THE ROLE OF POTASSIUM IONS IN

ANGIOTENSIN II-STIMULATED ALDOSTERONE SYNTHESIS.

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PUBLICATIONS.

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SUMMARY.

Angiotensin II (AII) stimulation of aldosterone synthesis entails depolarisation of the zona glomerulosa (ZG) cells. Since the ZG membrane potential is known to depend on the membrane permeability to potassium ions (K^+), this study investigated the relationship between ^{43}K efflux from freshly-isolated bovine ZG cells and basal and AII-stimulated aldosterone release.

In static incubations, ^{43}K efflux from unstimulated cells described a curve best fitted by two exponential functions with $t_{\frac{1}{2}}$ values of 47.7±1.7 and 14.2±0.6 min. Isotope from the faster-exchanging component appeared depleted by 50 min. AII (10^{-6} M) decreased the $t_{\frac{1}{2}}$ of the slower-exchanging component to 37.1±0.6 min and retarded efflux from the faster-exchanging one for 30 min. With ouabain present to prevent reuptake of the isotope, the slower-exchanging component $t_{\frac{1}{2}}$ was 28.4±1.1 min and the faster-exchanging component $t_{\frac{1}{2}}$ was 12.0±0.7 min. AII (10^{-6} M) reduced the former to 24.2±1.7 min ($p<0.01$) and retarded efflux from the latter.

Lower doses of AII also affected ^{43}K efflux biphasically but the point at which increased efflux from the slower component predominated was earlier for 10^{-6} M AII, about 30 min after the start of incubation, as compared to later than 50 min for 10^{-10} and 10^{-8} M.

^{43}K efflux from superfused cells identified only one component of cellular origin ($t_{\frac{1}{2}}$ 24.0±3.0 min) and isotope efflux from this was increased in parallel with

aldosterone production within 1 min of stimulation with AII 10^{-6} M. Increased efflux with AII stimulation derived from the slower-exchanging component in static incubations, therefore, the ^{43}K efflux from superfused cells may also derive from this component. Isotope from the faster-exchanging component may be depleted during perfusion prior to addition of the stimulus hence only the repolarising phase of ^{43}K efflux appeared to be affected by AII.

Manipulation of ZG cell membrane permeability to K^+ altered steroidogenesis. Increases in ^{43}K efflux caused by the ionophore valinomycin or the K^+ channel opener BRL 34915, reduced AII-stimulated aldosterone synthesis. Decreasing K^+ permeability with the ATP-dependent K^+ channel blocker glibenclamide shifted the AII dose-response curve to the left as did the Ca^{2+}-dependent K^+ channel inhibitor apamin. From the nature of the interaction of these agents with AII on ^{43}K loss, it is inferred that each acts separately and discretely on a channel in common with AII. Glibenclamide affects the faster- and apamin the slower-exchange component.

Blockade of voltage-dependent Ca^{2+} entry by verapamil not only decreased basal and AII-stimulated aldosterone output but also markedly decreased ^{43}K efflux. This was additive with the decrease in ^{43}K efflux seen with AII during the early part of incubation. When verapmil was added to cells from which isotope from the faster-exchange component was depleted, it still reduced ^{43}K efflux but, if applied simultaneously with AII, ^{43}K efflux was reduced less.

When added to cells preincubated with AII, verapamil had no significant effect on ^{43}K efflux or on aldosterone output. Verapamil appeared to affect the slower-exchange pool and aldosterone synthesis via a reduction in intracellular free Ca^{2+} ($[Ca^{2+}]_i$). Once AII-stimulated aldosterone production is established, Ca^{2+} entry via verapamil-sensitive channels appears to be of minor importance in maintaining steroidogenesis.

Blockers of Ca^{2+} release from intracellular stores, TMB-8 (10^{-4} M) and dantrolene ($2x10^{-4}$ M) abolished AII-stimulated aldosterone synthesis. The same dose of TMB-8 and $2x10^{-5}$ M dantrolene decreased ^{43}K efflux to a greater extent than AII (10^{-8}M) by 20 min and at 40 min the effect of either agent with AII was additive with that of the agent alone. However, this lower dose of dantrolene did not affect aldosterone synthesis.

Increasing the cell permeability to K^+ decreased AII-stimulated aldosterone synthesis probably by hyperpolarising the cells. Decreasing ^{43}K efflux, whether from the fast or slow exchange pool, increases both basal and AII-stimulated aldosterone synthesis assuming that $[Ca^{2+}]_i$ is high enough to maintain steroidogenesis.

The data presented here fit a model in which interaction of AII with its receptor closes K^+ channels to depolarise the cell membrane and promote Ca^{2+} entry. Glibenclamide is also able to close these K^+ channels and verapamil can reduce Ca^{2+} entry. Ca^{2+} entry by a means not sensitive to verapamil may also be increased by AII. Activation of the AII receptor also

induces inositol trisphosphate-mediated release of Ca^{2+} from intracellular stores which can be inhibited by TMB-8 and dantrolene. The raised $[Ca^{2+}]_i$ increases aldosterone synthesis and also opens Ca^{2+}-sensitive K^+ channels to repolarise the cell. These channels can be blocked by apamin.

ABBREVIATIONS.

ACTH	adrenocorticotropic hormone
AII	angiotensin II
4-AP	4-aminopyridine
ATP	adenosine 3',5'-triphosphate
BRL 34915	(+) 6 cyano-3, 4-dihydro-2, 2-dimethyl-trans-4 (2-oxo-1-pyrrolidyl)-2H-benzo[b] pyran-3-ol
BSA	bovine serum albumin
$[Ca^{2+}]_i$	intracellular free calcium ion concentration
$[Ca^{2+}]_i$	extracellular free calcium ion concentration
cAMP	cyclic adenosine 3',5'-monophosphate
dantrolene	(1-(5-(p-nitrophenyl)-fur-furyldene-amino) hydantoin sodium hydrate
IP_3	inositol 1,4,5 trisphosphate
$[K^+]_i$, $[K^+]_o$	as for calcium above
TEA	tetraethylammonium chloride
TMB-8	(8-(N,N-diethylamino) octyl-3,4,5-trimethoxybenzoate)
Val	valinomycin
ZF	zona fasciculata
ZG	zona glomerulosa
ZR	zona reticularis

CHAPTER 1: LITERATURE REVIEW.

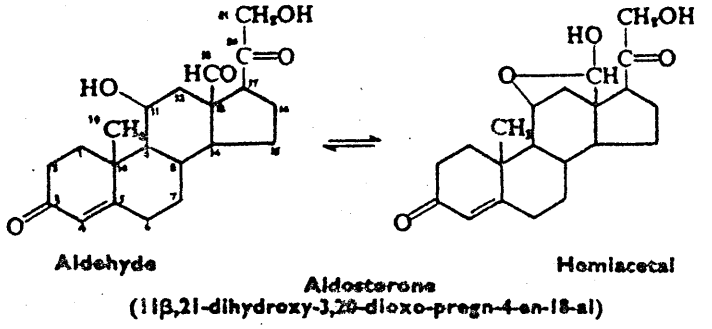
1.1. INTRODUCTION.

Aldosterone, (11ß,21-dihydroxy-3, 20-dioxo-pregn-4-en-18al) is one of the steroid hormones produced by the adrenal cortex. Figure 1.1a shows the structure of the two forms between which it can readily change. Like all steroids, it has four ring structures but aldosterone is unique in possessing an aldehyde group at carbon 18. In man and higher vertebrates it is the main mineralocorticoid, exerting effects at many sites in the body to maintain regulation of sodium and potassium balance.

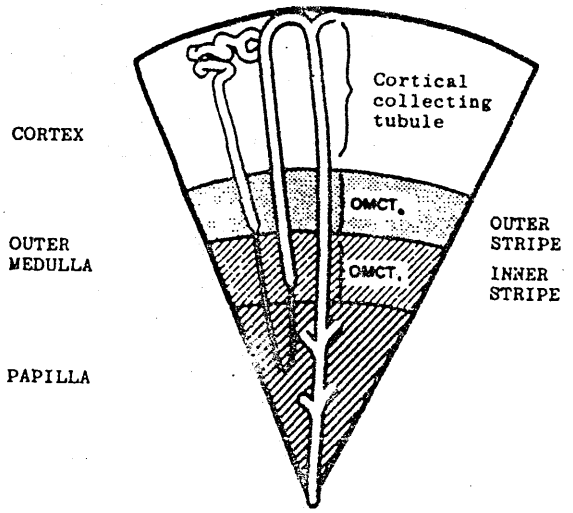
1.2. ACTIONS OF ALDOSTERONE.

The major site of aldosterone's action is on the principal cells of the kidney cortical collecting duct (Stokes et al 1981, figure 1.1b) to stimulate the mechanisms of Na^+ reabsorption and K^+ excretion. H^+ excretion is coupled to Na^+ reabsorption as an exchange mechanism in the cortical collecting duct (Stokes et al 1981, Kornandakieti and Tannen 1984) but in the medullary collecting duct it is mediated by a H^+ pump which can also be stimulated by aldosterone (Stone et al 1983). Water passively follows Na^+ movements, hence aldosterone is also involved in fluid balance.

Figure 1.1c summarises the intracellular actions of aldosterone. Much of the early work was done on the ventral skin and urinary bladder preparations from the



A.



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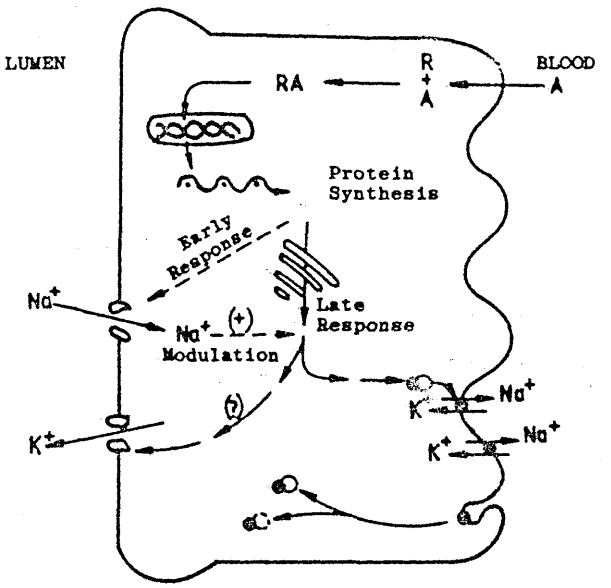


C.

Figure 1.1 Structure and site of action of aldosterone. A. Both forms which the molecule can adopt. (From Chattoraj, 1976). B. Site of the cortical collecting duct in the kidney in cross-section (from Stokes et al, 1981). C. Model of aldosterone action in the principal cell of the collecting duct. (From O'Neil, 1987).

marine toad (Bufo marinus) and showed that aldosterone raised Na^+ reabsorption by stimulating DNA-dependent synthesis of RNA and de novo synthesis of proteins (Edelman et al 1963, Crabbé and DeWeer 1964, Porter et al 1964, Sharp and Leaf 1964 and Fanestil and Edelman 1966). Experiments established that there was a latent period of 60-90 min between the application of aldosterone and the increased Na^+ reabsorption (Crabbé 1963) but that this was not due to a slow uptake of the hormone by the tissue (Edelman et al 1963). Having bound to cytoplasmic receptors, the aldosterone/ receptor complex migrates to the nucleus, (Fenastil and Edelman 1966), binds to chromatin and induces both transcription and synthesis of new messenger RNA. This produces a large number of, as yet, uncharacterised aldosterone-induced proteins by which the physiological effects are expressed. Some of these are involved in the supply of energy to the transporting mechanisms (reviewed by Edelman and Marver 1980).

The earliest effect is an increase in apical cell membrane Na^+ conductance (Fimognari et al 1967), which causes a net influx of Na^+ into the cell. This depolarises the apical cell membrane creating a driving force for K^+ excretion into the lumen. Also, the increased rate of Na^+ transport raises the turnover transport rate of the Na^+/K^+ pumps which increases the uptake of K^+ from the blood and secretion of Na^+ into the blood. This, combined with the depolarisation of the apical membrane favours K^+ secretion into the lumen. Following the onset of these changes in Na^+ and K^+ transport, the response, in adrenalectomised dogs

(Barger et al 1958) and adrenalectomised rats (Fimognari et al 1967), is increased to maximal levels 3 h after the injection of aldosterone.

Later, after 12-24 h, aldosterone increases in parallel both the synthesis of basolateral membrane Na^+/K^+ATPase and apical membrane conductance, possibly by increasing the number of K^+ channels (reviewed O'Neil 1987).

Prolonged exposure to aldosterone either experimentally induced or in clinical situations of hypersecretion, leads to metabolic alkalosis (Kassirer et al 1970, Hulter et al 1980). This phenomenon depends on the K^+ status of the kidney such that its severity increases with K^+ depletion. This means that although aldosterone stimulates Na^+ reabsorption in exchange for either K^+ or H^+, when K^+ is depleted, H^+ are preferentially secreted causing metabolic alkalosis (Mills et al 1960).

Although Na^+ and K^+ are exchanged in the principal cells this is not an obligatory one for one mechanism and the Na^+ and K^+ balance can be maintained independently over a wide range of their respective intakes (reviewed by Young 1988). Raised $[K^+]_o$ within the kidney tubule of itself increases the secretion of the ion into the distal nephron and, as noted above, during K^+ depletion H^+ is secreted in its stead.

1.3. SYNTHESIS OF ALDOSTERONE.

1.3.1. Zonation within the adrenal gland.

The mammalian adrenal cortex (shown in cross-section

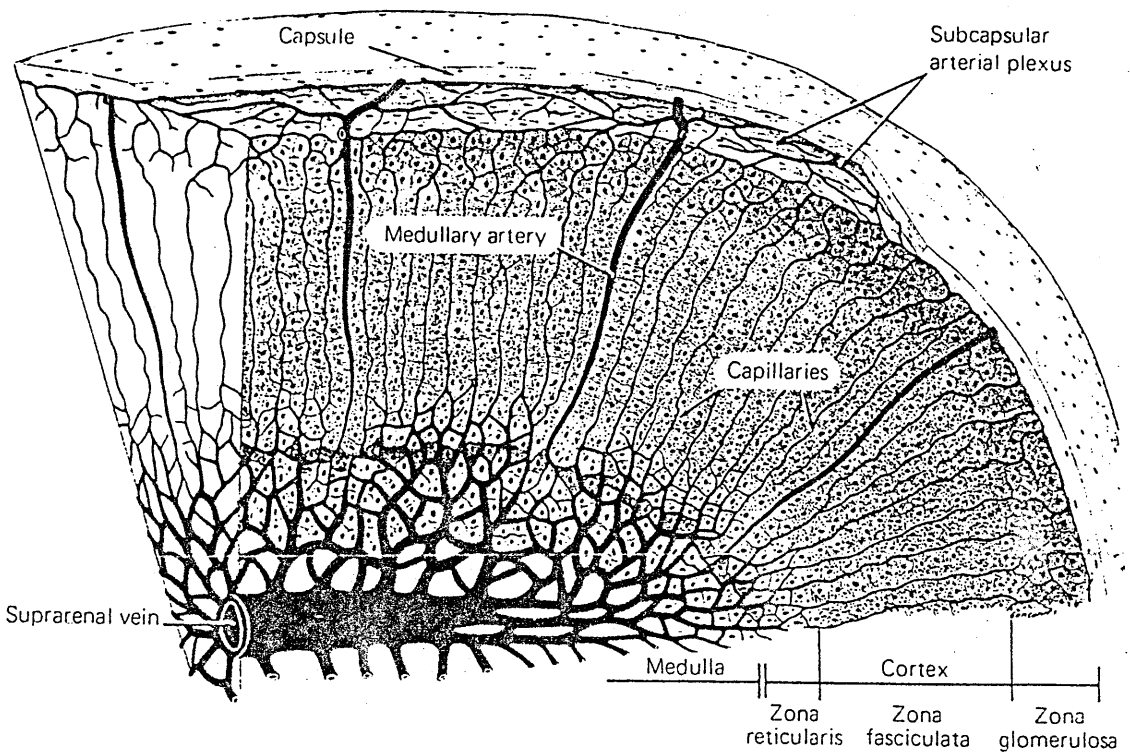


Figure 1.2. Diagram of the mammalian adrenal gland. (From Junquiera et al, 1986).

in figure 1.2) comprises three concentrically-arranged zones, each of which secretes a different combination of steroids. In non-mammalian species, these zones are less well-defined morphologically. Adrenocortical cells are continually renewed from subcapsular stem cells and are displaced inwards towards the medulla over about 104 days (Zajicek et al 1986). There is a common "core" set of reactions such that all the zones produce corticosterone, deoxy-corticosterone (DOC), progesterone, 18-hydroxy-corticosterone and 18-hydroxy-deoxycorticosterone. There are also specific conversions peculiar to the different regions of the cortex (Figure 1.3). The production of aldosterone is confined to the zona glomerulosa (ZG) and the zonae fasciculata/reticularis (ZF/R) are the main source of cortisol. An important species difference is that in the rat, 17α hydroxylation does not take place so that corticosterone, not cortisol, is the major gluco-corticoid. These inner regions of the cortex are also the sites of androgen precursor formation such as androstenedione (reviewed by Tait and Tait 1979). This means that early on in its life an adrenocyte secretes aldosterone, but later, on entering the (ZF) and the (ZR), it produces glucocorticoids and androgen precursors. About half of the adrenocytes die en route and the remainder are eliminated in the inner portion of the ZR by a process of aptosis (Wyllie et al 1973).

