velocity from that obtained at 10 nM Ca^{2+} (151 nmol/min/mg). However, this observation is from a single experiment conducted in triplicate at each Ca^{2+} concentration and, thus, any conclusions drawn must remain tentative.

As the reaction velocity of the KCl-soluble DAG kinase was determined in the presence of 2.0 mM *sn* -1-stearoyl-2-arachidonylglycerol and 2.0 mM ATP (both determined as near-saturating concentrations under identical assay conditions), it would appear that the V_{max} of the enzyme is not altered by increasing the free Ca^{2+} over the range 10^{-8} - 10^{-5} M. The effect of such Ca^{2+} concentrations on the K_m of the enzyme for either *sn* -1-stearoyl-2-arachidonylglycerol or ATP has still to be determined. Therefore, it cannot be concluded that physiological concentrations of Ca^{2+} have no effect on the KCl-soluble enzyme, especially as 10^{-5} M free Ca^{2+} was found to 10-fold reduce the K_m of the porcine 80 kDa DAG kinase for ATP in a similar *n* -octyl-β,D-glucopyranoside/PtdSer assay system (Sakane *et al.*, 1991).

In conclusion, no apparent alteration of the V_{max} of the KCl-soluble DAG kinase was observed as the Ca^{2+} concentration was increased through the

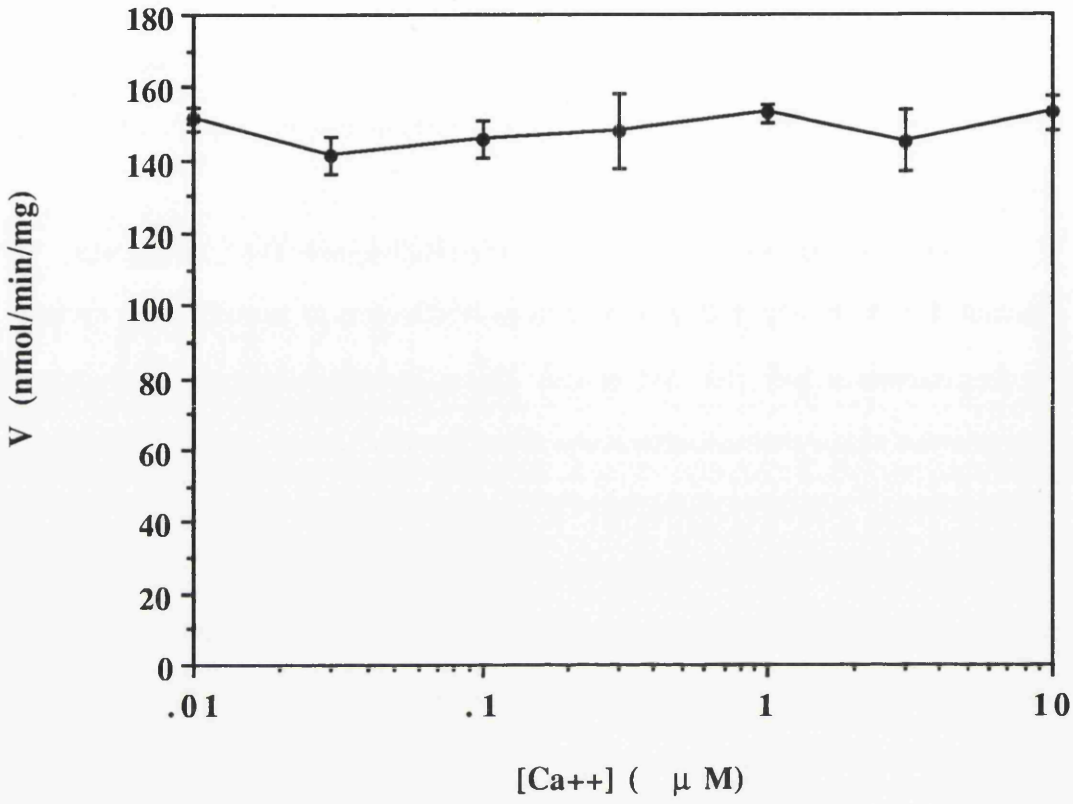

180
160
140
120
100
80
60
40
20
0
V (nmol/min/mg)
.01
.1
1
10
[Ca++] (μ M)

Fig 4.7. Effect of Calcium on the Reaction Velocity of the KCl-Soluble DAG Kinase.

20 μl of the post heparin-agarose preparation of the KCl-soluble DAG kinase (1.6 μg protein) was diluted to 50 μl with buffer E and assayed for DAG kinase activity. DAG kinase reaction velocity was determined in the presence 2.0 mM ATP, 2.0 mM (3.7 mol%) *sn*-1-stearoyl-2-arachidonylglyceol and the indicated free Ca^{2+} concentrations by the method described in Fig 4.2. The free Ca^{2+} concentrations in the reaction mixture were achieved by the inclusion of Ca^{2+} and 1.0 mM EGTA. The total Ca^{2+} required was calculated from the program React 2, a kind gift from Dr. G. Smith. The protein concentration of the DAG kinase preparation was determined by the Coomassie dye method (Section 2.2.3).

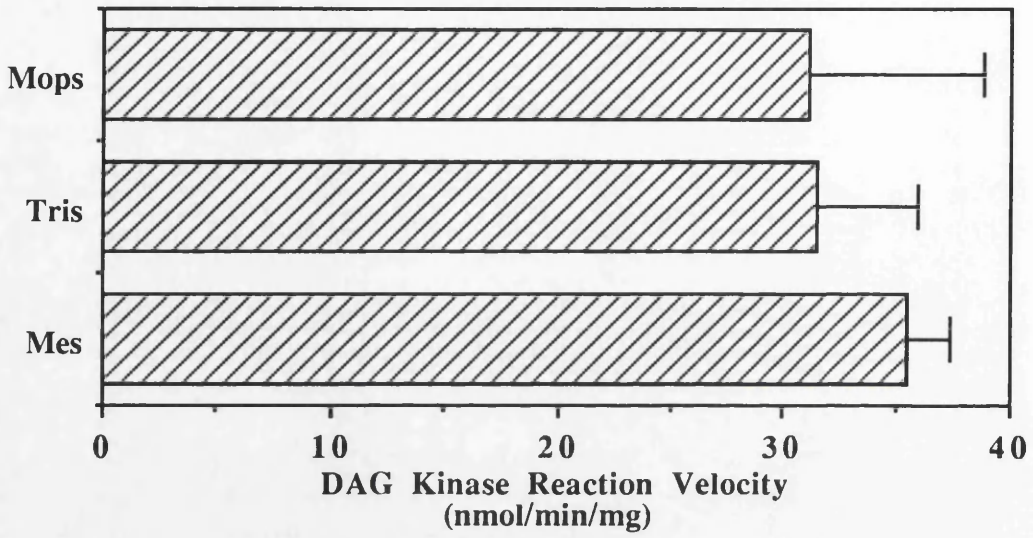
physiological range of this ion. Although, the K_m for either substrate was not determined, this would seem to indicate that the activity of the KCl-soluble DAG kinase of rat brain is not modulated by alterations in physiologically relevant free Ca^{2+}. This conclusion does, however, assume that the Ca^{2+} responsiveness of KCl-soluble enzyme is not dependent on any phospholipids or other membrane-associated activators distinct from PtdSer. This also assumes that the activity of KCl-soluble DAG kinase is not modulated by physiological Ca^{2+} concentrations via a second protein, for example calmodulin, as this was not investigated further.

4.8. pH Dependence of Activity.

A preliminary investigation was made into the effect of pH on the reaction velocity of the KCl-soluble DAG kinase. The enzyme's rate of reaction was measured over the range pH 5.0 to 9.0 using an *n*-octyl-β,D-glucopyranoside/PtdSer mixed micellar assay method modified from that described Section 2.4.3. The above range of pH in the reaction mixture was established with 50 mM Mes/NaOH over the range pH 5.0 to 7.2 and 50 mM Tris/HCl from pH 7.2 to 9.0, these buffers replacing the customary 50 mM Mops/NaOH, pH 7.2.

In the standard reaction mixture, replacing 50 mM Mops/NaOH, pH 7.2 with either 50 mM Mes/NaOH, pH 7.2 or 50 mM Tris/HCl, pH 7.2 resulted in no significant alteration of the KCl-soluble DAG kinase's reaction rate (Fig 4.8A). Reduced reaction velocities compared to those measured at pH 7.2 were observed at pH 5.0 (Fig 4.8B). Increasing the pH of the reaction mixture from pH 5.0 to pH 6.5 resulted in a progressive increase in reaction rates to levels similar to those measured at pH 7.2. Further increasing the pH from pH 6.5 to pH 8.0 resulted in no further significant increase in DAG kinase reaction velocity. However, further increasing the pH from 8.0 to 9.0 did result in reaction rates that were greater than those measured at pH 7.2. Therefore, the reaction rate of the KCl-soluble DAG kinase does not fluctuate in the physiological pH range. Below this range the KCl-soluble enzyme's activity is reduced, whereas above pH 8 the reaction

A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Tris
Mes
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10
20
30
40
DAG Kinase Reaction Velocity
(nmol/min/mg)

B

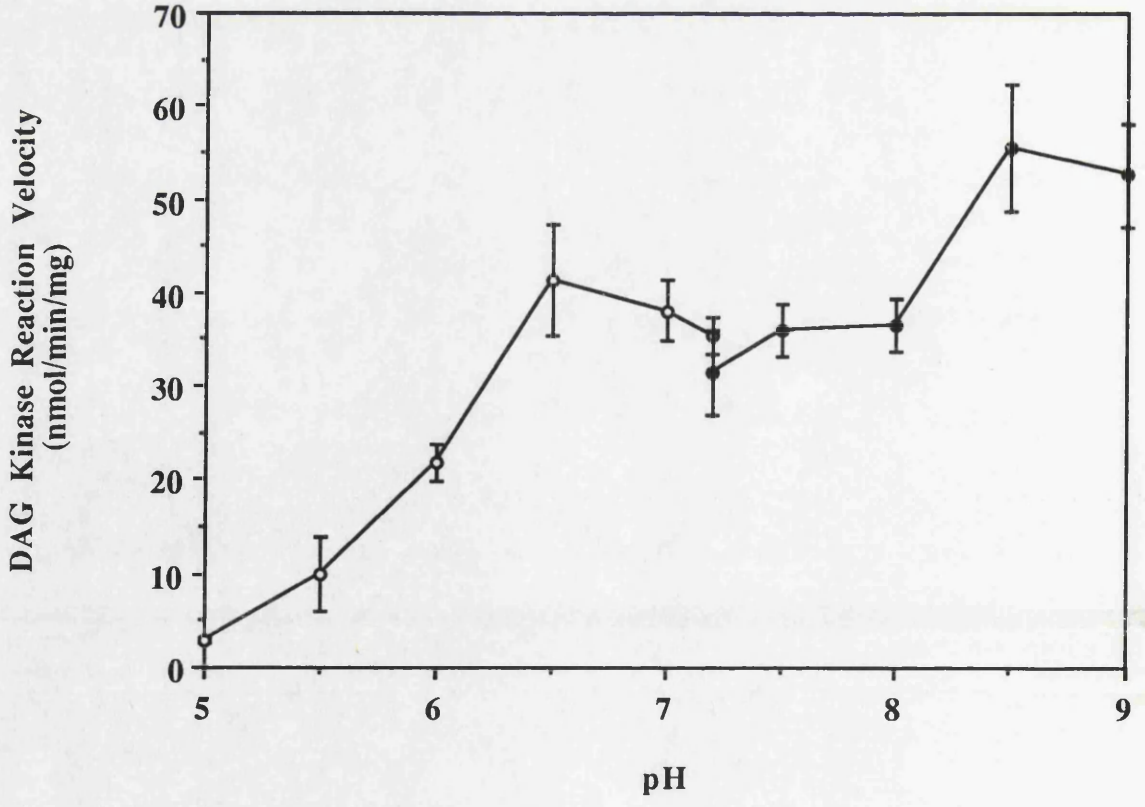

DAG Kinase Reaction Velocity
(nmol/min/mg)
70
60
50
40
30
20
10
0
5
6
7
8
9
pH

Fig 4.8A. Diacylglycerol Kinase Reaction Velocity at pH 7.2: Effect of Mes, Tris and Mops Buffers.

10 μl of the post heparin-agarose preparation of the KCl-soluble DAG kinase (0.8 μg protein) was diluted to 50μl with buffer E and assayed for DAG kinase activity. DAG kinase reaction velocity was determined in the presence 1.0 mM ATP and 2.0 mM (3.7 mol%, at 7.5 mol% phosphatidylserine) *sn*-1-stearoyl-2-arachidonylglycerol by the method described in Fig 4.2. The reaction velocity was also determined under identical conditions except for substitution of the 50 mM Mops/NaOH in the reaction mixture with 50 mM Mes/NaOH, pH 7.2 and 50 mM Tris/HCl, pH 7.2. After 35 min, the reaction was terminated and the [^{32}P]phosphatidic acid quantitated as described in Fig 4.2. The protein concentration of the DAG kinase preparation was determined by the Coomassie dye method (Section 2.2.3).

Fig 4.8B. pH Dependence of Diacylglycerol Kinase Reaction Velocity.

DAG kinase reaction velocity was determined with 10 μl of the post heparin-agarose preparation (diluted to 50 μl with buffer E). Unless otherwise stated, the reaction velocity was determined in the presence of 1.0 mM ATP and 2.0 mM (3.7 mol%) *sn* -1-stearoyl-2-arachidonylglycerol by the method described in Fig 4.2. The pH of the reaction mixture was established over the range pH 5.0 to 7.2, as indicated, with 50 mM Mes/NaOH and over the range pH 7.2 to 9.0, as indicated, with 50 mM Tris/HCl. The reaction was terminated after 35 min and the [^{32}P]phosphatidic acid quantitated as described in Fig 4.2. The protein concentration of the DAG kinase preparation was determined by the Coomassie dye method (Section 2.2.1). [O, 50 mM Mes/KOH; ●, 50 mM Mops/KOH]

velocity is enhanced (Fig 4.8B). This profile is different from the pH dependencies reported for the previously purified cytosolic and membrane-associated DAG kinase activities of rat brain. Both these enzymes displayed marked pH optima at 7.4, with reaction rates dropping rapidly above pH 7.5 (Kato & Takenawa, 1990). Similarly, the pH dependency of the KCl-soluble DAG kinase is also different from that of the 121 kDa cytosolic DAG kinase of rat liver, which displayed optimal rates between pH 8.0 and 8.5 (Kanoh & Ohno, 1981).

The profile of the pH dependency of the KCl-soluble DAG kinase's reaction velocity could indicate the presence of two enzyme activities, one with optimal activity at pH 6.5, the other with optimal activity at pH 8.5 (Fig 4.8B). This is unlikely as the enzyme preparation employed in this experiment was observed to elute as a single peak of activity both from heparin-agarose (Fig 3.9) and hydroxyapatite (Fig 3.10). This would indicate that the enzyme preparation contains a single DAG kinase activity. It is more likely, however, that the elevation of reaction rate observed above pH 8.0 is a result of the fourth ionisation of ATP. This suggests that the reaction velocity of the KCl-soluble DAG kinase is elevated when ATP is fully ionised.

4.9. Inorganic Phosphate: Effect on Determined Apparent K_m and V_{max} Values of KCl-soluble DAG kinase for ATP Substrate.

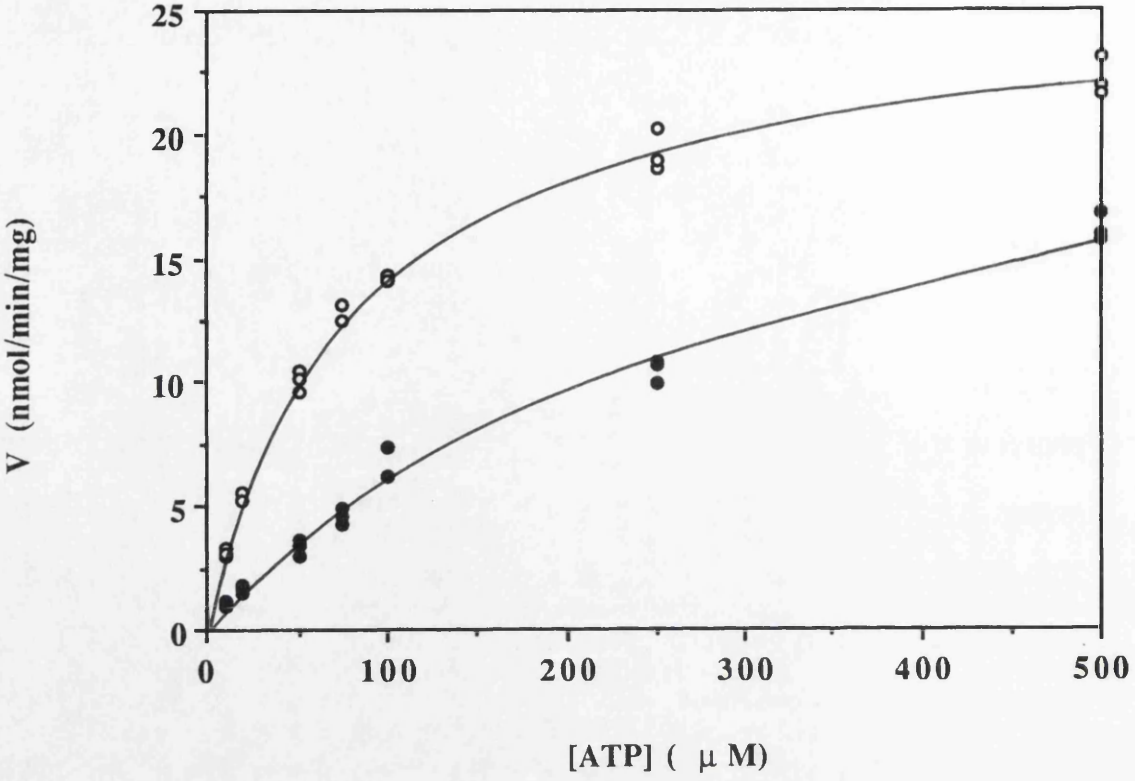
As discussed in Section 3.10, the inclusion of 20.0 mM potassium phosphate during measurement of the KCl-soluble DAG kinase rates of reaction was found to alter the measured rate from that determined in the absence of potassium phosphate. This was done in an attempt to assess what effect the presence of phosphate, used in the elution of KCl-soluble DAG kinase activity from hydroxyapatite, would have on the measurement of the DAG kinase activity present in the fractions obtained after hydroxyapatite chromatography. Using the deoxycholate/PtdSer dispersion assay, routinely employed in the measurement of DAG kinase activity in chromatography eluants, the presence of 20.0 mM

potassium phosphate was found to reduce the measured rate of reaction to 54% of that measured in the absence of potassium phosphate. To further characterise the effect of potassium phosphate on the rate of reaction of KCl-soluble DAG kinase, the effect of 20 mM potassium phosphate on the determined K_m and V_{max} values of the enzyme was examined.