How the expression of different types of steroids from the same cell is confined to various stages in its migration is not known. One possibility is that

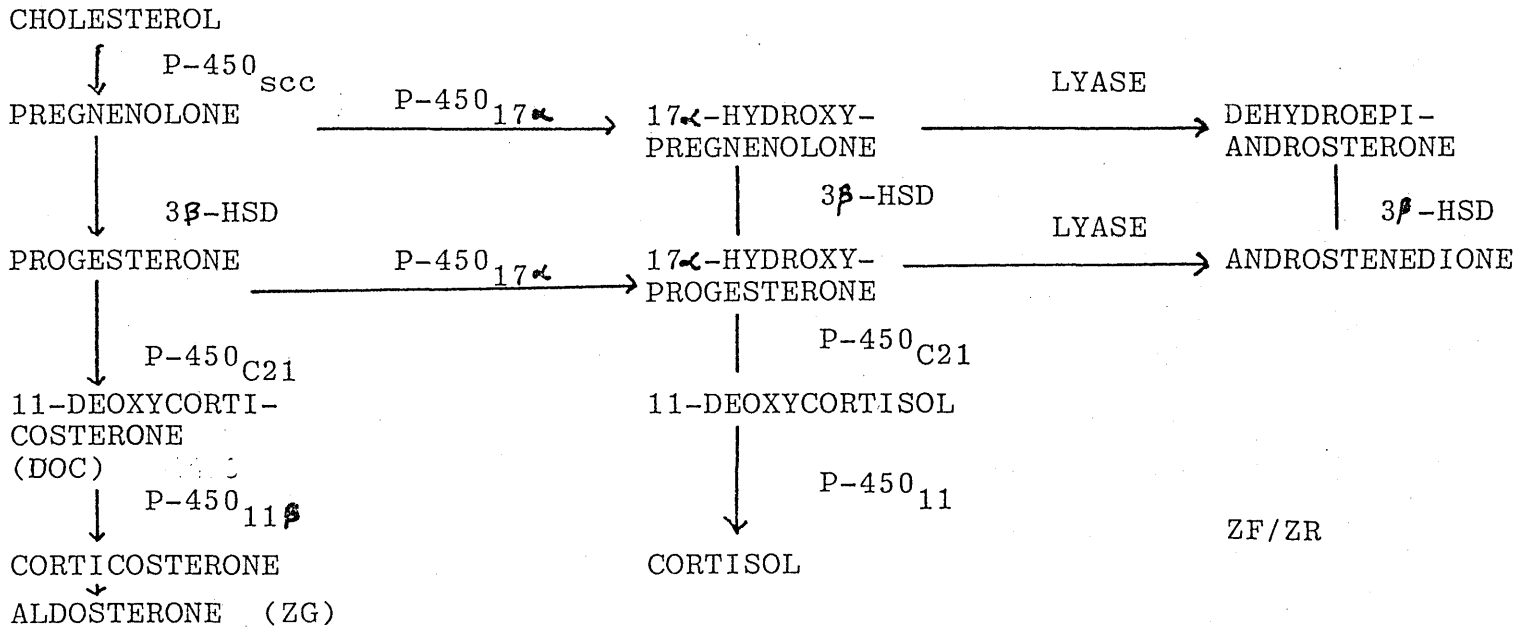


Figure 1.3. Steroidogenic pathways in the adrenal cortex. Lyase: 17,20-lyase, 3β-HSD: 3β-hydroxy steroid dehydrogenase. (Redrawn from Simpson et al, 1988)

steroids themselves interact with key cytochrome P-450 dependent enzymes and regulate their activity (see below). Steroids may reach very high concentrations in the inner portion of the adrenal cortex as a result of the unusual arrangement of the cortical capillaries which means that few arteries penetrate the cortex to supply the medulla without first supplying the cortex (see figure 1.2). Arterioles from the capsule give rise to capillaries that extend radially from the capsule through the cortex and unite in a plexus at the cortico-medullary boundary. This unusual centripetal arrangement of capillaries may result in cells of the inner cortex being exposed to blood containing high concentrations of steroids which have been secreted by the outer cortex, probably of the order of $6x10^{-5}$ M (reviewed by Hornsby 1989). This is about 100 fold higher than the normal range of $2\text{-}7x10^{-7}$ M for plasma cortisol which is the steroid produced in the greatest quantity in the normal human adrenal (Chattoraj 1976). However, during ACTH stimulation, the cortical vessels expand so that most adrenocortical cells are very close to a rich blood supply (Vinson et al 1985). This increased blood flow would not therefore allow the build-up of steroid gradients as proposed by Hornsby (1989).

However, high cortisol concentrations appear to contribute to the loss of 11ß hydroxylase activity and, in cultured adrenocortical cells, androstenedione in particular, behaves as a pseudosubstrate for 21 hydroxylase activity. Pseudosubstrates are compounds which, on binding to the substrate site of the

cytochrome P-450 containing enzymes, are not hydroxylated. Poor substrates, such as androstenedione with $P\text{-}450_{c21}$, or non-substrates such as norcamphor and fluorocarbons can act in this role. In "uncoupling" reactions, pseudosubstrates cause two NADPH molecules to be oxidised for every O_2 consumed compared to the usual one. Pseudosubstrate reactions also release oxidants such as superoxide (O^{2-}) from the cytochrome P-450 $(Fe^{2+}).O^{2-}$ complex which can damage the enzyme. For example, this can degrade the protein moiety and inactivate the haeme (reviewed by Hornsby 1989). Further recent work on the final steps in aldosterone synthesis suggest other mechanisms for enzyme activity alteration in the ZF/R with respect to the ZG (see below).

The cortical zones also differ in their sensitvity to various stimuli. This is probably due to the quantity and characteristics of their plasma membrane receptors as well as differences in amplification mechanisms involved in signal-secretion coupling. For example, in the ZG, adrenocorticotropic hormone (ACTH), AII and $[K+]_o$ rises of 3mM increase corticosterone output 2-3 fold whereas, in the ZF/R, only ACTH is active and stimulates corticosterone output 130 and 20 times respectively for these inner zones (reviewed by Tait et al 1980).

1.3.2. The biosynthetic path for aldosterone.

This is outlined in figure 1.4. Three groups of reactions are involved (reviewed Hall 1985b), a) cholesterol side chain cleavage, b) C_{21} hydroxylation

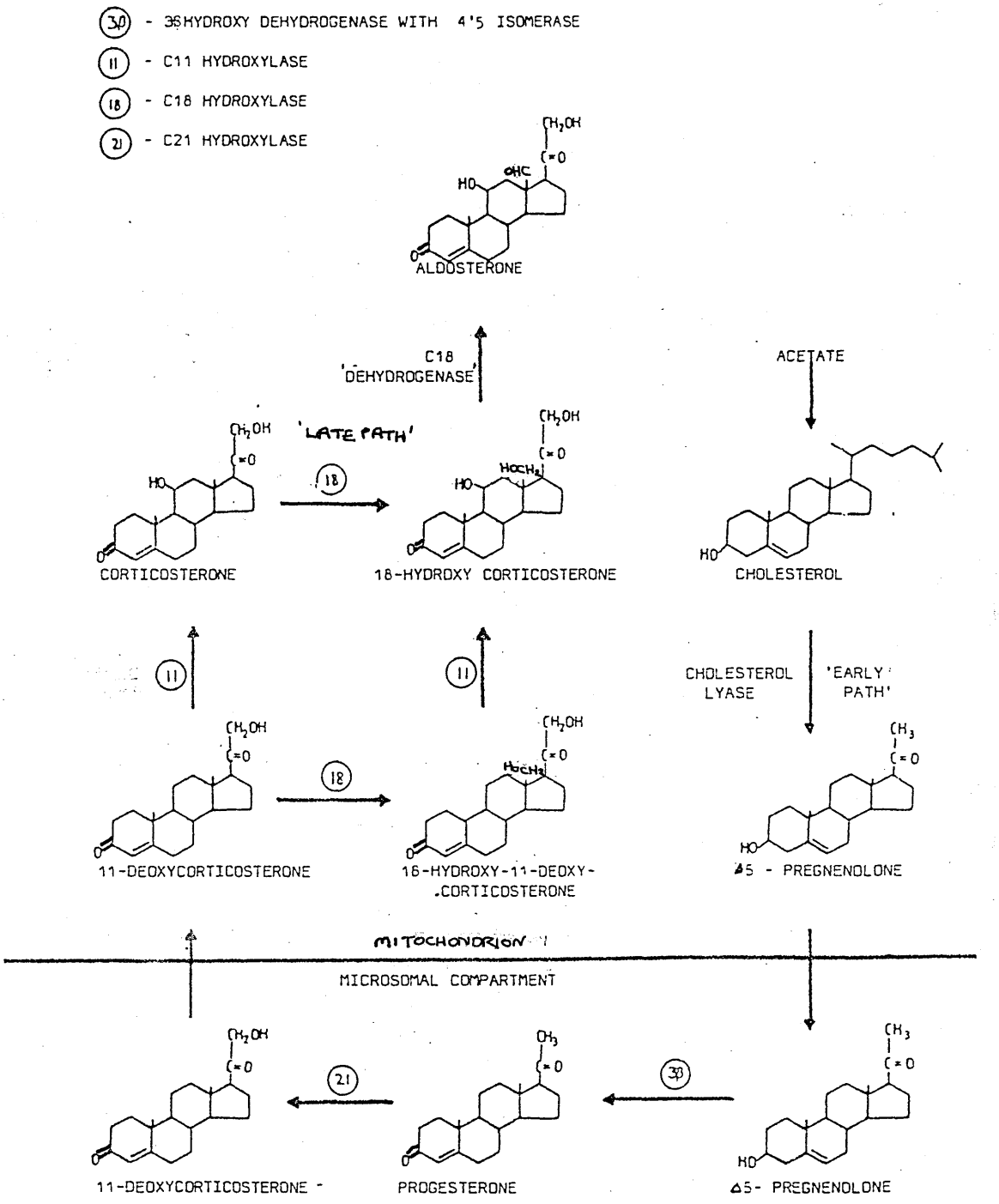


Figure 1.4. Biosynthetic pathway for aldosterone. (Redrawn from Chattoraj 1976).

and c) $C_{11\beta-18}$ hydroxylation. Each group is catalysed by a P-450 cytochrome-containing mixed-function oxidase complex (see below). $P\text{-}450_{scc}$ (cholesterol side-chain cleavage) and $P\text{-}450_{11\beta-18}$ are found in the mitochondrial inner membrane and $P\text{-}450_{c21}$ is sited in the microsomal compartment. It is therefore necessary for cholesterol and biosynthetic intermediates to move from the cytoplasm to the mitochondria then to the microsomes and then back to the mitochondria again. How the former transfers are accomplished is not known but disruption of the usual transport processes, the microfilaments, has been shown to decrease the conversion of 11-DOC to corticosterone (Feuilloley et al 1987). The mitochondrial hydroxylases comprise a flavoprotein (NADPH: adrenodoxin reductase) and an iron-sulphur protein adrenodoxin (reviewed by Hall 1985a). Reducing equivalents in the form of NADH are supplied to the enzyme complexes from the citric acid cycle and also by an electron transport system involving NADH-semidehydroascorbate reductase and ascorbic acid (Natarajan and Harding 1985,1987). The microsomal hydroxylase has only a flavoprotein NADPH:cytochrome P-450 reductase. All three complexes are mono-oxygenases in that they insert one oxygen atom into the substrate (reviewed by Hall 1985a, Gwynne and Mahaffee 1986), the other atom of molecular oxygen combines with H^+ to form water. Overall, the reaction can be summed up as follows:-

$$\text{Steroid-H} + \text{NADPH} + H^+ + O_2 = \text{Steroid-OH} + NADP^+ + H_2O.$$

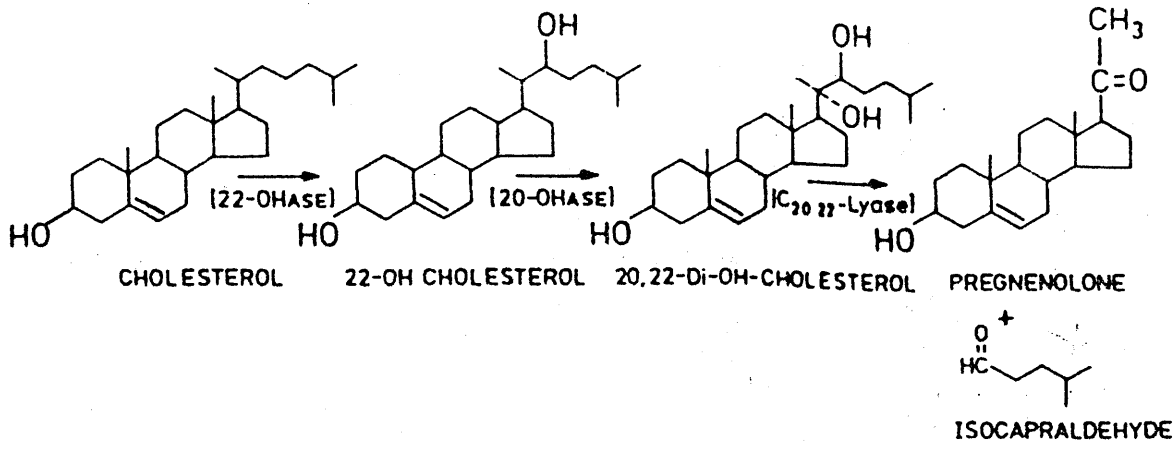


Figure 1.5. The reactions involved in the conversion of cholesterol to pregnenolone. (From Hall 1985b).

The conversion of cholesterol to pregnenolone requires three steps all catalysed by P-450$_{scc}$ and each requiring a molecule of NADPH and one of oxygen (Hall 1985a). Two of the steps are hydroxylations at the 20C and the 22R positions (figure 1.5) and the other is a lyase reaction which induces cleavage between these loci.

The conversion of pregnenolone to progesterone requires two distinct reactions; an isomerisation, in which the B ring is saturated and the A ring unsaturated (4-ene configuration) catalysed by a 5-4 isomerase, and the oxidation of the hydroxyl group at C_3 to an oxo-function which is controlled by 3ß-hydroxysteroid dehydrogenase (see figure 1.4). These enzymes are closely associated in the cell so that their respective reactions are linked. The so-called hydroxylation reactions at C_{21}, $_{11ß}$ and C_{18} are actually oxidation reactions like those involved in the side chain cleavage reactions. Oxidation of the C_{21} methyl group of progesterone is catalysed by the C_{21}-hydroxylase enzyme to produce 11-DOC. This becomes the substrate for 11ß hydroxylase and becomes converted to corticosterone and both 11-DOC and corticosterone are hydroxylated at C_{18}.