The ATP concentration dependence of the KCl-soluble DAG kinase reaction velocity was measured in the absence and presence of 20.0 mM potassium phosphate, using the *n*-octyl-β,D-glucopyranoside/PtdSer mixed micellar assay system, and the K_m and V_{max} values determined (Fig 4.9A). In the presence of 20.0 mM potassium phosphate, pH 7.2 the apparent K_m was found to fall from 330 μM to 76.0 μM when fitted to a Michaelis-Menten kinetic model. Fitting the data to this model revealed no significant alteration of the apparent V_{max} of the enzyme in the presence of potassium phosphate. Only a slight reduction of the determined V_{max} value from 26.4 nmol/min/mg to 25.4 nmol/min/mg in the presence of potassium phosphate was observed for the post-gel filtration preparation. These values closely correlate to those obtained by graphical Eadie-Hofstee analysis of the data (Fig 4.9B). This would seem to suggest that 20.0 mM potassium phosphate acts as an activator of the KCl-soluble DAG kinase in this assay system, acting by reducing the apparent K_m of the enzyme for ATP with no, or little, measurable effect on the V_{max} obtained in such an assay system.

A preliminary investigation showed this effect to be localised to the ATP binding site of the enzyme with no effect on the apparent K_m of the enzyme for *sn* -1-stearoyl-2-arachidonylglycerol substrate. At 2.0 mM ATP (K_m =330 μM) a concentration found to produce near maximal rates of reaction, with respect to ATP concentration, the presence of 20.0mM potassium phosphate was found not to affect the rate of reaction at both saturating and limiting concentrations of *sn*-1-stearoyl-2-arachidonylglycerol (3.8 and 0.38 mol%, respectively). However, at 50 μM ATP the presence of 20.0 mM potassium phosphate was found to increase

A


25
20
15
10
5
0
0
100
200
300
400
500
V (nmol/min/mg)
[ATP] (μ M)

B

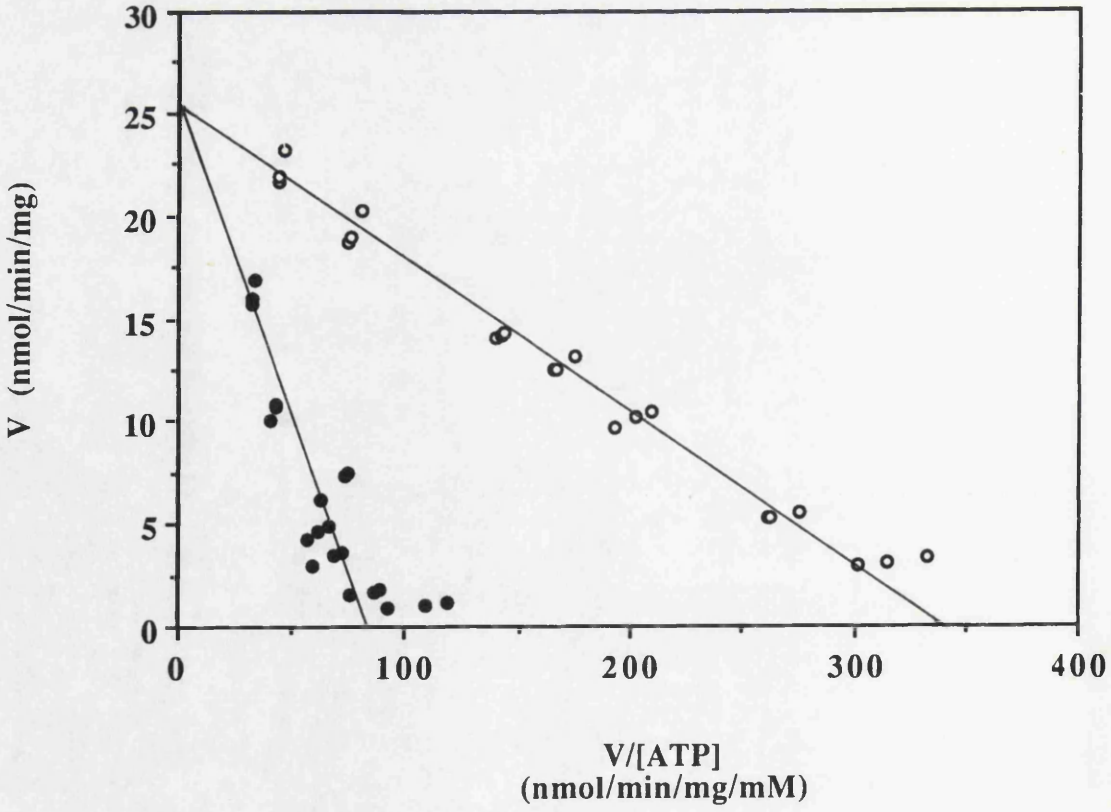

30
25
20
15
10
5
0
0
100
200
300
400
V (nmol/min/mg)
V/[ATP]
(nmol/min/mg/mM)

Fig 4.9. Effect of 20 mM Potassium Phosphate on the ATP Dependence of DAG Kinase Reaction Velocity.

A. Comparison of the ATP dependence of the KCl-soluble DAG kinase in the presence and absence of 20 mM potassium phosphate.

B. Eadie-Hofstee plot of the data shown in A.

20 µl (1.95 µg protein) of the post gel filtration preparation of the KCl-soluble DAG kinase was diluted to 40 µl with buffer E and assayed for DAG kinase activity. DAG kinase reaction velocity was determined in the presence or absence of 20.0 mM potassium phosphate with 2.0 mM (3.7 mol%) *sn*-1-stearoyl-2-arachidonylglycerol and the indicated concentrations of ATP by the method described in Fig 4.2. The potassium phosphate was delivered to the reaction mixture as 10 µl of a 10x stock solution (200 mM potassium phosphate, pH 7.2 in buffer E). The protein concentration of the DAG kinase preparation was determined by the Coomassie dye method (Section 2.2.3). [●, - 20 mM potassium phosphate; ○, +20 mM potassium phosphate]

the rate of reaction at both 3.8 and 0.38 mol% *sn* -1-stearoyl-2-arachidonylglycerol. Therefore, it would seem that 20.0 mM potassium phosphate, in the *n* -octyl-β,D-glucopyranoside/PtdSer assay system, acts on the KCl-soluble DAG kinase at the ATP-binding site, lowering the K_m of the enzyme for this substrate without altering the V_{max} of the enzyme or the K_m for the *sn* -1-stearoyl-2-arachidonylglycerol substrate. It is difficult to reconcile the inhibitory effect of 20.0 mM potassium phosphate on the enzyme in the deoxycholate/PtdSer dispersion assay system with this result. However, it may suggest an additional effect of phosphate on the DAG substrate when presented at the sub-maximal concentration of 200 μM, apart from the effect of phosphate in reducing the apparent K_m of the enzyme for ATP.

A phosphate concentration dependency of this activation of the KCl-soluble DAG kinase remains to be determined. Until this is undertaken, the effect of physiological concentrations of phosphate in brain, reported as 2.7 mM (Lehninger, 1982), will remain unkown. In addition, the K_i for phosphate acting as an inhibitor, and indeed the nature of the inhibition of the KCl-soluble DAG kinase in the dispersion assay system has still to be determined.

What is the physiological role of such an activation, i.e. the reduction of the K_m for ATP? It must of course be recognised that this effect may be secondary to the complex nature of the mixed micellar kinetic analysis of an enzyme with a lipid substrate. Mixed micellar systems allow kinetic characterisation of an enzyme towards lipid substrates or phospholipid activators without need for correlation to their solubility, orientation and, thus, presentation in an aqueous enviroment. It is, however, limited to being an artificial presentation of the physiological lipid substrates or activators and, thus, only limited a comparison can be made to the *in vivo* situation of the enzyme. This may explain the dual role of potassium phosphate as an activator in the mixed micellar assay system and as an inhibitor in the deoxycholate/PtdSer dispersion system. However, rather then being artefactual, the observed effect of potassium phosphate may identify the physiological interaction of another phosphorylated compound with KCl-soluble

DAG kinase, which may be eliciting an alteration of the enzyme's K_m for ATP. Moreover, such a physiological activator may do so at its physiological concentration, not at supraphysiological concentrations as appears to be the case for potassium phosphate. However, this is a tentative suggestion and requires further investigation.

4.10. Kinetic Characterisation of KCl-Solubilised Diacylglycerol Kinase: Summary.

After the preliminary characterisation of partially purified preparations of the KCl-soluble DAG kinase of rat brain, the following may be concluded: The K_m for *sn*-1-stearoyl-2-arachidonylglycerol (1.7 mol%) is similar to that obtained for the arachidonyl-specific DAG kinase activity of Swiss 3T3 fibroblast membranes (MacDonald *et al.*, 1988b) when assayed under similar conditions. The KCl-soluble DAG kinase did not display a similar degree of specificity towards this substrate over other *sn*-1,2-diacylglycerols, as reported for the Swiss 3T3 fibroblast DAG kinase activity (MacDonald *et al.*,1988b) and the arachidonyl-specific DAG kinase isozyme of baboon brain (Lemaitre *et al.*, 1990). The apparent K_m of the KCl-soluble DAG kinase for ATP (330 μM) was similar to the apparent K_m values for ATP obtained by other workers for pure or partially purified preparations of DAG kinase from various sources. The KCl-soluble DAG kinase was dependent on the presence of Mg^{2+} for activity, with maximal rates of reaction obtained at concentrations of 7.0 mM, or greater, total Mg^{2+}. The KCl-soluble DAG kinase activity did not require the presence of either Ca^{2+} or PtdSer for activity with the activity remaining insensitive to any alteration of PtdSer concentration or the range found to activate the membrane-associated DAG kinase activity of Swiss 3T3 fibroblasts. It was also insensitive to alteration of the Ca^{2+} concentrations *in vitro* over the range demonstrated physiologically to be able to activate the porcine 80 kDa DAG kinase. Finally and curiously, the presence of 20 mM potassium phosphate in the assay mixture resulted in the reduction of the

apparent K_m of the KCl-soluble DAG kinase for ATP from 330 μM to 76.0 μM without significant alteration of the determined V_{max} and without any apparent alteration of the interaction of the *sn*-1,2-diacylglycerol substrae with the kinase. Although the significance of physiological concentrations of phosphate have still to be determined, the role of such a specific alteration of the apparent K_m has still to reconciled.

Chapter 5

Concluding Discussion.

5.1. Characterisation of the DAG Kinase Involved in Receptor-Stimulated PtdIns(4,5)P_2 Turnover.

A specific DAG kinase isoenzyme is associated with the task of phosphorylating the *sn*-1,2-diacylglycerol produced on receptor-stimulated PtdIns(4,5)P_2 hydrolysis. PDGF stimulation of Swiss 3T3 fibroblasts in the presence of cell-permeable *sn*-1,2-didecanoylglycerol results in the preferential phosphorylation of the *sn*-1,2-diacylglycerol produced endogenously (MacDonald *et al.*, 1988a). This is despite *sn*-1,2-didecanoylglycerol proving to be a substrate in unstimulated cells (MacDonald *et al.*, 1988a). Employing the non-ionic detergent, *n*-octyl-β,D-glucopyranoside, in a mixed micellar assay system, the membrane-associated DAG kinase of Swiss 3T3 fibroblasts was observed to phosphorylate *sn*-1-stearoyl-2-arachidonylglycerol with a reaction rate at least 2-fold greater than that recorded with other naturally occuring long-chain *sn*-1,2-diacylglycerols also observed to accumulate on PDGF-stimulation, e.g. *sn*-1,2-dioleoylglycerol (MacDonald *et al.*, 1988b). In comparison, the cytosolic activity prepared from Swiss 3T3 fibroblasts exhibited no such elevated reaction rates towards *sn*-1-stearoyl-2-arachidonylglycerol (MacDonald *et al.*, 1988b). In contrast to the membrane-associated enzyme, the cytosolic DAG kinase catalysed the phosphorylation of *sn*-1,2-didecanoylglycerol with reaction rates greater than measured with *sn*-1-stearoyl-2-arachidonylglycerol. It remains to be defined if multiple DAG kinase isoenzymes are situated on the membrane of these cells. However, of the isoenzymes present within the cell, it is a solely membrane-associated activity that initiates the metabolism of PtdIns(4,5)P_2-derived *sn*-1,2-diacylglycerols (MacDonald *et al.*, 1988a). Furthermore, the DAG kinase that participates in this phosphorylation event is identifiable by a unique substrate specificity, catalysing the phosphorylation of *sn*-1-acyl-2-arachidonylglycerol with reaction rates several-fold greater than non-arachidonyl *sn*-1,2-diacylglycerols.

Furthermore, such catalytic specificity towards *sn*-1-acyl-2-arachidonylglycerol appears to be a wide-spread phenomenon. In rat brain

microvessels, exogenously added *sn*-1-palmitoyl-2-arachidonylglycerol is phosphorylated at a rate 1.7-fold greater than *sn*-1-palmitoyl-2-oleoylglycerol, the enhanced rate remaining in homogenised tissue (Hee-Cheong *et al.*, 1985). The reaction velocity of the DAG kinase activity present in the particulate and soluble fractions prepared from rabbit aortic endothelial cell homogenates increases 3.4-fold when *sn*-1-palmitoyl-2-oleoylglycerol replaces *sn*-1-palmitoyl-2-arachidonylglycerol as substrate (Severson & Hee-Cheong, 1986). Finally, an *sn*-1-acyl-2-arachidonylglycerol-selective DAG kinase activity can be resolved from the non-selective activities which are solubilised from baboon brain (Lemaitre *et al.*, 1990).

5.2. The DAG Kinase Linked to Receptor-Stimulated PtdIns(4,5)P_2 Turnover: Evidence of Substrate Specificity *in Vivo*.

sn-1,2-Diacylglycerol produced from PtdIns(4,5)P_2 hydrolysis differs in its subcellular site of accumulation from the sustained phase of *sn*-1,2-diacylglycerol generated from other phospholipd sources (see Section 1.4.7). It is, however, accepted that they are both produced at the same location, i.e. the plasma membrane, and only partition to a distinct cellular location after their production. Initially their metabolic compartmentalisation is not served by distinct intracellular location and must, therefore, be achieved by another mechanism.

The *sn*-1,2-diacylglycerol generated from stimulated hydrolysis of PtdIns(4,5)P_2 and other phospholipids are distinguishable by their acyl-chain composition and such molecular species distribution betrays the precursor phospholipid. This property was employed by Pessin and colleagues (1989, 1990) to identify the prevalent source of *sn*-1,2-diacylglycerol at various times after mitogenic stimulation of IIC9 fibroblasts. These workers observed PtdIns was the major source after 15 sec, while after 5 min and 60 min *sn*-1,2-diacylglycerol was almost exclusively produced from PtdCho hydrolysis. Additionally, they reported that although receptor-stimulated hydrolysis of PtdIns may be the major source,

PtdCho does contribute significantly to the stimulated levels of *sn*-1,2-diacylglycerol 15 sec after stimulation. These results indicated that the *sn*-1,2-diacylglycerol generated from PtdIns(4,5)P_2 hydrolysis shortly after receptor stimulation could be specifically removed, thus leaving a pool of *sn*-1,2-diacylglycerol with molecular species characteristic of other phospholipid sources. The possibility exists that the acyl-chain composition of a *sn*-1,2-diacylglycerol molecule identifies its requirement for DAG kinase catalysed phosphorylation and re-entry to the PI-cycle or, conversely, guarantees its exclusion from this route of phospholipid resynthesis and commits it to the resynthesis of PtdCho.

25 - 30 % of the total labelled *sn*-1,2-diacylglycerol in quiescent [^{3}H]glycerol-labelled Swiss 3T3 fibroblasts co-migrates with 16:0/18:1 and 18:0/18:2 diacylglycerols on HPLC analysis, while only 9.5 % of the *sn*-1,2-diacylglycerol popoulation migrated with *sn*-1-stearoyl-2-arachidonylglycerol (Divecha *et al.*, 1991). Also, Divecha *et al.* (1991) reported a 2-fold increase in *sn*-1,2-diacylglycerol concentration 5 min after bombesin stimulation of Swiss 3T3 cells and that the acyl-chain distribution within this expanded *sn*-1,2-diacylglycerol pool was unchanged. Although this is different to the previously reported *sn*-1-stearoyl-2-arachidonylglycerol predominance in PDGF-stimulated Swiss 3T3 cells (MacDonald *et al.*, 1988a), it is, however, noteworthy that Divecha *et al.* (1991) investigated the acyl-chain distribution amongst PtdOH after 5 min and observed a 50 % increase in the [^{3}H]glycerol-labelled *sn*-1-stearoyl-2-arachidonylglycero-3-phosphate. This proved to be the predominant labelled PtdOH they observed on bombesin stimulation and illustrated the marked differences between the molecular species profile of *sn*-1,2-diacylglycerol and PtdOH in bombesin-stimulated Swiss 3T3 fibroblasts. Together with their measurement of *sn*-1-stearoyl-2-arachidonylglycerol-3-phosphoinositol contributing to 25 % of the PtdIns pool within Swiss 3T3 cells, Divecha and colleagues (1991) postulated that *sn*-1-stearoyl-2-arachidonylglycerol was a significant component of *sn*-1,2-diacylglycerol derived from agonist-stimulated PtdIns(4,5)P_2 hydrolysis and that

DAG kinase did, indeed, selectively phosphorylate this acyl-chain molecular species.

Non-radiometric molecular species analysis of the PtdOH present in total cell lipid can be achieved by dephosphorylation of the PtdOH to *sn*-1,2-diacylglycerol followed by derivitisation and reverse-phase HPLC separation of the *sn*-1,2-diacylglycerol product (Lee *et al.*, 1991). Using this approach, the molecular species composition of the PtdOH in quiescent human SK-N-SH neuroblastoma cells was found to be similar to PtdCho, while the PtdOH generated 5 min after muscarinic stimulation more closely resembled the acyl-chain composition of PtdIns (Lee *et al.*, 1991). It was their conclusion that shortly after stimulation, PtdOH was formed solely by the phosphorylation of PtdIns-specific *sn*-1,2-diacylglycerols and not by the phosphorylation of *sn*-1,2-diacylglycerol formed on PtdCho hydrolysis. It should, however, be noted that alkaline phosphatase-catalysed dephosphorylation of PtdOH, as employed in the study of Lee and co-workers (1991), has been observed to dephosphorylate poly-unsaturated molecular species of PtdOH with greater efficiency than saturated acyl-chain molecular species (N. Thompson, personal communication). Obviously, the selectivity of the alkaline phosphatase-catalysed dephosphorylation may lead to erroneous identification of the phospholipid precursor of the muscarinic-stimulated levels of PtdOH in SK-N-SH cells. However, Lee and colleagues (1991) found that muscarinic-stimulation of SK-N-SH cells in the presence of $^{32}P_i$ resulted in the rapid labelling of PtdOH followed by labelling of PtdIns, suggesting a precursor-product relationship between PtdOH and PtdIns. Furthermore, the apparent precursor-product relationship would appear to confirm the original conclusion that the PtdOH formed 5 min after muscarinic-stimulation of SK-H-SH neuroblastoma cells is the product of enzymatic phosphorylation of phosphoinositide-derived *sn*-1,2-diacylglycerol (Lee *et al.*, 1991).