The final stage in the synthesis of aldosterone is incompletely understood. Comparison of the structures of aldosterone and its putative precursor, 18-hydroxy-corticosterone, suggests that a dehydrogenase enzyme system would be required (see figure 1.6). However, this precursor readily assumes a hemiketal structure, unfavourable to oxidation in this way, and the apparent

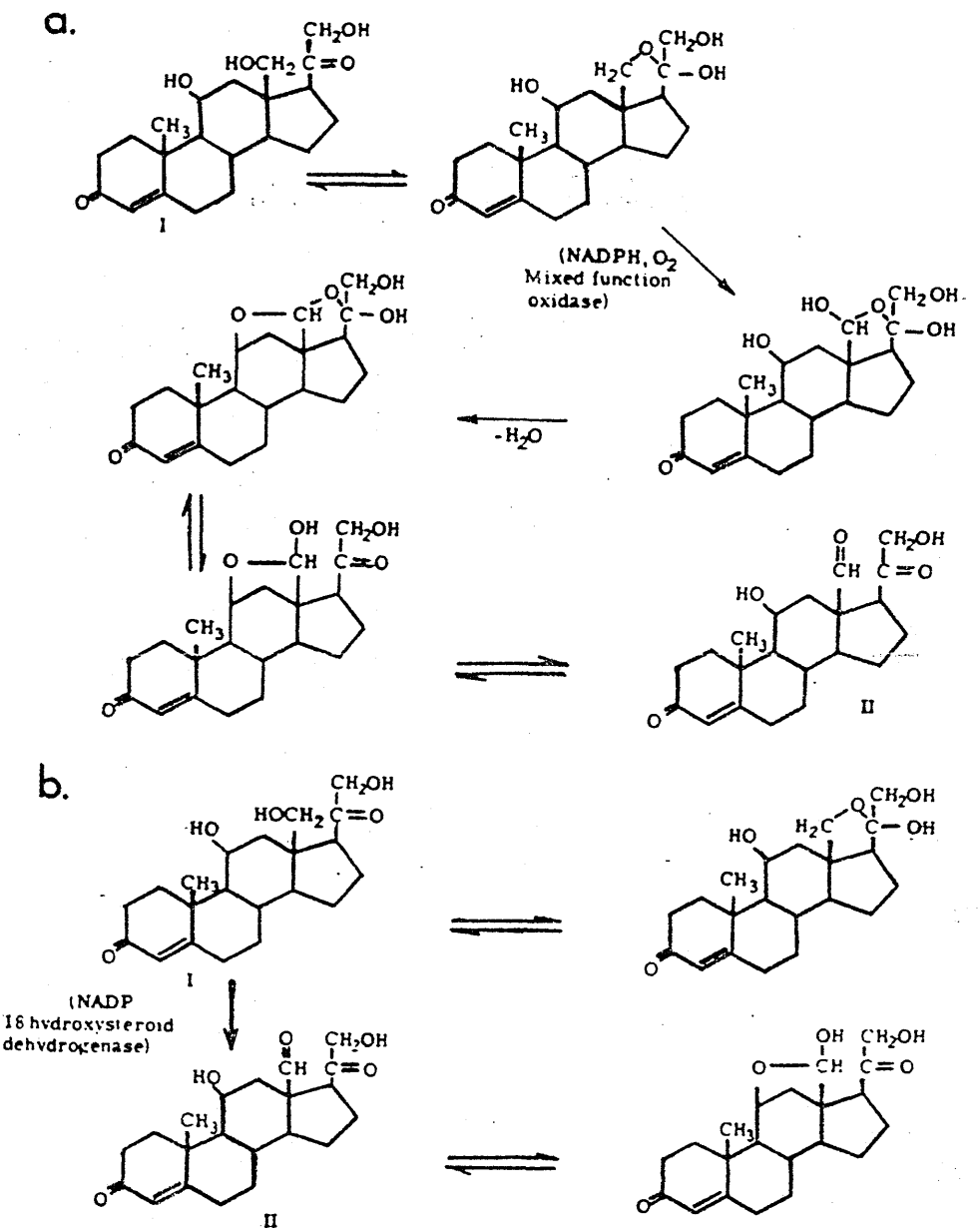


Figure 1.6. Possible biosynthetic pathways for the conversion of 18-hydroxycorticosterone (I) to aldosterone (II). (From Fraser and Lantos, 1978).

requirement for aerobic conditions in vitro is perhaps evidence for the involvement of a second mixed function oxidase introducing a second hydroxyl group at C18. This process could then be followed by dehydration (see figure 1.6) (reviewed by Fraser and Lantos 1978, Fraser 1983).

1.3.3. How aldosterone synthesis is confined to the zona glomerulosa: hypotheses.

It has been assumed that the enzyme catalysing the conversion of corticosterone or 18-hydroxycorticosterone to aldosterone is exclusive to the ZG. Recent studies using highly purified bovine adrenocortical cytochrome P-$450_{11\beta}$ hydroxylase from the ZF/R catalysed not only the hydroxylation of DOC at the C_{11} position, but also the conversion of corticosterone to 18 hydroxycorticosterone and aldosterone (Wada et al 1984, 1985, Ohnishi et al 1984). Similar results were confirmed with pig cytochrome P-$450_{11\beta}$ system (Yanagibashi et al 1986). Immunohistochemical studies on bovine adrenal cortex however, revealed that this cytochrome P-$450_{11\beta}$ is present in the mitochondria of both the ZF/R and the ZG (Mitani et al 1982, Sugano et al 1985). These findings apparently contradict the previous assumption that the aldosterone-synthesising enzyme is present only in the ZG cells. One possible explanation is that in the intact ZF/R the aldosterone synthetic activity of the P-$450_{11\beta}$ is inhibited, whereas that in the ZG is fully expressed. Further studies on the steroidogenic activity of detergent-solubilised mitochondria from pig and cattle ZF/R have

shown that the conversion of corticosterone to aldosterone in the presence of NADPH and the electron transfer components is catalysed at a rate comparable with that of ZG mitochondria (Ohnishi et al 1988). This suggested that the aldosterone synthesising activity of cytochrome P-$450_{11\beta}$ is inhibited in the intact ZF/R mitochondria but the inhibition can be overcome by detergent treatment. Phospholipids or lipidic extracts prepared from bovine adrenocortical mitochondria have been shown to increase the rates of aldosterone and 18-hydroxycorticosterone formation catalysed by purified cytochrome P-$450_{11\beta}$, while decreasing the conversion of 11-deoxycorticosterone to corticosterone (Wada et al 1984, 1985, Ohnishi et al 1984). Possibly, phospholipids surrounding the cytochrome P-$450_{11\beta}$ may influence the configuration of a steroid binding site in the cytochrome, affecting the rates of hydroxylation at different sites on the steroid skeleton. The active site of the cytochrome in the intact ZF/R mitochondria may be forced by phospholipids to take on a configuration that prevents further oxygenation of 18-hydroxycorticosterone at the C_{18} position. Adrenocortical calmodulin, in the presence of Ca^{2+}, also selectively inhibits the aldosterone synthetic activity of purified cytochrome P-$450_{11\beta}$ (Ohnishi et al 1986), so it is possible that a calmodulin-like protein factor could be present in the ZF/R mitochondria which prevents aldosterone synthesis.

Unlike the bovine or porcine adrenocortical mitochondria, those of rat ZF/R did not exhibit aldosterone synthesis after detergent solubilisation.

However, two different forms of rat cytochrome P-$450_{11\beta}$ were found in the rat adrenal cortex. A 49 kDa form found in the ZG and a 51 kDa form found in the ZF and in the ZG of rats with suppressed aldosterone synthesis. Since only the 49 kDa form was able to catalyse the final steps of aldosterone synthesis Lauber et al (1987) proposed that this form arises from post-translational processing of the 51 kDa form.

Studies of DNA from patients with inherited loss of the C_{18}-oxidation step show that both this condition and 11-ß hydroxylase deficiency may be caused by diferent mutations in the single gene for P-$450_{11\beta}$ (Globerman et al 1988). This, coupled with reports of 18-oxidation deficiency in patients with impaired 11ß hydroxylation argues for a multifunctional enzyme catalysing a series of steps from DOC to aldosterone.

Figure 1.7 summarises the three different hypotheses regarding the enzyme(s) involved in the biosynthetic steps between deoxycorticosterone and aldosterone.

1.3.4. Control points in the synthesis of aldosterone.

There are three sites at which steroidogenesis can be controlled:-

i) supply of cholesterol and its transport from lipid droplets to the inner mitochondrial membrane

ii) conversion of cholesterol to pregnenolone (the "early" path)

iii) conversion of corticosterone to aldosterone (the "late" path).

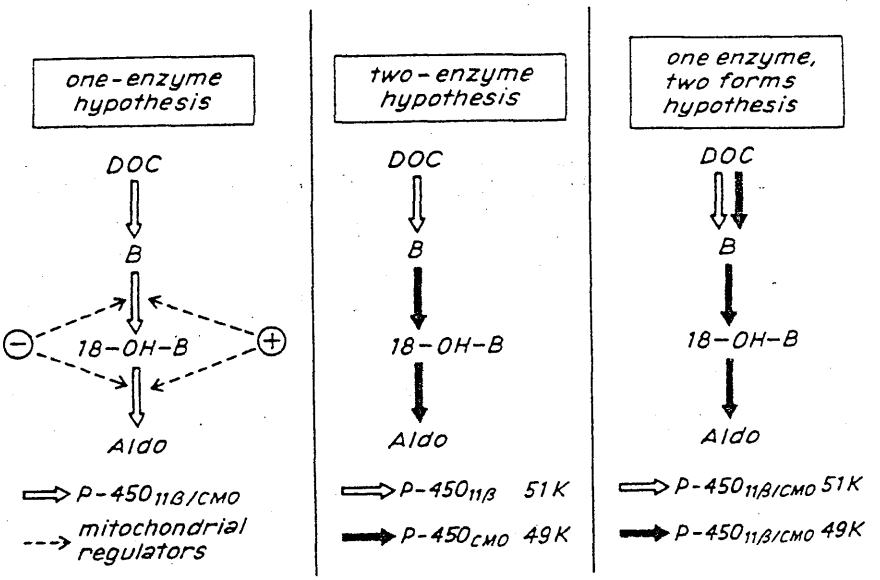


Figure 1.7. Three different hypotheses about the enzyme(s) involved in the biosynthetic steps between deoxycorticosterone (DOC) and aldosterone (Aldo).
(B) corticosterone, (18-OH-B) 18- hydroxycorticosterone.
(From Lauber et al, 1987).

1.3.4.(i) Supply of cholesterol.

Cholesterol is an obligate precursor in steroid production and, in the presence of adequate cholesterol substrate the rate-limiting, hormonally-regulated step is cholesterol side-chain cleavage (Gwynne and Mahaffee 1986). Specific inhibitors of this reaction are some synthetic cholesterol derivatives (Kreuger et al 1985) and aminoglutethimide (reviewed by Gower 1974).

Normally there are three potential sources of cholesterol:-

a) de novo synthesis from acetate

b) release from intracellular cholesterol ester stores

c) uptake of plasma lipoprotein cholesterol.

The latter two sources are more directly responsive to hormone stimulation since the increased activity of hydroxy- methylglutaryl (HMG) CoA reductase (the rate-limiting step enzyme in cholesterol synthesis) seems to be regulated mainly by cholesterol itself (reviewed Simpson et al 1987).

Two different paths for the uptake of lipoprotein cholesterol have been identified, one for low density (LDL) and one for high density lipoproteins (HDL). Figure 1.8 shows a model of LDL which is the major cholesterol-carrying lipoprotein in human plasma. The bulk of the cholesterol carried in LDL is located in an apolar core that contains approximately 1600 molecules of cholesterol per lipoprotein particle. Each molecule of cholesterol is esterified with a long chain fatty acid, the most abundant of which is linoleate. Surrounding the cholesteryl ester core is a polar coat comprising phospholipids, relatively small amounts of

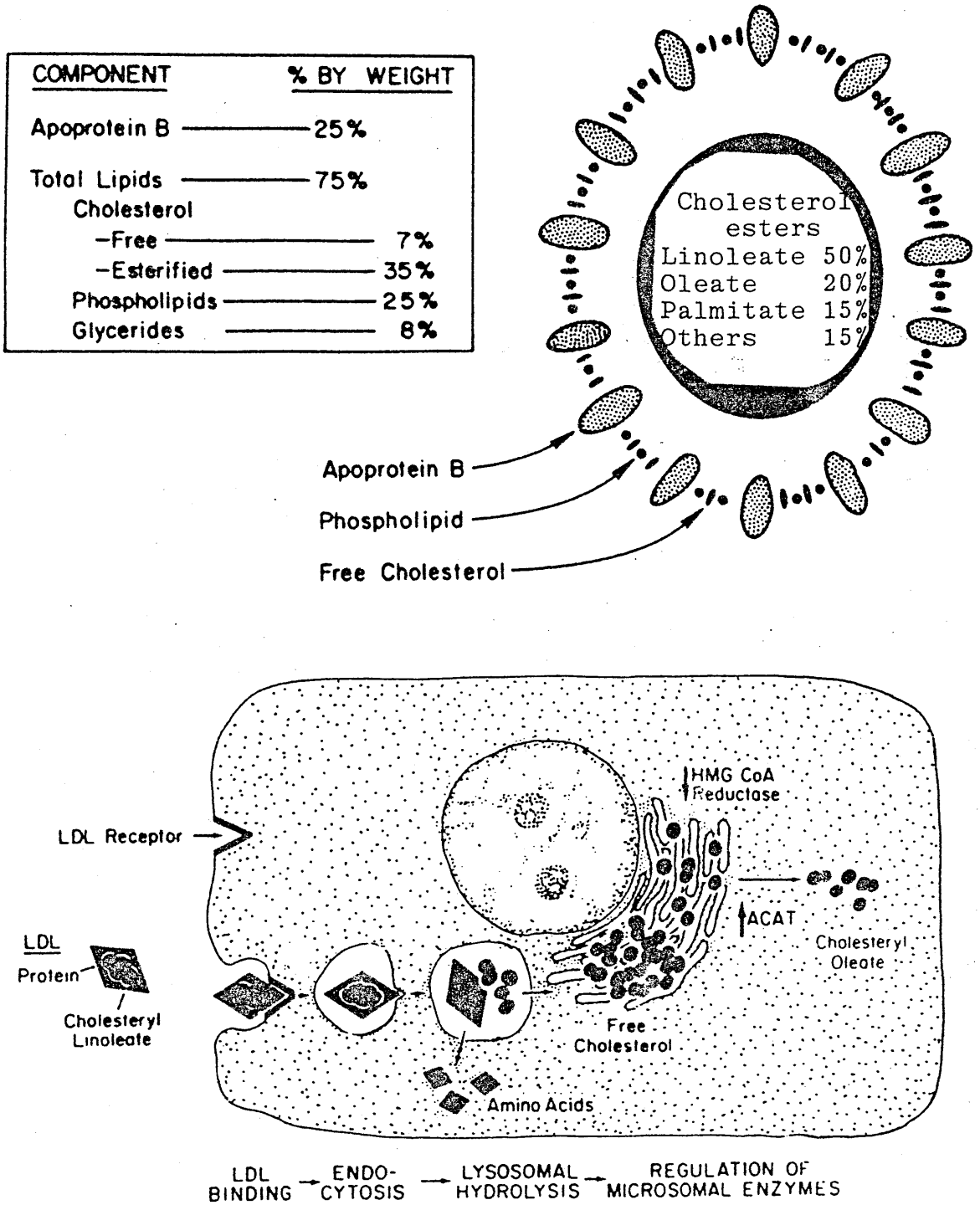


Figure 1.8. Structure and uptake mechanism of LDL. A. Schematic diagram of the structure and composition of plasma low-density lipoprotein. B. Steps in the LDL receptor pathway. (Both from Brown et al, 1979).

free cholesterol and a protein called apoprotein B. The structure of apoprotein B has not been elucidated but there is evidence to suggest that it is composed of multiple identical subunits each with a molecular weight of 25-35 kDa. Seven to ten of these subunits are associated to form a chain of about 240 kDa and two such chains are present in each LDL particle (reviewed by Brown et al 1979).

The sequence of reactions by which LDL is taken up by the cells and its cholesteryl esters hydrolysed has been called the LDL pathway. As shown in figure 1.8, the critical component of this path is a cell surface receptor which specifically binds the apoprotein component of LDL. In fibroblasts these receptors are located in discrete segments of the plasma membrane called coated pits (Andersen and Dietschy 1976, 1977). Approximately once every five minutes, each coated pit invaginates into the cell and pinches off to form a coated vesicle that carries the receptor-bound LDL to lysosomes. Within the lysosmes, the protein component of LDL is hydrolysed, the cholesteryl esters are cleaved by an acid lipase and the resultant free cholesterol is transported from the lysosome into the cellular compartment. The LDL receptor is recycled to the cell membrane (reviewed Brown et al 1979).

Cholesterol derived from LDL mediates three regulatory mechanisms which stabalise its intracellular concentration. First, the incoming cholesterol suppresses the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase), the rate-controlling step in cholesterol biosynthesis.

Second, the free cholesterol activates a microsomal acyl-coenzyme A:cholesteryl acyltransferase (ACAT), which esterifies any excess free cholesterol generated from LDL so that it can be stored as cholesteryl ester droplets. Finally, the free cholesterol derived from LDL suppresses the synthesis of LDL receptors thus preventing an overaccumulation of cholesterol (reviewed Brown et al 1979).

However, in rat steroidogenic tissues HDL is the preferred extracellular cholesterol donor (reviewed Gwynne and Strauss 1982). HDL particles resemble those for LDL but in man they contain apoproteins A-I and A-II which cannot bind to the LDL receptor. In some animals HDL contains apoprotein E which can bind to this receptor (Mahley 1988) but the HDL particle does not require endocytosis or lysosomal degradation to provide lipoprotein cholesterol for steroidogenesis or storage (reviewed by Gwynne and Strauss 1982). The LDL path has been identified in all steroidogenic tissues in all species tested but the HDL path has been identified in vivo only in the rat and mouse. In cultured sheep adrenal cells there is evidence for both paths (Durand et al 1987) but in freshly-isolated bovine cells incubated over 1h, HDL stimulates steroidogenesis whereas LDL is ineffective (Simpson et al 1989). In longer term incubations of cultured cells however, LDL is the preferred source of cholesterol (reviewewd Brown et al 1979).

Long term hormonal stimulation of steroidogenesis by, for instance, ACTH increases the number of LDL receptors and the rate of synthesis of sterol carrier

protein $_2$ (SCP_2) which transports cholesterol from the lipid stores to the mitochondria (Vahouny et al 1985). ACTH also stimulates P-450_{scc} activity by increasing cholesterol availability via activation of cholesteryl ester hydrolysis and regulation of ACAT (Shima et al 1972, Balasubramanian et al 1977.

1.3.4.(ii) Increased activity of the "early" and "late" paths.

Gene expression for all the cytochrome P-450 species in the steroidogenic pathway, as well as adrenodoxin is stimulated by the chronic action of ACTH (Kramer et al 1984, Simpson et al 1987 and reviewed by Simpson and Waterman 1988), an effect mediated by cyclic AMP (cAMP). Other factors acting by some, as yet, unspecified but independent mechanisms are required for the initial expression of steroid hydroxylase genes in the fetal adrenal which has minimal adenylate cyclase activity and therefore minimal cAMP production (John et al 1987). Increased production in rats of the P-450 enzyme which converts corticosterone to aldosterone has also been observed following both the resumption of K^+ intake following K^+ depletion and after Na^+ restriction (Meuli and Miller 1983,1984).