5.3. The DAG Kinase Linked to Receptor-Stimulated PtdIns(4,5)P_2 Turnover is Identified by its Subcellular Location?

It would appear that a specific component of total cellular DAG kinase activity is involved in the phosphorylation of *sn*-1,2-diacylglycerol produced on receptor-stimulated PtdIns(4,5)P_2 hydrolysis and, moreover, the activity responsible can be identified by its catalytic selectivity towards *sn*-1-acyl-2-arachidonylglycerol compared to other *sn*-1,2-diacylglycerols (see Section 5.1). Given that DAG kinase activity is ubiquitously distributed throughout the cell, can the *sn*-1-acyl-2-arachidonylglycerol-selective DAG kinase isoenzyme be localised to a subcellular compartment coincident with the site of PtdIns(4,5)P_2 hydrolysis ?

The generation of *sn*-1,2-diacylglycerol from receptor-stimulated PtdIns(4,5)P_2 hydrolysis is limited to the location of the receptor/G-protein/PtdIns-PLC complex, i.e. the plasma membrane. Transient accumulation of *sn*-1,2-diacylglycerol in the plasma membrane has been observed with a similar time-course to phosphoinositide hydrolysis and its longevity identical to the period of PtdOH formation in TRH-stimulated GH_3 pituitary cells (Rebecchi *et al.*, 1983; Martin *et al.*, 1990). This strongly indicates that the transient elevation of plasma membrane *sn*-1,2-diacylglycerol levels is a product of the rapid metabolism of PtdIns(4,5)P_2-derived second messenger in this intracellular location. However, the sustained accumulation of *sn*-1,2-diacylglycerol in the intracellular membranes of TRH-stimulated GH_3 cells (Martin *et al.*, 1990) could, in turn, represent the inability of DAG kinase to phosphorylate the *sn*-1,2-diacylglycerol constituting the sustained phase of elevated *sn*-1,2-diacylglycerol levels. This most likely represents a combination of DAG kinase being unable to catalyse the phosphorylation of the molecular species present in the sustained phase of elevated *sn*-1,2-diacylglycerol levels and also translocation of the *sn*-1,2-diacylglycerol from the site of its production away from the location of the activity delegated to its phosphorylation.

In agreement with the suggested location of such DAG kinase activity to the plasma membrane, PtdOH has been shown to accumulate preferentially in the plasma membrane on receptor activation (Seyfred & Wells, 1984). However, the identification of the DAG kinase isoenzyme involved in the stimulated turnover of PtdIns(4,5)P_2, i.e. the *sn*-1-acyl-2-arachidonylglycerol-selective enzyme, has not been achieved by identification of the differential localisation of individual isoenzymes to the plasma membrane. Previous investigations of the membrane-associated *sn*-1-acyl-2-arachidonylglycerol-selective isoenzymes in baboon brain and Swiss 3T3 membranes were performed with crude particulate fractions, without further localisation of the activity to a particular subcellular compartment (Lemaitre *et al.*, 1990; MacDonald *et al.*, 1988a, b). Therefore, subcellular fraction and localisation of individual isoenzymes to the plasma membrane, although of possible benefit in the identification of the activity responsible for phosphorylation of PtdIns(4,5)P_2-derived *sn*-1,2-diacylglycerol, has not, to date, been reported. It is noteworthy, however, that an *sn*-1-acyl-2-arachidonylglycerol-selective activity has been identified and solubilised from the dense membrane fraction of porcine testis homogenate (S. Gardner, A. Paterson & M.J.O. Wakelam, unpublished result).

5.4. Further Evidence of a Specific DAG Kinase Isoenzyme Dedicated to the Phosphorylation of PtdIns(4,5)P_2-Derived *sn*-1,2-Diacylglycerol.

Extraction of baboon brain homogenate with 2 % *n*-octyl-β,D-glucopyranoside/0.3 M KCl resulted in the solubilisation of three distinct DAG kinase isoenzymes that could be resolved by hydroxyapatite chromatography (Lemaitre *et al.*, 1990). Of these resolved activities, two exhibited similar rates of reaction with either *sn*-1,2-dioleoylglycerol or *sn*-1-stearoyl-2-arachidonylglycerol substrates when assayed by Triton X-100 or *n*-octyl-β, D-glucopyranoside mixed micellar assay methods. The third isoenzyme was, however, selective for its *sn*-

1,2-diacylglycerol substrate in either Triton X-100 or *n*-octyl-β,D-glucopyranoside assay systems, phosphorylating *sn*-1-stearoyl-2-arachidonylglycerol with a reaction rate 3-fold greater than that observed with *sn*-1,2-dioleoylglycerol (Lemaitre *et al.*, 1990). The truly selective nature of the *sn*-1-acyl-2-arachidonylglycerol-selective isoenzyme was further demonstrated by the preferential phosphorylation of *sn*-1-stearoyl-2-arachidonylglycerol when co-presented with *sn*-1,2-didecanoylglycerol in an *n*-octyl-β,D-glucopyranoside mixed micellar assay system (Lemaitre *et al.*, 1990). This is identical to the situation observed when *sn*-1-acyl-2-arachidonylglycerol is co-presented with *sn*-1,2-didecanoylglycerol to the DAG kinase activity of Swiss 3T3 cell lysates (MacDonald *et al.*, 1988a).

The *sn*-1-acyl-2-arachidonyl selective DAG kinases of Swiss 3T3 mouse fibroblasts and baboon brain are both membrane-associated, indicating a high degree of functional homology and most likely suggesting they perform identical roles within the stimulated cell (Lemaitre *et al.*, 1990). However, unlike the membrane-associated DAG kinase activity of Swiss 3T3 fibroblasts, multiple resolvable DAG kinase activities have been identified within the membrane-associated compartment of baboon brain (Lemaitre *et al.*, 1990). Of the three isoenzymes solubilised from the particulate fraction of baboon brain, only one displays the catalytic selectivity towards *sn*-1-acyl-2-arachidonylglycerol. This would seem to indicate that it is, indeed, a single membrane-associated isoenzyme that is active in catalysing the phosphorylation of *sn*-1,2-diacylglycerol generated on receptor-stimulated PtdIns(4,5)P_2 hydrolysis.

Unfortunately, it would seen that observation of such *sn*-1-acyl-2-arachidonylglycerol-specific DAG kinase activity is restricted by the method of solubilisation of the *sn*-1, 2-diacylglycerol substrate (Lemaitre *et al.*, 1990). In the study that identified the presence of a *sn*-1-acyl-2-arachidonylglycerol-specific DAG kinase activity in baboon brain homogenate, Lemaitre and co-workers demonstrated that such catalytic selectivity could only be observed if the *sn*-1,2-diacylglycerol substrate was solubilised in the non-ionic detergents, *n*-octyl-β,D-

glucopyranoside and Triton X-100, and not when dispersed with deoxycholate, an anionic detergent (Lemaitre *et al.*, 1990). Similarly, MacDonald *et al.* (1988a, b) employed an *n*-octyl-β,D-glucopyranoside mixed micellar assay method in their identification of an *sn*-1-acyl-2-arachidonylglycerol-selective DAG kinase in Swiss 3T3 fibroblast membranes. The method of *sn*-1,2-diacylglycerol presentation *in vitro* is, thus, extremely important in the observation of catalytic selectivity toward *sn*-1-acyl-2-arachidonylglycerol substrates. If the physiological behavior of DAG kinase towards *sn*-1,2-diacylglycerol substrates is to be observed, it is necessary to present the *sn*-1,2-diacylglycerol in as near a physiological setting as possible. It may not be surprising, therefore, that the *sn*-1-acyl-2-arachidonylglycerol-selective DAG kinase of baboon brain does not catalyse the phosphorylation of *sn*-1-stearoyl-2-arachidonylglycerol with greater reaction velocity than *sn*-1,2-dioleoylglycerol when dispersed at concentrations of 0.5 mM in the presence of 2.0 mM deoxycholate (Lemaitre *et al.*, 1990). However, *sn*-1-acyl-2-arachidonylglycerol-selectivity was observed when the *sn*-1,2-diacylglycerol substrate was solubilised as a minor component (7.4 mol%) of a *n*-octyl-β,D-glucopyranoside/PtdSer mixed micelle (Lemaitre *et al.*, 1990). Although not identical to the phospholipid bilayer of a cell membrane, this observation of *sn*-1-acyl-2-arachidonylglycerol-selectivity may indicate that *sn*-1,2-diacylglycerol intercalated in an *n*-octyl-β,D-glucopyranoside/PtdSer mixed micelle more closely approximates to the physiological presentation of *sn*-1,2-diacylglycerol within the cell membrane than *sn*-1,2-diacylglycerol dispersed in aqueous solution with 2.0 mM deoxycholate (c.m.c. = 5.0 mM).