Other factors which increase the activity, rather than the production, of the P-450 enzymes include intracellular pH ($[pH]_i$), AII and K^+ as well as ACTH. A fall in mitochondrial pH reduces the Kd for cholesterol binding to P-450_{scc} (Jefcoate 1982) as does cardiolipin (Pember et al 1983), whereas the other three stimulants

increase the activities of both the early and late paths (reviewed Tait et al 1980).

1.4. INTRACELLULAR MECHANISMS OF HORMONE ACTION.

Cellular responses to hormones are initiated by interaction with a receptor. Receptors can be located intracellularly, such as those for steroid hormones, vitamin D and thyroid hormones or in the plasma membrane such as those for neurotransmitters, peptide hormones and growth factors.

The signal transduction mechanisms used by activated membrane receptors are of four types:-

1. those increasing the hydrolysis of phoshatidyl-inositol phosphates and the formation of diacylglycerol (DAG) and inositol 1,4,5 trisphosphate (IP3).
2. those changing cAMP formation by stimulation or inhibition of adenylate cyclase activity.
3. those opening channels to permit the flux of specific ions through the plasma membrane to trigger responses.
4. those possessing tyrosine kinase activity. These are not pertinent to the current thesis and will not be discussed further.

1.4.1. The calcium messenger system.

This is a nearly universal mechanism by which extracellular messengers regulate cell function via the above transduction mechanisms (reviewed Rasmussen 1986). Ca^{2+} is an ideal intracellular messenger because its small size together with its double charge enables

it to bind tightly and with high specificity to target proteins and hence alter their activity (reviewed by Carafoli and Penniston 1985).

In order to switch activity on and off, the concentration of a messenger must vary greatly. $[Ca^{2+}]_i$ levels are kept low (<0.1-0.2 μM) with respect to the external medium (1mM), so that relatively small numbers of Ca^{2+} ions upon entering the cell raise $[Ca^{2+}]_i$ several fold. $[Ca^{2+}]_i$ is kept at a low level by active ATPase dependent pumps in the plasma membrane to extrude it in exchange for H^+ and by intracellular sequestration using similar pumps located in the membranes of organelles such as the mitochondria and endoplasmic reticulum (ER) (see figure 1.9). The mitochondria act as a large-capacity, low-affinity store which takes up Ca^{2+} only when $[Ca^{2+}]_i$ rises to >0.7μM. In contrast, the ER has a low capacity, but higher affinity for Ca^{2+} and "fine tunes" $[Ca^{2+}]_i$. Na^+/Ca^{2+} exchange, which uses the Na^+ concentration gradient to exchange $3Na^+$ for 1 Ca^{2+} in the plasma membrane and $2Na^+$ for 1 Ca^{2+} in the mitochondria, is also found in many cells as a means of reducing $[Ca^{2+}]_i$ (reviewed by Carafoli and Penniston 1985).

The cellular protein which most commonly mediates the Ca^{2+} message is calmodulin. This has four calcium binding sites and its properties vary according to the number of sites which are occupied. Such a variation in calmodulin's properties may be the reason why it is able to interact with a great number of different proteins. Calmodulin acts on enzymes which catalyse the formation (kinases) and destruction (phosphatases) of

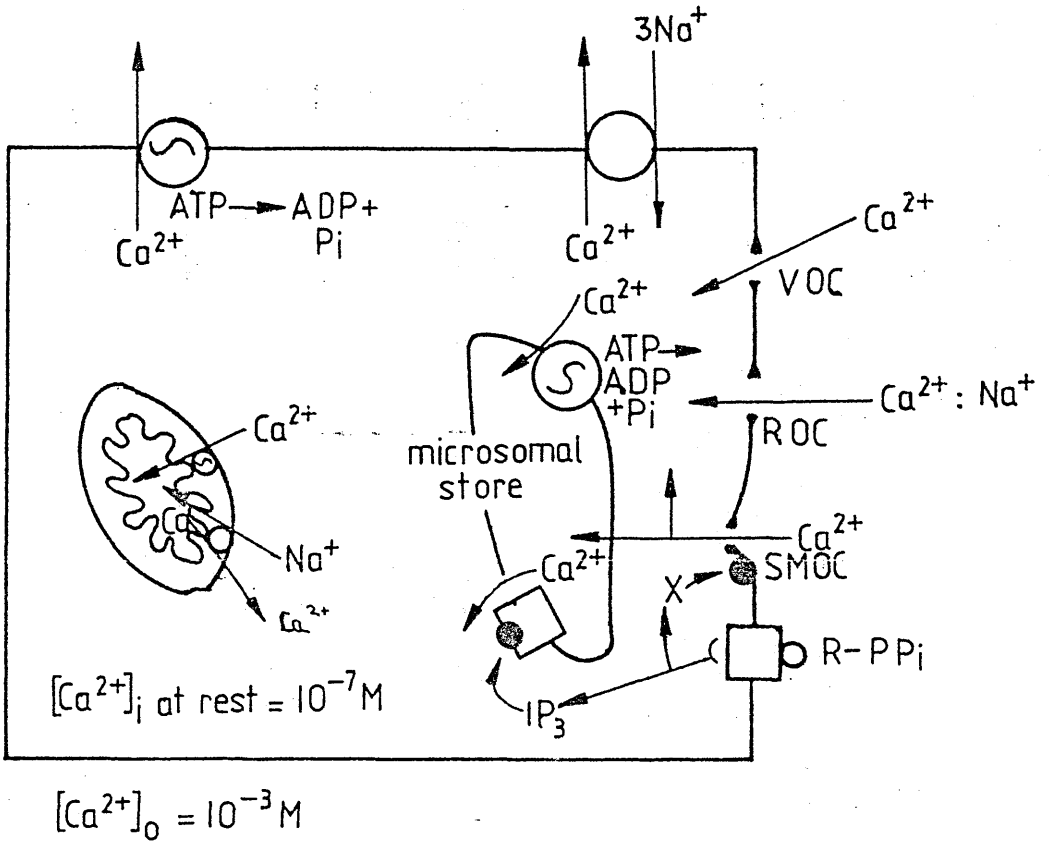


Figure 1.9. Schematic representation of processes involved in Ca^{2+} homeostasis. The Ca^{2+} pump and Na^{+}/Ca^{2+} exchanger are indicated at the top, Ca^{2+} channels of various types to the right and also a receptor coupled to phosphatidylinositol turnover (R-PPI). A second messenger (X) might cause opening of Ca^{2+} second messenger-operated channels (SMOCs). (Redrawn from Meldolesi and Pozzan, 1987).

phosphate bonds in biochemical molecules. Phosphorylation or dephosphorylation is often the trigger which alters the activity of an enzyme and hence calmodulin regulates many cell functions (reviewed by Carafoli and Penniston 1985). It also promotes Ca^{2+} extrusion, since rises in $[Ca^{2+}]_i$ allow calmodulin to associate with plasma membrane Ca^{2+}-ATPase (reviewed by Rasmussen 1986).

The duration of transient cellular responses like neuro-secretion and muscle contraction depend on the size of the Ca^{2+} message. In sustained responses, such as the increased aldosterone secretion following AII stimulation, an initial rise in $[Ca^{2+}]_i$ is often followed by a fall to a level at, or slightly above basal. However, the affinity for Ca^{2+} of the enzyme(s) or response elements involved in steroidogenesis is enhanced so that they remain activated at a lower $[Ca^{2+}]_i$ than was needed to initiate the response (reviewed by Rasmussen 1986).

1.4.2. The polyphosphatidyl inositol system.

Activation of receptors stimulates phospholipase C (figure 1.10) to catalyse the hydrolysis of phoshatidylinositol in the membrane to generate inositol 1,4,5 trisphosphate (IP_3) and diacylglycerol (DAG) (reviewed Berridge 1981, 1986). IP_3, being water-soluble, enters the cytosol and releases bound Ca^{2+} from intracellular stores. For some time, it has been assumed that the endoplasmic reticulum (ER) was the repository for IP_3-releasable Ca^{2+} in non-muscle cells (the sarcoplasmic reticulum (SR) in muscle).

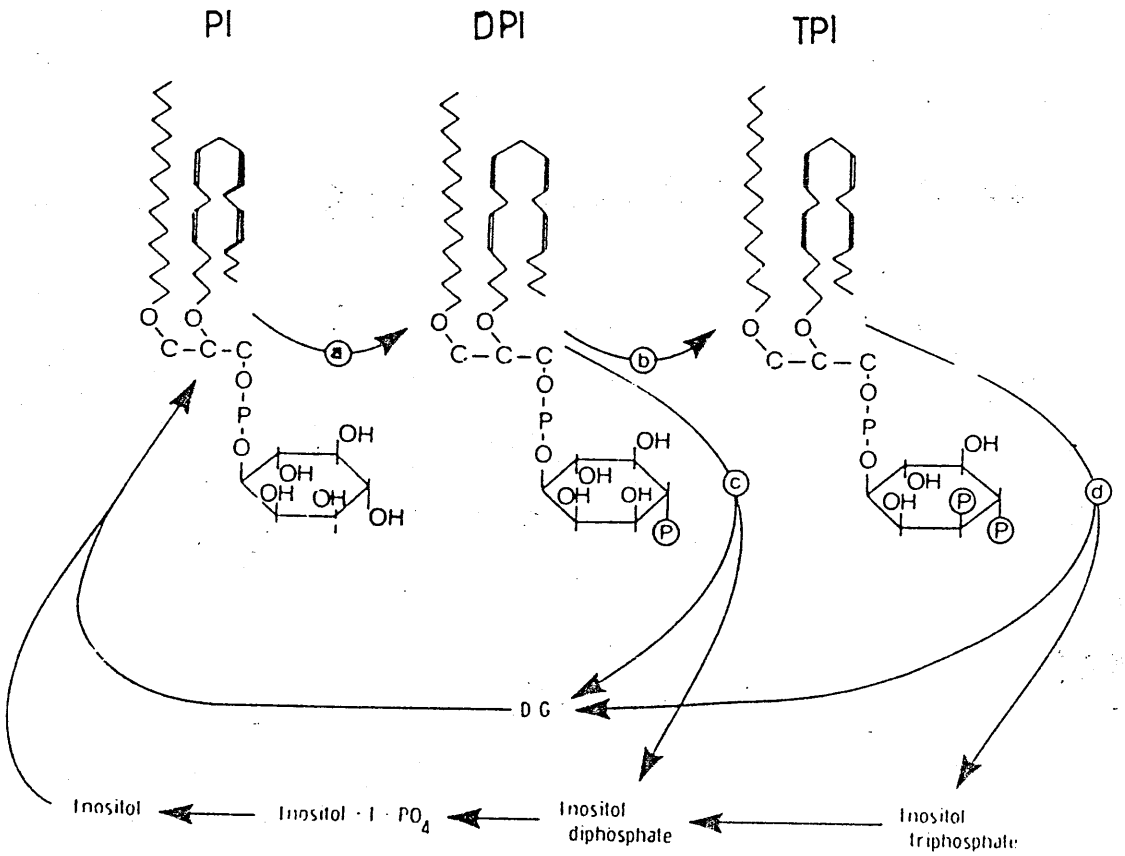


Figure 1.10. Major metabolic pathways for the polyphosphoinositides. Phoshatidylinositol (PI) is converted to phosphatidyl 4 phosphate (diphosphinositide, DPI) by a PI kinase (a). DPI is further phosphorylated to phosphatidylinositol 4,5 bisphosphate (triphosphoinositide, TPI) by a DPI kinase (b). There are corresponding phosphatases which remove these phosphate groups. In addition, there are phospholipase C-type enzymes (c and d) which remove the inositol headgroups of DPI and TPI to release inositol di- and trisphosphate respectively. Diacylglycerol (DG) remains in the membrane and resynthesised to PI. (From Berridge 1981).

However, recent work (Volpe et al 1988) provides good evidence for the existence in liver cells of other structures which appear to be discrete organelles called calciosomes. These possess a protein very similar, if not identical, to calsequestrin, a Ca^{2+}-binding molecule like calmodulin. In muscle, calsequestrin resides in the SR to aid Ca^{2+} accumulation (MacLennan et al 1983). If the Ca^{2+} binding properties of this calsequestrin-like protein are similar to the muscle calsequestrin then the capacity of the former store is estimated to be 0.2-0.4mmol/litre of cell volume (Volpe et al 1988). This would be sufficient to sustain the rapid $[Ca^{2+}]_i$ transients triggered by IP_3 (Pozzan et al 1982, Lew et al 1986). The initial rise in $[Ca^{2+}]_i$ released by IP_3 from intracellular stores is short-lived since the Ca^{2+} is rapidly sequestered by organelles (mainly non-mitochondrial) to maintain $[Ca^{2+}]_i$ below 0.1μm and the IP_3 quickly metabolised.

1.4.2.(i) Initial phase of cellular response.

In addition to releasing Ca^{2+} from intracellular stores, hormone-receptor interactions also stimulate the uptake of extracellular Ca^{2+} (figure 1.11). Initially, the transient rise in $[Ca^{2+}]_i$ activates calmodulin-dependent protein kinase which then catalyses the phosphorylation of a subset of cellular proteins responsible for further cellular responses. In a addition, calmodulin activates a plasma membrane Ca^{2+} pump. Simultaneous increases in Ca^{2+} uptake and release raises Ca^{2+} in the submembrane domain. This is also the

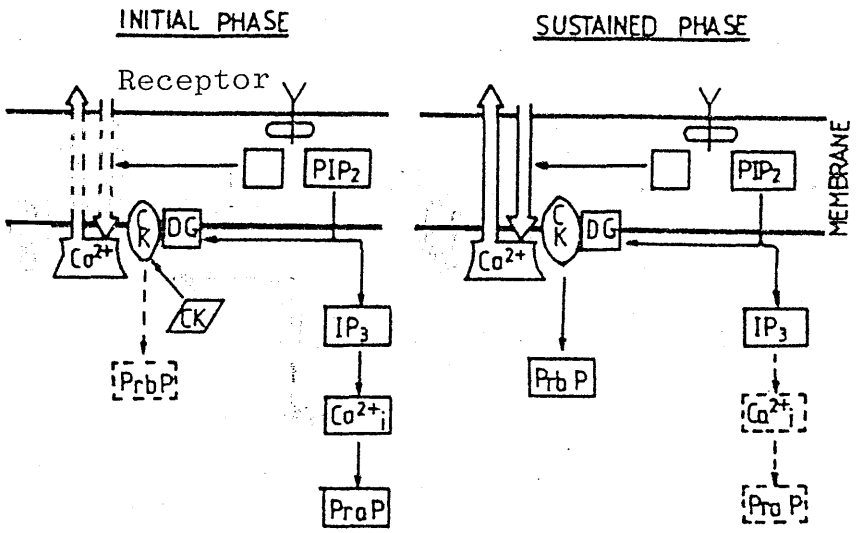


Figure 1.11. Model of the roles of diacylglycerol and inositol 1,4,5 trisphosphate in the Ca^{2+} messenger system.(Redrawn from Rasmussen 1986).

KEY

C K	protein kinase C (Ca^{2+}-sensitive form)
CK	" (Ca^{2+}-insensitive ")
DG	diacylglycerol
PIP_2	phosphatidylinositol 4,5 bisphosphate
PraP, PrbP	phosphorylated cellular proteins
———	major path
– – – –	minor path

site of DAG release and together, Ca^{2+} and DAG are though to regulate the activity of protein kinase C.

1.4.2.(ii) Sustained phase of cellular response.

During the sustained phase of the response, $[Ca^{2+}]_i$ returns to near basal levels and the activity of calmodulin-dependent protein kinase falls, so that this subset of cellular proteins becomes dephosphorylated. However, the increases in plasma membrane Ca^{2+} cycling and in DAG are sustained and keep protein kinase C activated. In this phase phosphorylation of a second subset of proteins controls the sustained cellular response (reviewed Rasmussen 1986).