5.5. DAG Kinase Assay Methodology Affects Measured Reaction Velocities.

5.5.1. DAG Kinase Reaction Velocities are Influenced by the *sn*-1,2-Diacylglycerol Substrate Environment in an Isoenzyme-Specific Manner.

Generally, it has been demonstrated that individual DAG kinase isoenzymes display different rates of reaction depending on the method of *sn*-1,2-diacylglycerol substrate presentation *in vitro*. Presentation of *sn*-1,2-diacylglycerol either dispersed in aqueous solution or fully solubilised in a mixed micellar system employing detergent at concentrations above its c.m.c. to individual DAG kinase isoenzymes has been found to result in greatly altered reaction velocities. The soluble DAG kinase activity of rat brain can be separated to two distinct activities by heparin-agarose chromatography (Strathopoulos *at al.*, 1990). Of these two activities, Strathopoulos *et al.* (1990) observed that one catalyses the phosphorylation of *rac*-1,2-dioleoylglycerol with similar rates of reaction in the presence of either 1.0 mM deoxycholate (c.m.c. = 5.0 mM) or 73 mM *n*-octyl-β,D-glucopyranoside (c.m.c. = 25 mM). The second soluble DAG kinase reported by these workers, however, phosphorylates *rac*-1,2-dioleoylglycerol in the presence of 1.0 mM deoxycholate with a rate 11-fold greater than that observed when the *sn*-1,2-diacylglycerol substrate was solubilised in 73 mM *n*-octyl-β,D-glucopyranoside. The first of the two rat brain soluble kinases apparently catalyses the phosphorylation of *sn*-1,2-diacylglycerol with equal velocity, regardless of substrate environment, whereas, the second isoenzyme displays increased reaction velocity towards *sn*-1,2-diacylglycerol dispersed in the presence of anionic detergent below its c.m.c. compared to *sn*-1,2-diacylglycerol fully solubilised in the mixed micellar environment provided by a non-ionic detergent above its c.m.c..

Coco-Maroney and Macara (1989) observed that the measured cytosol:membrane distribution of DAG kinase activity in Swiss 3T3 fibroblasts altered markedly depending on assay detergent conditions. Rates of reaction in both cytosolic and membrane fractions prepared from these cells was greatest when the *sn*-1,2-dioleoylglycerol substrate was present with 1.0 mM deoxycholate, with the cytosolic activity possessing greatest specific activity (Coco-Maroney & Macara, 1989). In the presence of 52 mM *n*-octyl-β,D-glucopyranoside and absence of exogenous phospholipid, the cytosolic activity was again present with

greatest specific activity. However, in the presence of exogenous PtdSer or PtdCho (both at 6.9 mol%) the cytosolic and membrane-associated activities possessed identical specific activities, although, much reduced compared to the specific activities observed for both the cytosolic and membrane-associated activities when measured in the presence of PtdSer and deoxycholate (Coco-Maroney & Macara, 1989).

Clearly, DAG kinase isoenzymes maintain a specific requirement for the method of *sn*-1,2-diacylglycerol solubilisation/presentation *in vitro* in attaining maximal rates of phosphorylation and, by the same token, selectivity towards individual *sn*-1,2-diacylglycerol molecular species. This, however, obscures the physiological reaction that each DAG kinase isoenzyme catalyses, as the detergent and phospholipid conditions that can be employed to measure DAG kinase activity with the greatest rates of reaction may not necessarily allow the isoenzyme to display substrate selectivity. For example, the membrane-associated DAG kinase of Swiss 3T3 fibroblasts has a specific activity of 148 pmol/min/mg when measured in the presence of 1.0 mM deoxycholate, 2.0 mM *sn*-1,2-dioleoylglycerol, 2.0 mM PtdSer, yet the specific activity for the same enzyme preparation is only 67 pmol/min/mg when the deoxycholate is replaced by 52 mM *n*-octyl-β,D-glucopyranoside (Coco-Maroney & Macara, 1989). The *sn*-1-acyl-2-arachidonylglycerol-specific DAG kinase activity of baboon brain, however, catalyses the phosphorylation of *sn*-1,2-dioleoylglycerol and *sn*-1-stearoyl-2-arachidonylglycerol with similar rates of reaction in the presence of deoxycholate/PtdSer, but, phosphorylates *sn*-1-stearoyl-2-arachidonylglycerol with a reaction rate 3-fold greater than *sn*-1,2-dioleoylglycerol when assayed with 73 mM *n*-octyl-β,D-glucopyranoside/7.4 mol% PtdSer (Lemaitre *et al.*, 1990). This provides a clear example where the *sn*-1-acyl-2-arachidonylglycerol selectivity of a particular enzyme is compromised when the reaction rate has been measured by a method that provides the greatest reaction velocity.

Employing identical *n*-octyl-β,D-glucopyranoside detergent conditions to those utilised in the characterisation of the Swiss 3T3 membrane-associated DAG kinase, MacDonald *et al.* (1988b) employed DAG kinase prepared from *E. coli.* and observed no increased rate of phosphorylation towards *sn*-1-acyl-2-arachidonylglycerol compared to non-arachidonyl *sn*-1,2-diacylglycerols. The requirement for *sn*-1,2-diacylglycerol substrate presentation in an *n*-octyl-β,D-glucopyranoside mixed micellar assay system would appear not to result in artefactual *sn*-1-acyl-2-arachidonylglycerol specificity but, rather, permit the phenomenon, whereas presentation of *sn*-1,2-diacylglycerol as a dispersion in the presence of deoxycholate does not. This observation is further consolidated by non-arachidonyl-specific DAG kinase activities catalysing the phosphorylation of *sn*-1-stearoyl-2-arachidonylglycerol, *sn*-1,2-dioleoylglycerol and *sn*-1,2-didecanoylglycerol with similar rates of reaction under identical assay conditions to those employed in the characterisation of the *sn*-1-acyl-2-arachidonylglycerol-specific activities (MacDonald *et al.* 1988b; Lemaitre *et al.*, 1990).

5.5.2. DAG kinase Reaction Velocities are Influenced by Phospholipid.

Phospholipid has been reported to modulate the activity of DAG kinase *in vitro*. For example, the porcine 80 kDa cytosolic DAG kinase has been reported to be activated by PtdCho, lyso-PtdCho, PtdEtn, PtdSer and sphingomyelin (Kanoh *et al.*, 1983), while the 121 kDa cytosolic activity of rat liver is not affected by the presence of PtdSer, PtdCho, PtdEtn and phosphatidylglycerol *in vitro* (Kanoh & Ohno, 1981). As with the observation of *sn*-1-acyl-2-arachidonyl-selective activity this is unlikely to represent the physiological requirements of DAG kinase for phospholipid. Rather, such modulation of DAG kinase activity *in vitro* is more liable to represent alteration of the *sn*-1,2-diacylglycerol's environment within the assay system.

Dispersed *sn*-1,2-diacylglycerol has been employed by many workers in the measurement of phospholipid modulation of DAG kinase reaction velocity (Kanoh & Ohno, 1981; Kanoh *et al.*, 1983; Sakane *et al.*, 1989) and *sn*-1,2-diacylglycerol dispersed with deoxycholate employed for the same purpose by others (Ide & Weinhold, 1982; Lin *et al.*, 1986; Kato & Takenawa, 1990). Yet, the porcine cytosolic 80 kDa DAG kinase poorly utilises dispersed *sn*-1,2-diacylglycerol as substrate compared to *sn*-1,2-diacylglycerol dispersed with 1.0 mM deoxycholate or 0.5 mM PtdCho (Kanoh *et al*, 1983). Furthermore, the porcine DAG kinase reaction velocity was also observed to be increased when the *sn*-1,2-diacylglycerol substrate was dispersed in other anionic amphiphiles, e.g. 300 μM PtdSer and 10 μM - 20 μM sphingosine. However, at greater concentrations the DAG kinase reaction velocity returned to levels similar to those obtained in the presence of dispersed *sn*-1,2-diacylglycerol alone (Sakane *et al.*, 1989). This evidence may suggest that *sn*-1,2-diacylglycerol dispersed with anionic amphiphiles is more accessible as substrate than dispersed *sn*-1,2-diacylglycerol alone. Also, it may suggest that the previously reported phospholipid modulation of DAG kinase activity *per se* is artefactual and, alternatively, represents the increased suitability of *sn*-1,2-diacylglycerol as substrate for the kinase when dispersed with amphiphiles, such as phospholipids. This is qualified by the measurement of increased rates of phosphorylation catalysed by the porcine 80 kDa DAG kinase when *sn*-1,2-dioleoylglycerol is solubilised in 50 mM *n*-octyl-β,D-glucopyranoside (c.m.c. = 25 mM, [*sn*-1,2-dioleoylglycerol] = 8 mol%) compared to those recorded with *sn*-1,2-dioleoylglycerol dispersed at a concentration of 0.5 - 1.0 mM in the absence of amphiphilic activator (Sakane *et al.*, 1991). Attention must, therefore, be paid to the environment of the *sn*-1,2-diacylglycerol substrate and the effect added amphiphiles may have on the nature of the water/*sn*-1,2-diacylglycerol interface before increased reaction velocity is attributed to a physiological modulation of the activity by phospholipid.