1.4.3. The adenylate cyclase system.

There are hormones whose actions are mediated by changes in cAMP. Receptors which, when occupied, cause increases in cAMP levels are termed R_s and those which bring about decreases in cAMP are termed R_i. At the centre of the coupling process between the receptors and the altered adenylate cyclase activity are two signal-transducing proteins which bind magnesium and guanine nucleotides (figure 1.12). One, G_s, mediates the effects of R_s, the other, G_i, mediates the effects of Ri receptor binding. Both are heterotrimers consisting of a major subunit (α) of variable molecular weight (52 or 42 kDa for G_s and 40-41 kDa for G_i) and two smaller, tightly-coupled subunits (β and γ) which differ little between tissues and species but some heterogeneity may exist. Both G_i and G_s bind and hydrolyse GTP. The adenylate

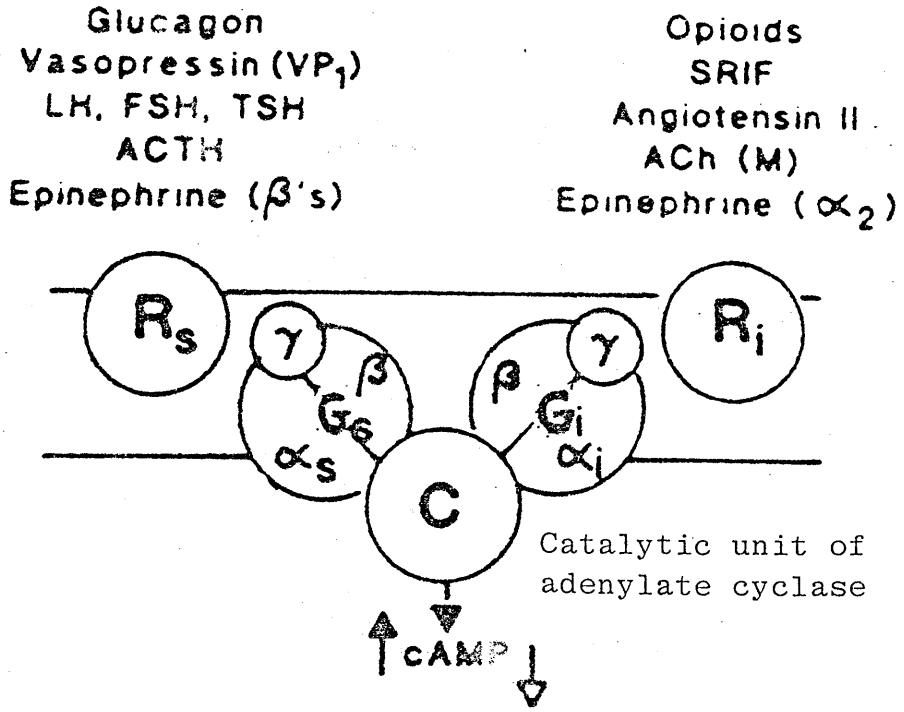


Figure 1.12. Basic constituents of a hormone-sensitive adenylate cyclase system. R_s and R_i stimulatory and inhibitory receptors which interact with G_s and G_i respectively. Both G proteins comprise three subunits, and act as Mg binding regulatory components. $Alpha_s$ is a substrate for cholera toxin, $alpha_i$ the substrate for Pertussis toxin. (From Birnbaumer et al 1985).

cyclase forms cAMP and Mg^{2+} pyrophosphate from Mg^{2+} ATP (reviewed Birnbaumer and Brown 1985). Addition of a stimulating hormone to an adenylate cyclase system causes a stimulation of cAMP production via the activation of G_s. An inhibitory hormone stimulates G_i resulting in inhibition of cyclase activity and a fall in cAMP production.

Cholera toxin causes the ADP-ribosylation of the α subunit of G_s and inhibits its GTPase thereby permenantly stimulating adenylate cyclase. Pertussis toxin ADP-ribosylates the α subunit of G_i but this blocks its effects on adenylate cyclase so removing the inhibitory action of G_i.

The activity of adenylate cyclase along with that of phosphodiesterase, the enzyme responsible for cAMP degradation by hydrolysis, determines the cellular content of cAMP which in turn modulates the flow of information through the protein kinase C and IP_3 branches of the Ca^{2+} messenger system (see figure 1.13). The mechanism by which cAMP influences these remains to be defined but it may involve sensitivity modulation of the kinases or the inhibition of phosphoprotein phosphatases (Rasmussen 1986).

1.4.4. Modulation of transmembrane ion fluxes.

A typical resting cell maintains differences in ionic concentrations across its plasma membrane as illustrated in figure 1.14. $[K^+]_i$ is high with respect to the medium and $[Na^+]_i$ and $[Ca^{2+}]_i$ are low. The membrane is permeable to K^+ so the ion tends to leak out down its concentration gradient. This transfers

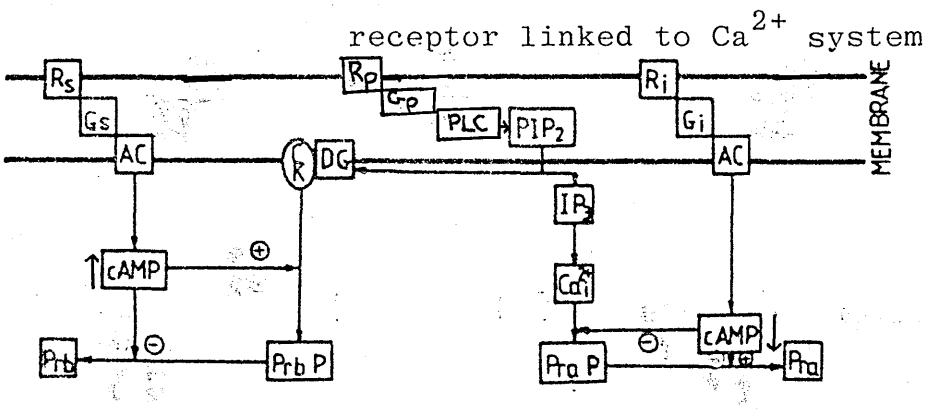


Figure 1.13. Model of the role of the cyclic AMP messenger system in regulating the responsiveness of a cell to activation through the Ca^{2+} messenger system. (Redrawn from Rasmussen 1986).

KEY

AD	adenylate cyclase
CK	protein kinase C
DG	diacylglycerol
G_i, G_s, G_p	nucleotide regulatory proteins
PIP_2	phosphatidylinositol 4,5 bisphosphate
Pra, Prb	dephosphorylated cellular proteins
PraP, PrbP	phosphorylated " "
R_i, R_s, R_p	receptors
PLC	phospholipase C
⊕	stimulation
⊖	inhibition

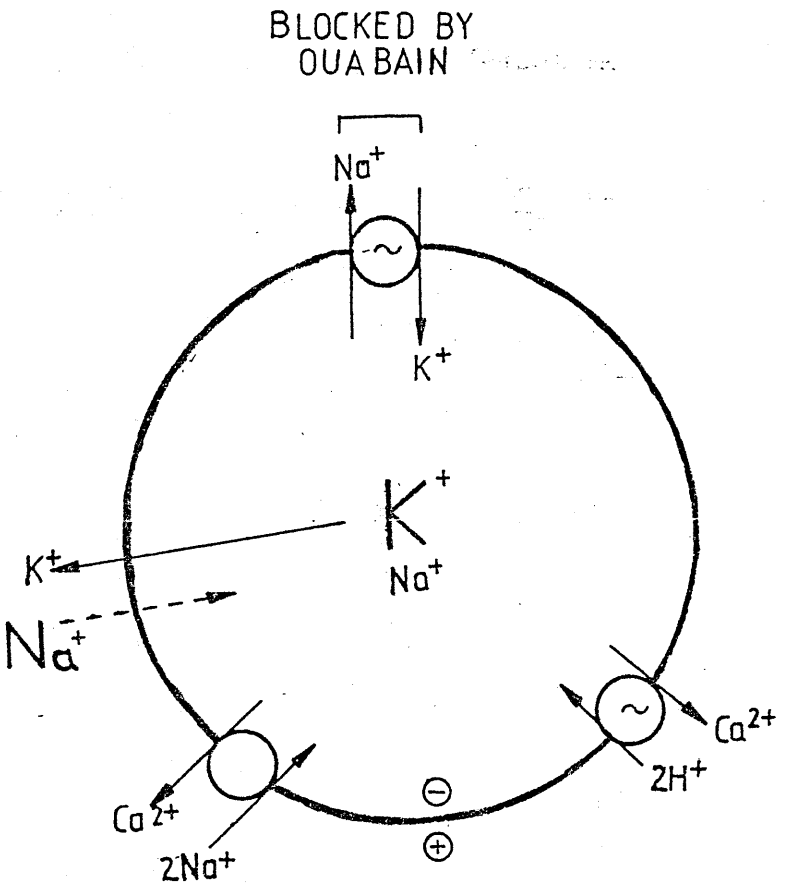


Figure 1.1.4. Diagram to show the movements of the major cations of a cell at rest. ∿ Denotes ATP-dependent pumps.

positive charge out of the cell leaving the inside negative with respect to the outside. Since the K^+ leak channel is slightly permeable to Na^+, this ion enters the cell down its concentration gradient rendering the inside less negative. This would encourage more K^+ to leave but the ionic gradients for these ions are maintained by the Na^+/K^+ ATPase. The membrane potential at which K^+ exit and entry are in balance is the resting membrane potential and for ZG cells this is about -70 mV (Quinn et al 1987a).

The high concentration gradient for Ca^{2+} means that if the plasma membrane were permeable, this ion would leak into the cell. However, as mentioned earlier, low intracellular levels are maintained by Ca^{2+} ATPase and by the Na^+/Ca^{2+} exchanger.

Perturbations of these ionic distributions can be effected by receptor-operated and voltage-dependent channels. Of major importance for non-excitable cells such as adrenocortical cells are those channels which affect K^+ and Ca^{2+} movements across the cell membrane.

1.4.4.(i) K^+ channels.

These can be divided into Ca^{2+}-activated and ATP-sensitive K^+ channels (reviewed by Petersen et al 1986, Blatz and Magleby 1987). The former channels are found in adrenal chromaffin, muscle, epithelial and exocrine acinar cells and are controlled by neurotransmitters such as acetylcholine or hormones e.g. cholecystokinin in acinar cells. ATP-sensitive K^+ channels have been found in cardiac myocytes and insulin-secreting pancreatic ß cells, both of which are

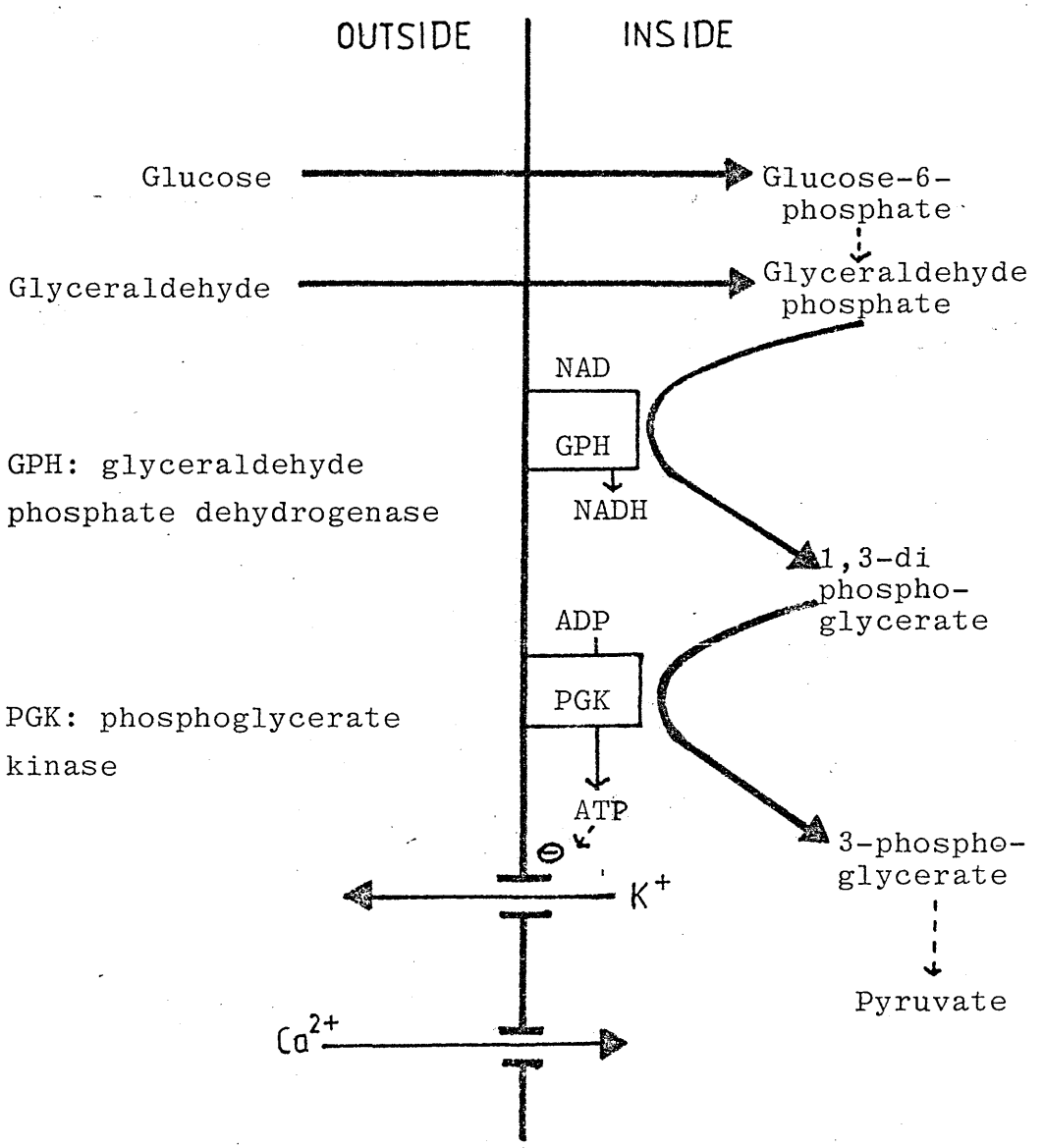


Figure 1.15. Schematic diagram of the mechanism by which glucose or glyceraldehyde closes K^+ channels in insulin-secreting cells. (Redrawn from Petersen et al, 1986).

controlled by glucose metabolism (reviewed by Petersen et al 1986, Escande et al 1988). These ß cells K^+ channels, under normoglycaemic conditions are open. Increases in extracellular glucose promote glycolysis which in turn increases the level of ATP within the cell (see figure 1.15). This closes the ATP-sensitive K^+ channel so that less K^+ leaves the cell and hence the membrane depolarises and opens voltage-dependent Ca^{2+} channels. The resulting rise in $[Ca^{2+}]_i$ triggers insulin release. In contrast, ATP-sensitive K^+ channels in cardiac myocytes are normally closed due to the high levels of intracellular ATP ($[ATP]_i$). When $[ATP]_i$ falls, the channels open and the heart muscle relaxes due to the hyperpolarisaton of the membrane. This relaxation allows $[ATP]_i$ levels to rise so that these K^+ channels resume their normal open position. ß cell and myocyte K^+ channels also differ in their voltage sensitivity, such that ß cell channels are relatively unaffected by membrane depolarisation whereas cardiac cells channels tend to open more readily. Direct action of G proteins to open ATP-sensitive K^+ channels has also been noted (Logothetis et al 1987).

The three types of Ca^{2+}-activated K^+ channel are designated BK or "maxi", SK (small) and OK (other), (reviewed by Blatz and Magleby 1987). These, with their conductances and inhibitors, are shown in the top part of figure 1.16. The channels are opened by increased Ca^{2+} at the inner membrane surface which, for BK channel activation, can arise from membrane depolarisation which opens voltage-dependent Ca^{2+} channels (denoted VCa in figure 1.16). The SK and OK

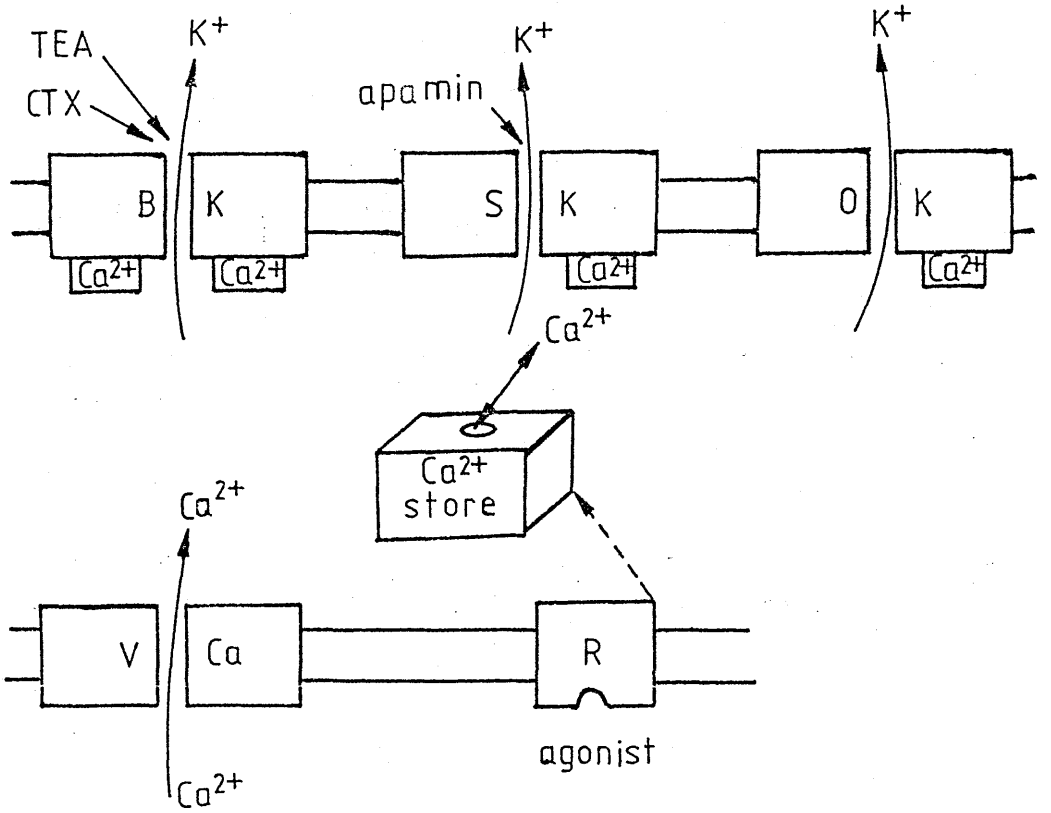


Figure 1.16. Schematic diagram of Ca^{2+}-activated K^+ channels. (Redrawn from Blatz and Magleby, 1987).

CTX	charybdotoxin
TEA	tetraethylammonium
VCa	voltage-dependent Ca^{2+} channel.

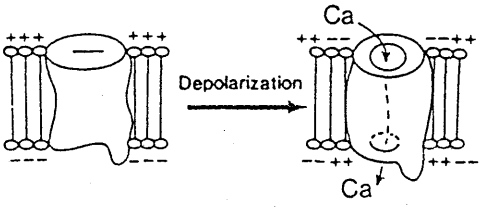
channels are more dependent on receptor-initiated release of Ca^{2+} from intracellular stores.

1.4.4.(ii) Ca^{2+} channels.

These channels can be voltage-dependent or receptor-operated (reviewed by Meldolesi and Pozzan 1987, Hosey and Lazdunski 1988). Three groups of the voltage-dependent channels (see figure 1.17), have been categorised. (i) T type channels are rapidly and transiently activated by very small depolarisations from negative membrane potentials. They are insensitive to the dihydropyridines. (ii) L type channels are activated at less negative membrane potentials (-30 mV) and, with their high conductance and longer opening, they are primarily involved with the regulation of $[Ca^{2+}]_i$. Inactivation is controlled by two mechanisms; a time- and voltage-regulated mechanism and a $[Ca^{2+}]_i$-dependent one. L channels are regulated by hormones and neurotransmitters and the channel molecule itself has been shown to be a substrate for both cAMP- and Ca^{2+}-dependent protein kinases and G proteins which can either increase or decrease their period of opening. Characteristically, dihydropyridines bind to these channels and either inhibit (nitrendipine) or promote (BAY K 8644) Ca^{2+} entry. (iii) N channel conductance and duration of opening are intermediate between those of the L and T channels.

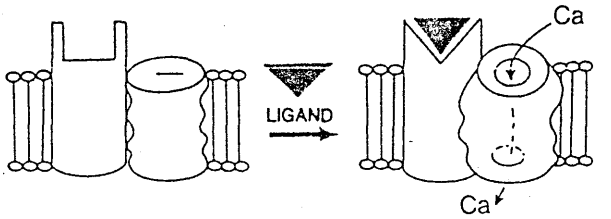
Receptor-operated Ca^{2+} channels (ROCs) are of two kinds. Those in which the receptor and channel functions coexist in one molecule or in two closely-associated molecules so that receptor

VOLTAGE DEPENDENT Ca CHANNELS



	T TYPE	N TYPE	L TYPE
Activation	> -70 mv	> -30 mv	> -10 mv
Conductance	Tiny	Moderate	Large
	Transient	Transient	Long-lasting
Sensitive to			
DHPs	No	No	Yes
ω -CT	No	Yes	Yes

RECEPTOR-OPERATED Ca CHANNELS



EXAMPLES	RECEPTOR	CELL
	ATP, Vasopressin	Smooth muscle
	Mitogens	Lymphocytes
	Parathyroid hormone	Osteosarcoma
	f-Met-Leu-Phe	Neutrophils
	NMDA	Neurons

Figure 1.17. Hypothetical models of the different types of Ca^{2+} channels. (From Hosey and Lazdunski, 1988).

ω - CT ω - conotoxin

activation and channel opening appear intimately interconnected and second messenger operated channels (SMOCs), which respond to second messenger molecules generated within the cell. An example of a ROC is the nicotinic acetylcholine receptor, although transporting mainly Na^+ and K^+ also allows Ca^{2+} entry under physiological conditions. In PC12 cells, an adrenal medullary line, activation of the nicotinic receptor causes a rise in $[Ca^{2+}]_i$ sufficient to evoke a secretory response. However, this response has not been seen in other cell types (reviewed by Meldolesi and Pozzan 1987).

In a wide variety of cell types, receptor activation is often associated with a voltage-independent increase in the Ca^{2+} permeability of the cell membrane. This effect might be due to the opening of channels triggered by second messengers generated by receptor activation. To date, direct evidence of SMOCs has been obtained in T lymphocytes and neutrophils only. Receptors involved in SMOC activation are those coupled across the cell membrane to hydrolysis of PIP_2 by a G protein similar to G_s and G_i (reviewed by Meldolesi and Pozzan 1987). The sequence of events following receptor activation has been described earlier (figure 1.11). Receptors involved in SMOC activation are thought to include M_1 muscarinic, α_1 adrenergic, H_1 histaminergic and those for bradykinin, vasopressin and AII. As can be seen, there seems to be a great similarity in the properties of the SMOC and L-type Ca^{2+} channels. It has been suggested by Kojima et al (1985b), that the voltage-dependent and

receptor-operated Ca^{2+} channels (in ZG cells), may be identical molecules exhibiting two modes of operation. Certainly, K^{+}- and AII-induced aldosterone secretion can be inhibited equally by nitrendipine provided that the release of Ca^{2+} from intracellular stores by AII is blocked (Kojima et al 1985c).

1.5. ANGIOTENSIN II STIMULATION OF ALDOSTERONE SYNTHESIS.

AII is a specific agonist of aldosterone secretion and its normal plasma concentration is about 3×10^{-11} mol per litre. It is derived from the glycoprotein angiotensinogen in two steps. First the proteolytic enzyme renin, secreted principally from cells in the kidney juxtaglomerular apparatus, cleaves a decapeptide, angiotensin I, from the amino terminal of angiotensinogen. Angiotensin I is further processed by the angiotensin-converting enzyme (ACE) which removes the two carboxy terminal amino acids to give AII. Although AII is the principal circulating peptide hormone, AIII (des Asp' AII) is also able to stimulate aldosterone synthesis.

The major functions of AII are to regulate cardiovascular and renal homeostasis. Until recently, emphasis had been placed on the circulating endocrine renin-angiotensin system (figure 1.18). In this system renin, secreted by the kidney, acts on angiotensinogen released from the liver in the circulation. Recently, it has become clear that separate local renin-angiotensin systems are present within many tissues

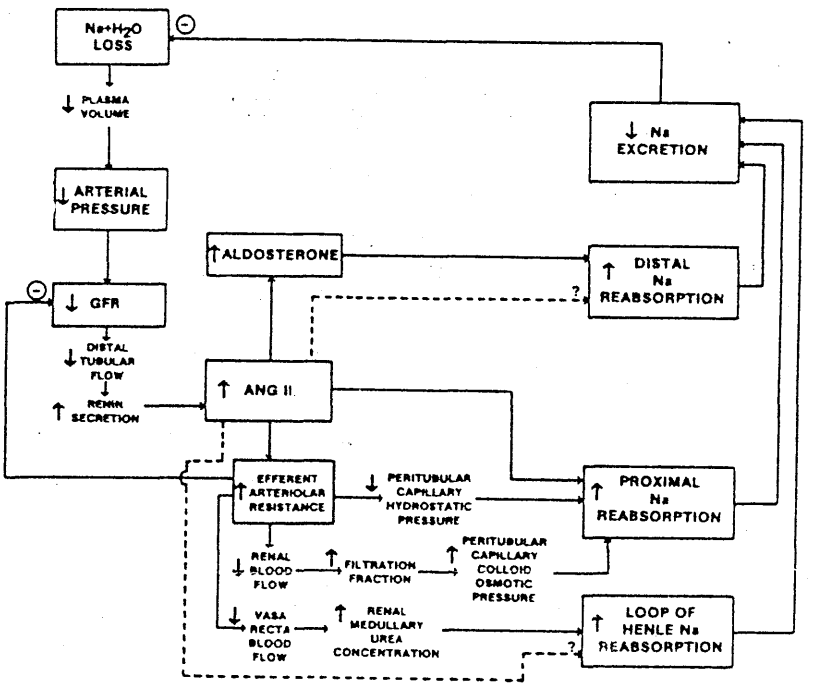


Figure 1.18. The Renin-angiotensin system. Feedback control of sodium balance, arterial pressure and glomerular filtration rate (GFR) by AII (ANG II). (From Hall et al, 1986).

such as blood vessels, heart, kidney, brain, adrenal and gut (reviewed by Dzau 1988). Potentially, these may exert powerful autocrine and paracrine influences on local tissue function.

AII is an extremely potent vasoconstrictor of arterial smooth muscle cells and acts systemically to raise blood pressure and intrarenally to decrease filtration at the glomerulus. It also stimulates aldosterone synthesis in the adrenal cortex secretion.

The AII holoreceptor is a glycoprotein of molecular weight 125 kDa. There is a size heterogeneity among AII binding sites between species and target tissues ranging between 55-79 kDa. These variations probably reflect differences in carbohydrate content of individual receptor sites. The adrenal AII receptor, as in other tissues, is coupled to G_i (see below). In the rat ZG the receptor affinity Kd is 1.0-1.2 nM and the binding capacity is 36-38,000 sites per cell (Bradshaw and Moore 1988). Although most of AII's action occurs via its interaction with cell membrane receptors there is growing evidence that AII can be taken up by the cells and enters the nuclei to regulate DNA synthesis (Re et al 1984). Changes in AII concentration in vivo, produced by manipulation of dietary Na^+ and K^+ intake, administration of AII and treatment with ACE inhibitors all change AII receptor characteristics. Within the physiological range of AII concentrations, receptor affinity changes over the short term and the number of sites in the long term with affinity returning to normal. The overall effect is of increased binding of AII to the adrenal with increased levels of AII and

decreased binding at lowered concentrations of the peptide, an example of positive feedback control. Na^+ depletion lowers the response threshold and steepens the dose response curve of increased aldosterone secretion to AII stimulation (Hollenberg et al 1974, Oelkers et al 1974, Williams 1978 and Swartz et al 1980). Upregulation of AII binding in salt restriction could partly explain the increase in sensitvity of ZG in this situation for it can occur without any change in the AII levels (Oelkers et al 1974). In contrast, binding of AII to smooth muscle receptors is decreased in salt depletion and K^+ loading corresponding to the lowering of its pressor activity (reviewed Tait et al 1980).

Activation of AII receptors in the ZG appears to affect two different G proteins (figure 1.19). One, which is G_i, may explain the inhibitory action of AII at supramaximal doses on the aldosterone response and probably reflects a low-affinity interaction between the hormone-receptor complex and G_i which lowers cAMP production and hence steroid release (reviewed Catt et al 1988, Spät 1988).

AII receptor-mediated stimulation of steroidogenesis is exerted through a different, pertussis-insensitive, G protein which couples the activated receptors to phospholipase C (Enyedi et al 1986). The ensuing hydrolysis of PIP_2 activates the two pathways outlined in figure 1.11. but which involve further inositol polyphosphate metabolism to higher and lower phosphorylated forms of IP_3. Release of Ca^{2+} from an intracellular store may not be a function

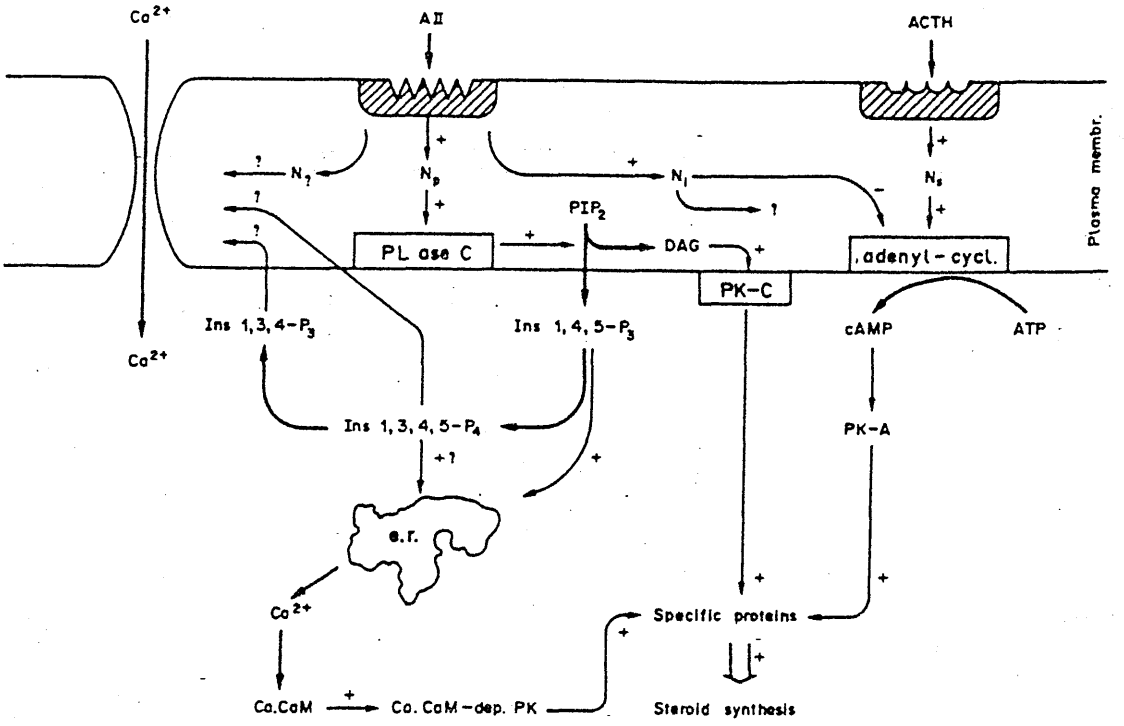


Figure 1.19. Signal transducing mechanisms in ZG cells stimulated with AII or ACTH. Thick lines are metabolic routes, thin lines for effects. N_i, N_p, N_s and $N_?$ are G proteins. PIP_2, phophatidylinositol 4,5-bisphosphate; DAG, diacylglycerol. Adenyl-cycl., adenylate cyclase, PK, protein kinase, CaM, calmodulin and Ins, inositol. (From Spät 1988).

confined to IP_3 alone. Microsomal fractions of bovine adrenocortical cells (prepared from the whole cortex) possess high and low-affinity binding sites for inositol 1,3,4,5 tetrakisphosphate which are clearly distinct from those for IP_3 (Enyedi and Williams 1988). What role is played by this (and possibly some other) higher inositol polyphosphate is not known but in sea urchin eggs it promotes Ca^{2+} entry across the cell membrane (Parker and Miledi 1987).

Direct monitoring of $[Ca^{2+}]_i$ using the Ca^{2+}-sensitive photoprotein aequorin (reviewed by Blinks et al 1976) and more recently the fluorescent dyes quin 2 and fura 2 (reviewed Rink and Pozzan 1985, Grynkiewicz et al 1985) has become a powerful technique for investigating intracellular signalling. Studies in glomerulosa cells show differing dynamics for the ion depending on whether cell populations or isolated cells are used.

In population studies, using freshly isolated bovine cells, AII (10^{-10} to 10^{-8} M), produces rapid rises in $[Ca^{2+}]_i$, which are due to release from intracellular stores and also to uptake from the extracellular medium. $[Ca^{2+}]_i$ changes are also dose dependent, reaching a peak within 2 min of stimulation before falling to basal levels within 10 min. The responses are maximal at 10^{-8} M AII (Capponi et al 1984). Similarly, in aequorin-loaded bovine cells, 10^{-9} M AII produces a sharp peak in $[Ca^{2+}]_i$ which falls to basal levels; the entire effect occurring over 1 min (Kojima and Ogata 1986). In superfused bovine cells also loaded with quin 2, the steroid response which follows the observed

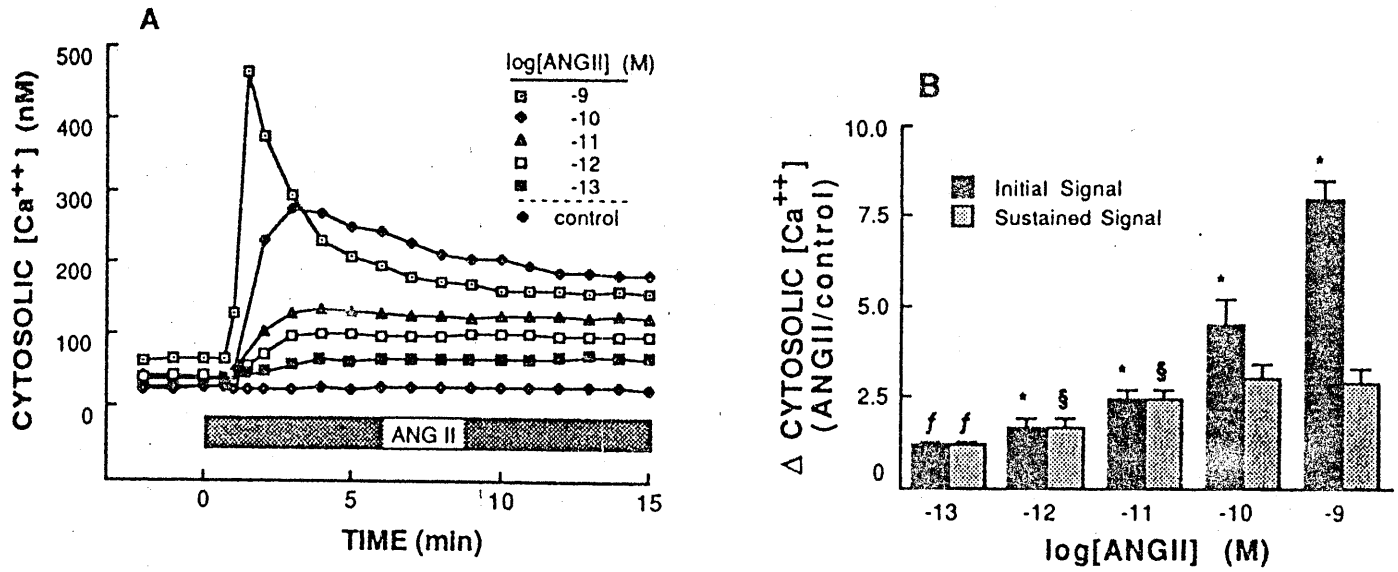


Figure 1.20. Dose-relationships between AII and $[Ca^{2+}]_i$. The latter was measured by fura-2 fluorescence in ZG cells. Temporal changes in $[Ca^{2+}]_i$ observed in response to increasing doses of AII during a representative experiment are shown in A. Panel B shows mean initial (0-5 min) and sustained (15 min) changes in $[Ca^{2+}]_i$ produced by AII determined from the superfused monolayers in A. Vertical bars represent 1 SE. $^{f}P<0.05$ vs control, $^{*}P<0.05$ vs initial change in $[Ca^{2+}]_i$ invoked by next lower dose of AII. $^{S}P<0.05$ vs sustained change in $[Ca^{2+}]_i$ invoked by next lower dose of AII. (From Kramer 1988).

changes in $[Ca^{2+}]_i$ is apparent within 2 min of an AII challenge (Capponi et al 1984).