It may, however, be possible to draw conclusions on the modulation of DAG kinase activity by phospholipid if both the phospolipid and *sn*-1,2-diacylglycerol are solubilised in the mixed micellar enviroment provided by a detergent above its c.m.c. This approach has been successfully employed in the characterisation of the lipid requirements of both rat brain PKC and the 14 kDa DAG kinase of *E. coli* (Hannun *et al.*, 1985, 1986; Walsh & Bell, 1986a, b). Employing such mixed micellar methodology, PtdSer was identified as an activator of the porcine 80 kDa DAG kinase in the presence of 10 μM Ca^{2+}, although it is slightly inhibitory in the absence of free Ca^{2+} (Sakane *et al.*, 1991a). Such activation of the porcine DAG kinase in the presence of both PtdSer and Ca^{2+} was the result of approximately 4-fold greater V_{max} and 10-fold reduced K_m for ATP (Sakane *et al.*, 1991a). Similarly, employing mixed micellar assay methodology, MacDonald and co-workers found PtdSer activated the *sn*-1-acyl-2-arachidonylglycerol-selective DAG kinase of Swiss 3T3 fibroblast membranes by elevating the V_{max} and decreasing the apparent K_m for *sn*-1,2-diacylglycerol (MacDonald *et al.*, 1988b). Cardiolipin and phosphatidylglycerol were also observed to activate the Swiss 3T3 membrane-associated enzyme under mixed micellar conditions, but to a lesser extent than PtdSer (MacDonald *et al.*, 1988a).

The effect of phospholipid on DAG kinase activity *in vivo* has still to be approached and extrapolation of *in vitro* data to the physiological state is limited by the methodology available at present for the solubilisation/presentation of *sn*-1,2-diacylglycerol in aqueous solution. The use of mixed micellar methodology eliminates the possibility that alteration of DAG kinase reaction velocity is a product of altered solubility or orientation of *sn*-1,2-diacylglycerol and has been employed to identify some modulatory role of phospholipids for the Swiss 3T3 and porcine 80 kDa DAG kinases (see above). The interpretation of such data must be qualified. The PtdSer-dependent Ca^{2+} activation of the porcine enzyme is of unknown function within the cell and follows a shorter time-course than the Ca^{2+}-dependent translocation of 80 kDa DAG kinase immunoreactivity from the cytosolic

to particulate fraction of porcine thymocyte homogenate (Sakane *et al.*, 1991a). Also, the PtdSer-dependency of the Swiss 3T3 enzyme can be reduced by elevating the *sn*-1,2-diacylglycerol concentration within the mixed micelle, thus resulting in the reduction of the K_a from 0.56 mol% (with 3 mol % *sn*-1-stearoyl-2-arachidonylglycerol) to 0.24 mol% (with 6 mol% *sn*-1-stearoyl-2-arachidonylglycerol). This may indicate that the enzyme has a non-specific requirement for glycerolipid, rather than a specific dependence on PtdSer for increased reaction rates.

5.6. An *sn*-1-Acyl-2-Arachidonyl-Selective DAG Kinase Has Not Been Purified?

Identification of the DAG kinase isoenzyme involved in the metabolism of PtdIns(4,5)P_2-derived *sn*-1,2-diacylglycerol has been prevented by the specific detergent and phospholipid requirements of the *sn*-1-acyl-2-arachidonylglycerol-specific enzyme (see Section 5.5). To date, several DAG kinases have been purified to homogeneity (Kanoh *et al.*, 1983; Kato & Takenawa, 1990; Yada *et al.*, 1990). Several other DAG kinases have been partially purified or separated from other isoenzymes (Kanoh & Ohno, 1981; Lin *et al.*, 1986; Besterman *et al.*, 1986b; Sakane *et al.*, 1989; Lemaitre *et al.*, 1990; Kahn and Besterman, 1991; Inoue *et al.*, 1992). Unfortunately, the kinetic characterisation of these enzymes has been wholly performed with dispersed *sn*-1,2-diacylglycerol/deoxycholate methods and not with the *n*-octyl-β,D-glucopyranoside mixed micellar method employed in the identification of *sn*-1-acyl-2-arachidonylglycerol-selective activities in Swiss 3T3 fibroblasts and baboon brain (MacDonald *et al.*, 1988a; Lemaitre *et al.*, 1990). Not surprisingly, none of the purified DAG kinases has been reported to phosphorylate *sn*-1-acyl-2-arachidonylglycerol with reaction velocities greater than other *sn*-1,2-diacylglycerols. This does not rule out the possibility that an *sn*-1-acyl-2-arachidonylglycerol-selective enzyme has been purified already, it hasn't, however, been identified. This is especially pertinent to the 150 kDa DAG kinase purified

from the membranes of rat brain (Kato and Takenawa, 1990), as MacDonald and co-workers located the DAG kinase active in the phosphorylation of receptor-generated *sn*-1,2-diacylglycerol in Swiss 3T3 cells to the membrane-associated fraction (MacDonald *et al.*, 1988a). However, until the detergent and phospholipid requirements of each individual enzyme are characterised this possibility remains unanswered.

5.7. The Substrate Specificity of the DAG Kinase Involved in Stimulated Phosphoinositide Tunover is Not Limited to *sn*-1-Acyl-2-Arachidonylglycerol.

To date, the DAG kinase active in the phosphorylation of PtdIns(4,5)P_2-derived *sn*-1,2-diacylglycerol has been characterised by its phosphorylation of *sn*-1-acyl-2-arachidonylglycerol with reaction rates greater than other long-chain *sn*-1,2-diaclgycerols, e.g. *sn*-1,2-dioleoylglycerol (MacDonald *et al.*, 1988a). However, present molecular species analysis is capable of separation and identification of *sn*-1,2-diacylglycerols previously unobserved in stimulated cells. MacDonald *et al.* (1988a) did not identify 18:0/20:3(n-9) or 16:0/20:3(n-9) diacylglycerol in PDGF-stimulated Swiss 3T3 cells, yet, they are significant components of the PtdIns(4,5)P_2-derived *sn*-1,2-diacylglycerol that is present 5 min after carbachol-stimulation of SK-N-SH cells (Lee *et al.*, 1991). However, together with *sn*-1-stearoyl-2-arachidonylglycerol and *sn*-1,2-dipalmitoylglycerol, 18:0/20:3(n-9) and 16:0/20:3(n-9) molecular species contribute towards greater than 55 mol% of the the PtdOH formed shortly after muscarinic stimulation (Lee *et al.*, 1991). Other *sn*-1,2-diacylglycerol products of receptor-mediated PtdIns(4,5)P_2 hydrolysis, distinct from *sn*-1-acyl-2-arachidonylglycerol, are also phosphorylated in reactions catalysed by the DAG kinase involved in stimulated PtdIns(4,5)P_2 turnover. It would seem likely that the DAG kinase linked to stimulated phosphoinositide hydrolysis catalyses the phosphorylation of most, if not all, the *sn*-1,2-diacylglycerol molecular species derived from PrtdIns(4,5)P_2.

This may not be a surprising conclusion as, typically, the agonist-elevated levels of *sn*-1,2-diacylglycerol at times shortly after stimulation (15 sec - 25 sec) are composed of molecular species characteristic of both PtdIns(4,5)P_2 and PtdCho hydrolysis (Pessin & Raben, 1989; Pessin *et al.*, 1990; Pettit & Wakelam, 1993). However, after more prolonged stimulation, for example 5 to 60 min, the molecular species present in the stimulated levels of *sn*-1,2-diacylglycerol are mainly, if not totally, representative of PtdCho hydrolysis (Pessin & Raben, 1989; Lee *et al.*, 1991; Pettit & Wakelam, 1993), suggesting quite selective removal of PtdIns(4,5)P_2-derived *sn*-1,2-diacylglycerol.

Although the ability of the DAG kinase isoenzyme, active in initiating the metabolism of PtdIns(4,5)P_2-derived *sn*-1,2-diacylglycerol, to selectively phosphorylate *sn*-1-acyl-2-arachidonylglycerol is diagnostic of this particular enzyme, its catalytic selectivity towards other molecular species characteristic of PtdIns(4,5)P_2 hydrolysis, e.g. *sn*-1,2-diacylglycerols with unsaturated fatty acids with 3 or more double bonds in the *sn*-2-position (Pessin & Raben, 1989; Pettit & Wakelam, 1993) must be borne in mind.

For the purposes of the discussion within this thesis, although it is acknowledged that the DAG kinase linked to receptor-stimulated PtdIns(4,5)P_2 turnover is active in catalysing the phosphorylation of many molecular species of *sn*-1,2-diacylglycerol typical of PtdIns(4,5)P_2 hydrolysis, the DAG kinase identified in this role will be refered to as the '*sn*-1-acyl-2-arachidonylglycerol-selective DAG kinase'.