A more complex pattern of $[Ca^{2+}]_i$ changes has been observed in superfused monolayers of cultured bovine glomerulosa cells loaded with fura 2 (Kramer 1988, see figure 1.20). The threshold concentration of AII was 10^{-14} to 10^{-13} M and produced a gradual rise in [Ca2+]i which reached a plateau after 3-5 min that was 1.2 times higher than the basal $[Ca^{2+}]_i$ (figure 1.21a). This elevation of $[Ca^{2+}]_i$ was sustained for the entire duration of the AII stimulus (up to 30 mins). The maximal $[Ca^{2+}]_i$ change, evoked by 10^{-9} M AII, caused an immediate eightfold rise in $[Ca^{2+}]_i$ which decayed within 5 minutes to a sustained level 2.5 to 3 times the control value (see figure 1.20a). Intermediate concentrations of AII showed a dose-dependent increase in peak height and a gradual transition in the $[Ca^{2+}]_i$ profile from that seen at low doses to those at maximum AII stimulation. The associated increase in aldosterone production correlated most closely to the height of the initial Ca^{2+} peak and at 10^{-10} to 10^{-9} M AII there was a clear dissociation between aldosterone output and the size of the sustained $[Ca^{2+}]_i$ level (figure 1.20b).

A yet more complex pattern of $[Ca^{2+}]_i$ changes is revealed by observations of single rat glomerulosa cells loaded with fura 2 (Quinn et al 1988b figure 1.21). The mean peak $[Ca^{2+}]_i$ increase is similar at all AII concentrations over the range of $5x10^{-12}$ to $5x10^{-8}$ M but there are marked differences in the $[Ca^{2+}]_i$ kinetics between these doses. A dose-dependent delay for the onset of the $[Ca^{2+}]_i$ response is observed

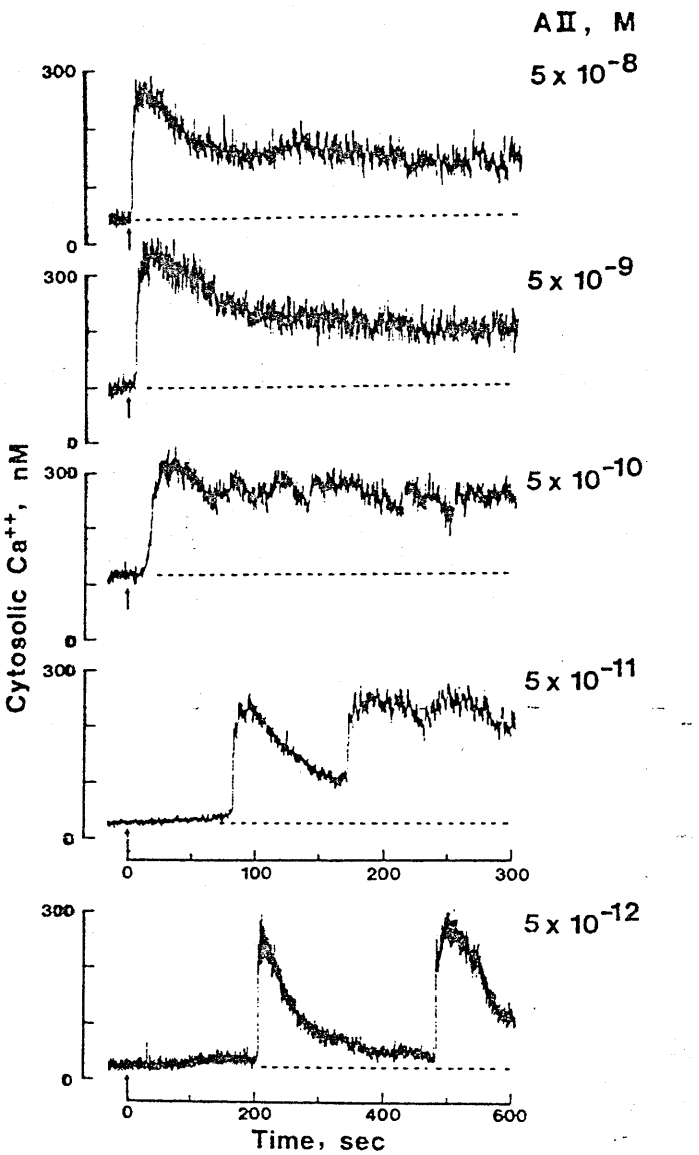


Figure 1.21. Time courses of the $[Ca^{2+}]i$ response to different AII doses. Traces are from 5 different ZG cells during puffer perfusion of AII. Duration of stimulation was 5 min except for 10 min application at $5x10^{-12}$ M AII. Arrows indicate onset of stimulation and dashed line is an extension of the basal $[Ca^{2+}]i$. (From Quinn et al 1988b).

ranging from 2.6±0.3 s at $5x10^{-8}$M to 181±27 s at $5x10^{-12}$ M. Following this delay, cells usually respond with a sharp increase in $[Ca^{2+}]_i$ which is complete within 15 s. At low AII concentrations ($5x10^{-12}$ and $5x10^{-11}$ M) the initial peak of $[Ca^{2+}]_i$ is followed by large oscillations in $[Ca^{2+}]_i$. At higher doses of AII these appear to fuse.

The explanation for such differences in $[Ca^{2+}]_i$ noted by these workers is twofold. One is that fura 2, having a greater sensitivity and brighter fluorescence than quin 2 and even more so than aequorin, is used at much lower concentrations in the cells than either of the other indicators. It will, therefore, not buffer and attenuate the $[Ca^{2+}]_i$ to the same extent (Quinn et al 1988b, Kramer 1988). The transience of the $[Ca^{2+}]_i$ peak in the AII stimulated cells loaded with aequorin (Kojima and Ogata 1986), is probably due to its much greater buffering of the Ca^{2+} (reviewed by Rink and Pozzan 1985).

Another factor is that freshly isolated cells appear much less sensitive to AII than cultured cells i.e. the threshold is three orders of magnitude higher for the former with respect to the latter. Also, their resting $[Ca^{2+}]_i$ is 2 to 4 times higher than that of cultured cells making changes in $[Ca^{2+}]_i$ more difficult to observe (Kramer 1988). Despite the differences observed in $[Ca^{2+}]_i$ changes, it is agreed that the initial rise in $[Ca^{2+}]_i$ is a result of release from intracellular stores by IP_3 and that the sustained rise is a result of Ca^{2+} influx from the extracellular medium (Capponi et al 1984, Kojima and Ogata 1986,

Quinn 1988b, Kramer 1988). Further, the oscillatory nature of the $[Ca^{2+}]_i$ signal seen by Quinn et al has been suggested by them to be a means by which AII may modulate aldosterone output via the sensitivity of the effector proteins involved in steroidogenesis. Those proteins located further away from the sources of $[Ca^{2+}]_i$ will be affected less than those which are closer due to the limited diffusion of Ca^{2+} through, and binding within, the cytosol.

1.6. ACTH STIMULATION OF GLOMERULOSA CELLS.

ACTH is synthesised in the anterior pituitary by cells which are stimulated by corticotropic releasing hormone (CRH) from the hypothalamus (Sawchenko et al 1984). Circulating ACTH binds to high affinity receptors on the adrenocortical cell surface to stimulate cortisol secretion in the main (Buckley and Ramachandran 1981) but also aldosterone and adrenal androgens. Only cortisol, however, exerts negative feedback control on ACTH synthesis and achieves this by suppressing transcription of the hormone gene in the pituitary as well as inhibiting CRH formation. ACTH is secreted in episodic bursts giving the characteristic circadian rythmn where morning plasma ACTH concentrations exceed those at evening by twofold. In nocturnal animals this is reversed so that peak ACTH levels occur during the major period of fasting (Miyabo et al 1980).

ACTH raises cAMP levels via the adenylate cyclase system but also stimulates Ca^{2+} influx at lower doses

of the hormone than those needed to activate adenylate cyclase (Kojima et al 1985b, Lymangrover et al 1982). Certainly there is a requirement for Ca^{2+} uptake for ACTH-stimulated protein synthesis (Leier and Jungman 1973) and in the ZF/R, Ca^{2+} must enter the cells for ACTH-activated steps before and after adenylate cyclase stimulation (Davies et al 1985). However, although there is a requirement for Ca^{2+} in the action of ACTH, Ca^{2+} release from intracellular stores does not appear to be a prerequisite of ACTH-stimulated steroidogenesis (Iida et al 1986, Kojima and Ogata 1986, Underwood et al 1988). Hence some form of co-operative interaction between cAMP and Ca^{2+} is envisaged (on the lines of figure 1.13). This probably involves calmodulin since the calmodulin inhibitor trifluoroperazine inhibits the steroidogenic response to the cAMP analogue, dibutyryl cAMP (Davies et al 1985). These interactions somehow regulate the level of phosphorylation of regulatory proteins in steroidogenesis via the induction and increased phosphorylation of inhibitor-1 (Iyer et al 1988). This is a cytosolic heat-stable protein which inhibits phosphoprotein phosphatase activity in both cytosol and mitochondria only after it has itself been phosphorylated by cAMP-dependent protein kinase. This has the effect of increasing phosphorylation of the regulatory protein(s) and hence steroidogenesis (Iyer et al 1988).

1.7. POTASSIUM ION ACTIVATION OF GLOMERULOSA CELLS.

Rises in $[K^+]_o$ produce a small increase in adenylate cyclase activity in ZG cells therefore its action may be considered to be slightly cAMP-dependent (Reviewed by Tait et al 1980, Kojima et al 1985c). Earlier work had indicated that $[K^+]_o$ had no effect on adenylate cyclase (Fujita et al 1979). However, its main mode of action is to open voltage-dependent Ca^{2+} channels in the cell membrane thereby enhancing Ca^{2+} influx (Kojima et al 1985a). The voltage-dependent Ca^{2+} entry blocker nifedipine lowers free $[Ca^{2+}]_i$ even in unstimulated ZG cells (Capponi et al 1984) when $[K^+]_o$ is only 3mM; therefore continuous Ca^{2+} influx via these channels may be necessary to maintain basal $[Ca^{2+}]_i$. Influx of Ca^{2+} is an absolute requirement for K^+-stimulated steroidogenesis (Foster et al 1981) and mobilisation of Ca^{2+} from intracellular stores via the IP_3 system was thought not to occur (Farese et al 1983). Surprisingly, recent work using microspectrophotometry shows rises in polyinositol phosphate metabolism of the same magnitude as those produced by AII (Underwood et al 1988). It is likely that these latest data may give a more accurate picture of the cells' response to the ion. Previous work on phosphoinositide turnover has relied on detecting the relative increases in $[^3H]$ inositol or ^{32}P phosphate (from ATP), incorporation into phosphatidylinositol and inositol phosphate groups. These methods appears not to label all available inositol and its derivatives as there is an intracellular pool not readily accessible

under non-stimulating preincubation conditions. Coupled with the inability to allow for uptake of unlabelled inositol which may occur in vivo, earlier results have produced conflicting patterns in the levels of phosphoinositide turnover. Microspectrophotometry allows the absolute concentrations of the cells' phosphoinositides to be assayed and the method of phosphate determination employed by Underwood et al (1988) is more sensitive with a standard curve range of 0.1-1.2 ng as opposed to 0.5-4.0 µg used by other workers (Farese et al 1979).

Despite AII and K^+ having potentially very similar intracellular signalling events, fluorescent monitoring of $[Ca^{2+}]_i$ changes in single cells shows the pattern of responses observed in K^+ and AII stimulation and hence their underlying mechanisms to be very different (Connor et al 1987, Quinn et al 1988a,b).

1.8. POSSIBLE FINAL COMMON PATH OF AII AND ACTH ACTIONS.

Since the glomerulosa cell mitochondria recognise factors generated by AII (cAMP-independent) and ACTH (cAMP-dependent) paths, a common intermediate is thought to exist, despite differences in signal transduction mechanisms (Solano et al 1987). This idea has been tested by stimulating isolated mitochondria from glomerulosa and fasciculata cells with mitochondria-free fractions from either cell type which have been previously stimulated with AII or ACTH (activated fractions). The activated fractions produced

substantial increases in net progesterone synthesis in mitochondria from unstimulated cells of both types. Inhibitors of arachidonic acid release and metabolism blocked corticosterone production in fasciculata cells stimulated with ACTH. They also blocked the formation of activated fractions. Non-activated fractions behaved as though they were activated when arachidonic acid was added. These findings suggest that ACTH and AII increase arachidonic acid release from phospholipids and an activation of its conversion to leukotriene products (Solano et al 1987) see figure 1.22 for the pathway. A specific mediator role for arachidonic acid via the lipoxygenase path has been noted for AII-induced aldosterone secretion (Nadler et al 1987) and increases in its 12 HETE (hydroxyeicosatetraenoic acid) metabolite are seen under these conditions (Natarajan et al 1988a,b). Studies in isolated rat adrenal cells have shown that incubation in the presence of arachidonic acid or lipoxygenase inhibitors reduced ACTH-stimulated corticosterone synthesis (Jones et al 1987). This inhibitory effect was also seen in the presence of leukotriene A_4 (LTA_4), 15HETE and 5HETE. Reduction of intracellular levels of LTA_4 by glutathione stimulated corticosterone output. Jones et al suggested that the inhibitory protein lipomodulin, which is induced by glucocorticoids, is phosphorylated by a cAMP-dependent protein kinase. This decreases lipomodulin suppression of arachodonic acid release thereby increasing its metabolism to lipoxygenase intermediates and products. Thus, ACTH-stimulated corticosterone release may involve LTA_4-mediated