5.8. The *sn*-1-Acyl-2-Arachidonylglycerol-Selectivity of the KCl-Soluble Diacylglycerol Kinase of Rat Brain.

Since the initiation of this project it has become apparent that several DAG kinase activities are present within the cell and careful consideration of the assay conditions is required in order that catalytic selectivity towards *sn*-1-acyl-2-arachidonylglycerol may be observed. As discussed in Section 5.5, the *sn*-1-acyl-

2-arachidonyl-selective DAG kinase would appear to require quite specific assay conditions in order that it may display catalytic selectivity similar to that observed in Swiss 3T3 fibroblasts. A mixed-micellar assay system employing *n*-octyl-β,D-glucopyranoside and PtdSer has been succesfully employed to identify *sn*-1-acyl-2-arachidonyl-selective catalytic enzymes Swiss 3T3 mouse fibroblasts and baboon brain (MacDonald *et al.*, 1988a, b; Lemaitre *et al.*, 1990). Under identical assay conditions, the KCl-soluble DAG kinase, partially purified in the work towards this thesis, would appear to display no catalytic selectivity towards *sn*-1-acyl-2-arachidonylglycerol and, thus, would seem not to be the DAG kinase involved in receptor-stimulated PtdIns(4,5)P_2 turnover in rat brain. As discussed in Section 4.3.2, it should be noted that this study was conducted without a positive contol, such as the *sn*-1-acyl-2-arachidonylglycerol-selective DAG kinase of Swiss 3T3 fibroblast membranes (MacDonald *et al.*, 1988b). However, subsequent to the completion of the work towards this thesis, an *sn*-1-acyl-2arachidonylglycerol-selective DAG kinase from porcine testis membranes was characterised within this laboratory while employing identical assay conditions (S.D. Gardner, personal communcation). This may still identify the need for different assay conditions in order that such selectivity may be observed with the rat brain KCl-soluble enzyme as individual isoenzymes have proven to retain specific detergent and phospholipid requirements (see Section 5.5). Alternatively, it may identify the loss of a regulatory factor during the purification of the KCl-soluble DAG kinase. The 110 kDa DAG kinase of rat brain purified to homogeneity by Kato & Takenawa (1990) was recovered with greater than 100 % yield on ATP-agarose chromatography, suggesting the separation of the catalytic activity from a modulatory factor or subunit. Typically, the DAG kinases purified to date exist as single polypeptide chains on SDS-PAGE analysis and no precedent exists for the involvement of a regulatory subunit in the modulation of DAG kinase reaction velocities. However, Sakane and co-workers (1989) did report the purification of a DAG kinase with apparent molecular mass of 150 kDa from porcine thymus cytosol. SDS-PAGE

analysis of the purified protein resulted in the resolution of two proteins (75 and 50 kDa). The nature of this heterodimeric DAG kinase preparation was not investigated further. Other regulatory factors, distinct from regulatory subunits, may also be lost on successive chromatographic steps. Given the modulatory role of phospholipids on the reaction rates catalysed by DAG kinase *in vitro* (see Section 5.5.2), the possibility may exist that the *sn*-1-acyl-2-arachidonyl-selectivity displayed by the mouse fibroblast and baboon enzymes requires the presence of a phospholipid distinct from PtdSer. Of the two *sn*-1-acyl-2-arachidonyl-selective enzymes previously reported, both had been examined in crude homogenates and lysates or detergent extracts. Neither enzyme had undergone multiple chromatographic steps which would remove the enzyme from a phospholipid/detergent mixed micellar environment (MacDonald *et al.*, 1988a, b; Lemaitre *et al.*, 1990). This may be pertinent, as the DAG kinase of *E. coli* displays altered *sn*-1,2-diacylglycerol substrate concentration dependence in the presence of phospholipid, when assayed in a non-ionic detergent mixed micellar system (Walsh & Bell, 1986b). These workers observed the *sn*-1,2-dioleoylglycerol substrate concentration dependence of the *E. coli* DAG kinase to be highly cooperative, with a Hill Coefficient of 4.4 - 4.5 in the absence of phospholipid, whereas in the presence of phosphatidylglycerol, double-reciprocal plots of the *sn*-1,2-diacylglycerol concentration dependence were linear at all concentrations of phospholipid examined (Walsh & Bell, 1986b). Protection of the *E. coli* DAG kinase from inactivation during reconstitution by incubation with cardiolipin (Loomis *et al.*, 1985) led to the suggestion that delipidation was at least partly responsible for the loss of enzyme activity during inactivation (Walsh & Bell, 1986a). To avoid such problems, membranes prepared from an *E. coli* strain that over-expressed the DAG kinase 100-fold were employed to investigate the enzyme's *sn*-1,2-diacylglycerol substrate and phospholipid co-factor dependence (Walsh & Bell, 1986a, b). The possibility that the *sn*-1-acyl-2-arachidonylglycerol-selectivity of mammalian DAG kinase isoenzymes could similarly alter during

purification remains to be investigated. Irrespective of these possibilities, the KCl-soluble DAG kinase discussed within this thesis would appear not to be *sn*-1-acyl-2-arachidonyl-selective.

5.9. An Alternative Strategy to the Purification of the *sn*-1-Acyl-2-Arachidonylglycerol-Selective DAG Kinase.

Of the DAG kinases purified to date, none have been reported to selectively phosphorylate *sn*-1,2-diacylglycerol characteristic of PtdIns(4,5)P_2 hydrolysis with increased rates of reaction, compared to substrates typical of PtdCho hydrolysis. As discussed in Section 5.5, this may indicate the use of improper assay methodology during such assessment. Alternatively, it may simply indicate that all the purifications, to date, involve isoenzymes distinct from the *sn*-1-acyl-2-arachidonylglycerol-selective enzyme. As the majority of purifications involve cytosolic enzymes, this may not be a surprising finding due to localisation of the *sn*-1-acyl-2-arachidonylglycerol-selective enzyme to the particulate fraction (MacDonald *et al.*, 1988a, b; Lemaitre *et al.*, 1990). To date, in addition to the purification reported within this thesis, only two membrane-associated DAG kinases have been purified, from rat brain and *Drosophila melanogoster* head, and neither appear to display selectivity amongst *sn*-1,2-diacylglycerol substrates (Kato & Takenawa, 1990; Inoue *et al.*, 1992, respectively).

The purification of membrane-associated DAG kinase has, typically, employed solubilisation of the enzyme by increasing ionic strength and not detergent solubilisation (Kato & Takenawa, 1990; Inoue *et al.*, 1992; Section 3.2.1, this thesis). The continued association of DAG kinase activity with the membranes of rat brain after treatment with 2.0 M NaCl has been reported (Kato & Takenawa, 1990). However, the use of non-ionic detergent above its c.m.c. has been reported to completely solubilise the membrane associated DAG kinase from Swiss 3T3 fibroblast membranes (MacDonald *et al.*, 1988a) and the use of non-ionic detergent above its c.m.c., in conjunction with increased ionic strength, has

resulted in the complete solubilisation of the membrane-associated activity of baboon brain (Lemaitre *et al.*, 1990). It is noteworthy that both reports highlighting the requirement for non-ionic detergent to achieve fully efficient solublisation of membrane-associated DAG kinase are concerned with the successful identification of *sn*-1-acyl-2-arachidonylglycerol-specific activities. This could easily be interpreted as the identification of the *sn*-1-acyl-2-arachidonylglycerol-selective DAG kinase as an integral membrane-associated protein, which requires detergent for solubilisation. Whereas, the salt-solubilised DAG kinases purified to date represent extrinsically-associated proteins without catalytic selectivity towards *sn*-1,2-diacylglycerol substrates.

Indeed, since the termination of the work conducted towards this thesis, work within this laboratory has identified such an *sn*-1-acyl-2-arachidonylglycerol-selective DAG kinase. Employing a plasma-membrane enriched particulate fraction prepared from porcine testis, it has been possible to solubilise a membrane-associated DAG kinase activity with non-ionic detergent. Moreover, this enzyme catalyses the phosphorylation of *sn*-1-stearoyl-2-arachidonylglycerol with greater rates of reaction than those observed with *sn*-1,2-dioleoylglycerol (S. Gardner, A. Paterson & M.J.O. Wakelam, unpublished results).

5.10. Purification of the *sn*-1-Acyl-2-Arachidonylglycerol-Selective DAG Kinase: Long-Term Aim of Project.

The ultimate aim of purifying *sn*-1-acyl-2-arachidonylglycerol-selective DAG kinase was to provide protein sequence data that would allow the construction of oligonucleotide probes and screening of rat brain cDNA libraries in order that a full length cDNA could be isolated and sequenced. Comparison of the cDNA sequence determined for the *sn*-1-acyl-2-arachidonylglycerol-selective DAG kinase to those previously determined for the porcine 80 kDa DAG kinase (Sakane *et al.*, 1990) and human white blood cell DAG kinase (Schaap *et al.*, 1990) would allow the initiation of structure/function studies and the possible identification of protein

motifs common to all DAG kinases, e.g. the *sn*-1,2-diacylglycerol binding site. Furthermore, such structure function studies would possibly allow the identification of motifs unique to the *sn*-1-acyl-2-arachidonylglycerol-selective DAG kinase and may identify the nature of the catalytic selectivity characteristic of the isoenzyme. Additionally, the information gained from these comparative studies of the isolated cDNA may allow the isolation of further related cDNA coding for other DAG kinase isoenzymes after the construction of oligonucleotide probes to regions of homology shared between the DAG kinases.

Finally, the purification of the *sn*-1-acyl-2-arachidonylglycerol-selective DAG kinase would allow the production of antisera. The antisera would possibly be raised from direct immunisation with the protein if the yield on purification was great enough or, alternatively, the antisera would be raised against peptides corresponding to sequence data generated during protein sequencing of the purified enzyme or deduced amino-acid sequence generated during the sequencing of cDNA isolated after oligonucleotide probing of cDNA libraries. Nevertheless, generation of antisera against the *sn*-1-acyl-2-arachidonylglycerol-selective DAG kinase would allow further investigation of the enzyme's role in the agonist-stimulated or quiescent cell by means of a range of immunological techniques, e.g. immunoprecipitation, uncoupling of DAG kinase activity in permeabilised cells, immunohistochemistry, etc.

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