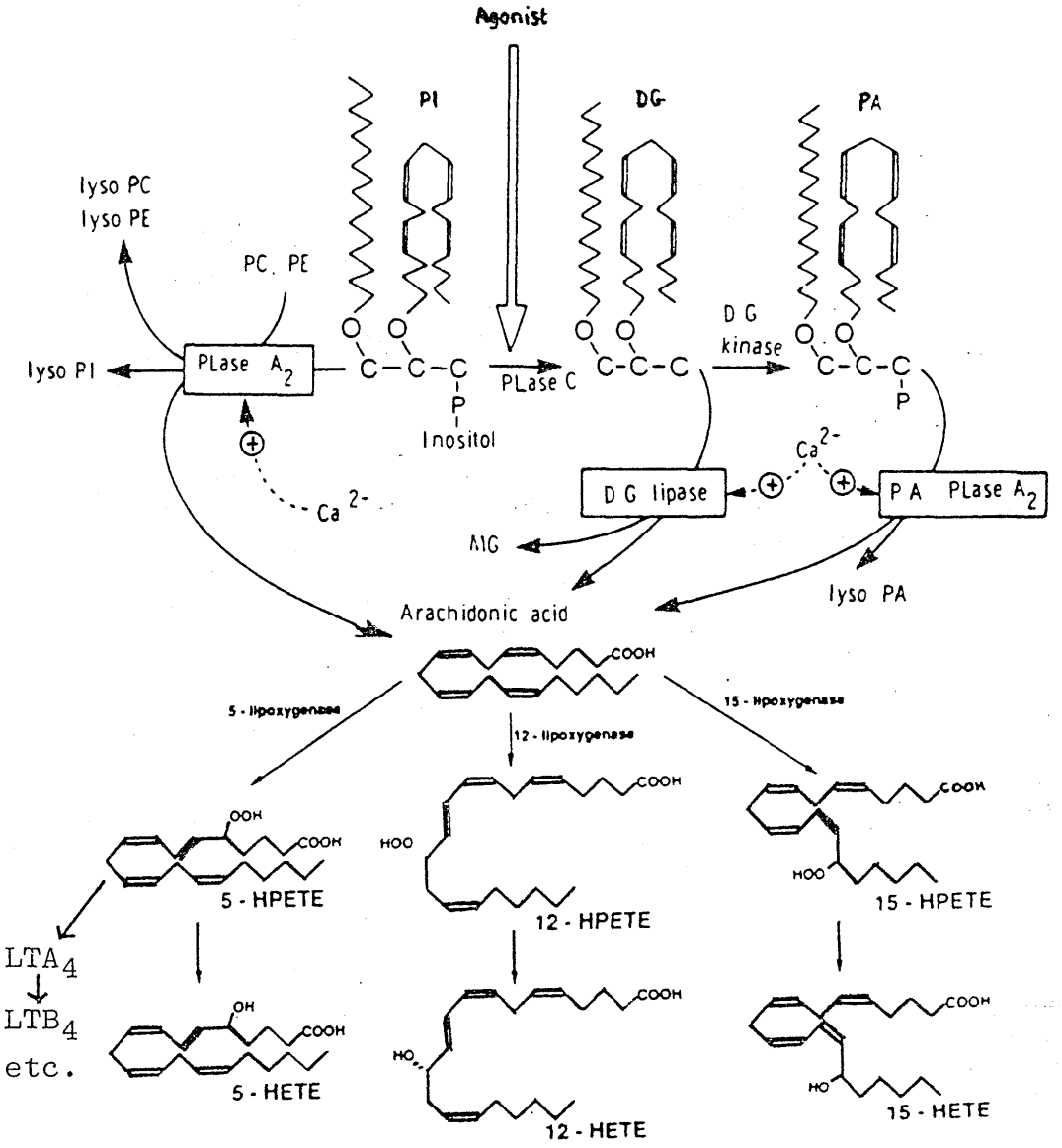


Figure 1.22. Pathway generating arachidonic acid and its metabolites. LT: leukotrienes.
HETE; hydroxyeicosatetraenoic acid, HPETE; hydroperoxy-eicosatetraenoic acid, DG; diacylglycerol, PA; phosphatidic acid, PC; phosphatidylcholine, PE; phosphatidylethanolamine, PI; phosphatidylinositol, PLase; phospholipase, MG; monoacylglycerol. (Composite diagram; top half from Berridge 1981 and lower part from Spector et al, 1988).

inhibition of the lipoxygenase path. However, the 5HPETE (hydroperoxyeicosatetraenoic acid) metabolite has been reported to increase ACTH-stimulated corticosterone release in this preparation (Hirai et al 1985). Much further work is needed to elucidate importance of such a path and to define any role it may have in K^+-stimulated aldosterone release.

1.9. CELL MEMBRANE MECHANISMS FOR ALTERING $[Ca^{2+}]_i$.

As discussed earlier the three major secretagogues of aldosterone are dependent in various ways on Ca^{2+} for their actions. Not only are the steroidogenic responses to these and cAMP inhibited by blockers of the voltage-dependent Ca^{2+} channel (Balla et al 1982, Chartier and Schiffrin 1987, Davies et al 1985 and Shiebinger et al 1986) but all three agents promote Ca^{2+} uptake via this channel (Kojima et al 1985). These observations suggest that stimulation of steroid synthesis is universally, if not exclusively, linked to changes in membrane potential. Indeed, ACTH, cAMP, AII and K^+ have all been shown to depolarise the plasma membrane of adrenocortical cells (Natke and Kabela 1979, Lymangrover et al 1982).

The resting potential of adrenocortical cells is dependent upon the permeability of the cell membrane to K^+ (Matthews 1967, Quinn et al 1987a). It follows, therefore, that depolarisation could arise from decreases in K^+ permeability. Decreases in passive (ouabain-insensitive) ^{43}K uptake and/or efflux have been described in bovine ZF cells treated with ACTH,

cAMP and AII (Kenyon et al 1985). Similarly, decreases in membrane potential and K^+ permeability are known to mediate the insulin release promoted by glucose in pancreatic ß cells (Henquin 1980). However, despite electrophysiological evidence to support the idea that aldosterone synthesis by glomerulosa cells is regulated in a similar way (Payet et al 1987), other studies (Lobo and Marusic 1986) have suggested that the superfused rat glomerulosa cell becomes hyperpolarised when stimulated, since AII caused an increase in rubidium (^{86}Rb) efflux, (used as a tracer of K^+).

1.10. PURPOSE OF THE PRESENT STUDY.

The purpose of the current work is to address the following questions:-

i) Can the discrepancy in K^+ efflux characteristics of stimulated glomerulosa cells as measured in the above methods be reconciled? Are they in fact indications of separate, but interrelated, processes involved in the depolarisation and subsequent repolarisation of the cell membrane ?

ii) How do manipulations of cell membrane permeability to cations affect steroidogenesis ?

iii) In what way are changes in K^+ permeability related to changes in $[Ca^{2+}]_i$?

The studies have been carried out using freshly isolated bovine glomerulosa cells in either static incubations or on superfusion columns. ^{43}K was the isotope of choice partly because of its availability

and partly because there is evidence that ^{86}Rb, which is often used as a qualitative tracer for K^+ movements, has a significantly slower efflux rate than ^{43}K (Putney 1976). The relatively short half-life of ^{43}K (22.4 h) also allows measurements of aldosterone by radio-immunoassay in the same samples as ^{43}K has previously been measured.

CHAPTER 2. MATERIALS AND METHODS.

2.1. MATERIALS.

2.1.(i). Reagents

The following chemicals used in this study, with their suppliers, are:-

ACTH (Synacthen), Ciba Laboratories, Horsham, W. Sussex, UK.

Aldosterone-3-cmo-(2[125]I) iodohistamine (370 kBq/100µl), $^{45}CaCl_2$ and cortisol-3-cmo-(2[125]I) iodohistamine (925 kBq/ 250µl), Amersham International plc, Amersham, UK.

Aldosterone antiserum, a kind gift from Prof. Dr. Th. J. Benraad, Division of Endocrinology, Dept. of Internal Medicine, University of Nijmegen, The Netherlands.

4-Aminopyridine, apamin, corticosterone, di-n-butylphthalate, γ globulin, ouabain octahydrate, tetraethylammonium chloride, TMB-8, trilostane, Triton X-100 and valinomycin, Sigma Chemical Co., Poole, Dorset, UK.

Angiotensin II (human), Cambridge Research Biochemicals, Cambridge, UK.

Boric acid, D-glucose, $NaHCO_3$ and NaCl (analytical grades), Formachem, Strathleven, Scotland, UK.

Bovine serum albumin (fraction V), ICN Biomedicals Ltd. (UK), Immuno-Biologicals Division, High Wycombe.

BRL 34519, Beechams Pharmaceuticals, UK.

$CaCl_2$ (1 M analytic volumetric solution), charcoal

(Norit GSX chloride-free) and all other inorganic reagents BDH Chemicals Ltd., Poole, Dorset, UK.

Collagenase (179 μ/mg), Worthington Biochemical Corporation, Freehold, New Jersey, USA.

Cortisol antiserum (sheep), Scottish Antibody Production Unit, Law Hospital, Carluke, Lanarkshire, Scotland, UK.

Dantrolene sodium, Norwich Eaton Pharmaceutical Company.

Dextran T-70 and Percoll, Pharmacia Fine Chemicals AB, Uppsala, Sweden.

Dinonylphthalate, Fluka AG, Switzerland.

Glibenclamide, Hoechst UK Ltd., Hounslow, Middlesex, UK.

^{43}KCl, MRC Cyclotron Unit, Hammersmith Hospital, London, UK.

Medium 199 (Modified) powder (with Earles' salts and glutamine, without $NaHCO_3$), Flow Laboratories, Irvine, Scotland, UK.

Methanol (Proanalysis grade), May and Baker, Dagenham, UK.

TRIS ((Hydroxymethyl) amino methane), Boehringer Mannheim GmbH. W. Germany.

Verapamil hydrochloride (i.v.), Abbott Laboratories Ltd. Queensborough, Kent. UK.

2.1.(ii). Disposables

Biogel P-2 (100-200 mesh), Bio-Rad Laboratories, Richmond, California, USA.

Nylon gauze (100 and 56 μm), Henry Simon, Stockport, UK.

2.1.(iii). Equipment

ß Counter, LKB Wallac 1217 Rackbeta, LKB-Produkter AB, Bromma, Sweden.

IEC Centra 8R Centrifuge, Damon/IEC division, Needham HTS, Massachusetts, USA.

Superfusion columns, a kind gift from Dr. B.C. Williams, Western General Hospital, Edinburgh, UK.

ɣ Counter, LKB Wallac or LKB 1280, LKB-Produkter as above. Also NE-1612 turbo, Nuclear Enterprises Ltd, Sighthill, Edinburgh, Scotland, UK.

Microcentrifuge, "Microcentaur", MSE SCientific Instruments, Crawley, W. Sussex, UK.

Gilson Minipuls 2 peristaltic pump, Gilson Medical Electronics Inc., Middleton, WI, USA.

Fraction collector FC 203, Gilson, as above.

Waterbath, "Gallenkamp Shakerbath", Gallenkamp, Loughborough, Leicesterhire, UK.

2.1.(iv). Solutions

Krebs-Ringer bicarbonate contained (in mmols/l); Na^+ 145, K^+ 3.75, Ca^{2+} 2.54, Mg^{2+} 1.15, Cl^- 128.3, HCO_3 24.8, PO_4^{2-} 1.15,and SO_4^{2+} 1.15. The solution was gassed with 95% O_2/5% CO_2 then glucose and BSA added at 2mg/ml.

Medium 199 was modified to lower the K^+ content by diluting powdered medium 199 to 2/3 normal strength with distilled water and adding various salt solutions so that the final ionic composition was identical to that of the Krebs-Ringer bicarbonate above. The solution was gassed with 95% O_2/5% CO_2 then glucose and

BSA added to give final concentrations of 2 mg/ml each.

Oil separation.

Cells were separated from the supernatant by centrifugation through a mixture of di-n-butyl phthalate and dinonylphthalate (SG 1.015).

Borate Buffer.

For one litre; 8.25g boric acid plus 2.7g NaOH adjusted to 7.5 with HCl.

Charcoal solution.

This contained 0.5% Dextran T 70 and 0.5% methanol-washed charcoal in borate buffer.

Stock solutions of angiotensin II and apamin were stored at -20 C at a concentration of $2x10^{-4}$ M in the following buffer; NaCl 150, TRIS 5 mmol/l adjusted to pH 7.4 with HCl then BSA added as 2%.

The following agents (table 2.1) were made up as fresh stocks in the following solvents then further diluted with medium 199 except for ouabain which was added to the incubation tube then the solvent evaporated off. Suitable dilutions of vehicle were added to control incubations.

Agent	Solvent	Concentration (M)
ACTH	saline	8.3×10^{-5}
4-Aminopyridine	medium 199 (re pH)	4.0×10^{-2}
BRL 34915	ethanol	1.0×10^{-2}
Corticosterone	methanol	1.0×10^{-2}
Dantrolene sodium	methanol	2.0×10^{-2}
Glibenclamide	methanol	6.6×10^{-3}
Ouabain	methanol	4.0×10^{-3}
TEA	medium 199 (re pH)	2.0×10^{-1}
TMB-8	medium 199	1.0×10^{-4}
Trilostane	methanol	1.0×10^{-2}
Valinomycin	methanol (5:1 with H_2O)	6.0×10^{-4}
Verapamil	saline	5.3×10^{-5}

Table 2.1.

2.2. METHODS

2.2.(i). Cell isolation and purification.

6-7 Bovine adrenal glands were transported in ice-cold 0.9% saline from the local abbattoir. Once dissected free of fat, 500μm slices of the outermost region of the cortex were finely minced with scissors then digested by 60 mg collagenase in a volume of 35 ml Krebs Ringer bicarbonate containing a further 750mg bovine serum albumin (BSA), (Haning et al 1979, Kenyon et al 1985). Disaggregation was effected by drawing the material up and down through a 5ml pipette thirty times after 20 and 40 min of collagenase treatment. The pipette tip was cut to a diameter of 3mm in the first instance but was replaced by one of 1.5mm for the second occasion. The collagenase digest was filtered first through a coarse nylon sieve (tea strainer) then a 100μm nylon gauze to remove undigested tissue. After centrifugation (500xg, 10 min) the cells were resuspended in modified medium 199 and centrifuged once more. Broken cells and red blood cells were removed by separation on a discontinuous density gradient comprising 20, 30, 40 and 75% isotonic Percoll in modified medium 199 (1750xg, 10 min). The band of cells at the 30-40% Percoll interface was washed once more and resuspended in medium 199.

2.2.(ii). Superfusion studies.

Cells (2-3x 10^6) were incubated in medium 199 containing 1-3 MBq ^{43}K/ml for 2h at 37 C. 1ml aliquots of this ^{43}K-loaded suspension were pumped onto a

1.2x1.0 cm column of Biogel at rates of 1 or 0.5 ml/min (Gilson Minipuls peristaltic pump) as indicated using non-radioactive medium (see diagram 2.1). Fractions of the eluate were collected over timed intervals (Gilson FC 203). Radioactivity in each fraction was measured using a γ spectrometer (LKB Wallac or 1280) and aldosterone was measured by radioimmunoassay (see below). At the end of an experiment any radioactivity remaining in the column and tubing was collected by flushing with distilled water and lysing the cells with 0.1% Triton X-100.

2.2.(iii). Static incubations.

Cells were pre-incubated as above with ^{43}K, centrifuged (500g, 10 min) and resuspended in medium 199 (1-5x10^6 cells/ ml). At timed intervals, 100 or 200µl aliquots of this suspension were added to a larger volume of medium 199, containing the various drugs or agents and incubated at 37°C. Samples (100 or 200µl) of these incubations were withdrawn and cells separated from medium by immediate centrifugation (30s, 12,000g) (MSE "Microcentaur") through a layer of oil. After freezing the separated mixture in methanol and dry ice, the centrifuge tubes were cut in half through the oil interface. Radioactivity in the cells and supernatant was measured separately. At the end of some experiments the remaining cell incubation mixture was centrifuged (10,000g, 10 min) and the supernatant removed and stored at -20°C for later steroid analysis.

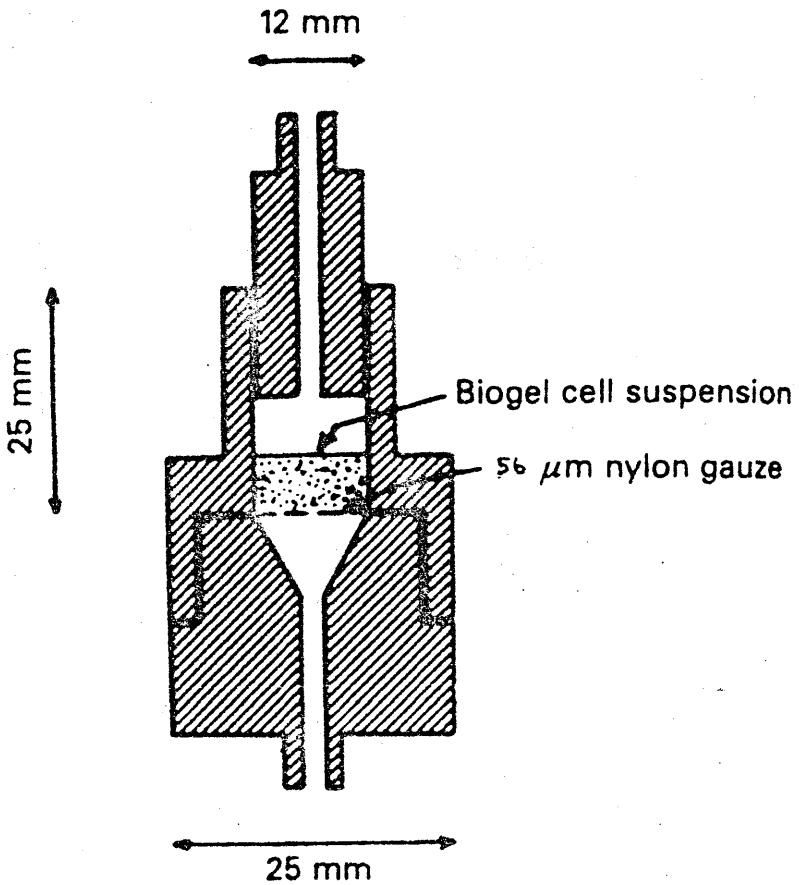


Figure 2.1. Diagram of a superfusion column.
(Altered from Drury et al 1986).

2.2.(iv). Steroid assays.

After allowing 10 days for the radioactive decay in incubations performed in the presence of the ^{43}K ($t_{\frac{1}{2}}$ 22h), aldosterone and less frequently, cortisol, were measured in the supernatants of incubations by direct radioimmunoassay as follows:-

Aldosterone assay.

Duplicate assay standards were made up in 20µl methanol (containing per 10 or 20µl; 0.32, 0.63, 1.25, 2.5, 5, 10, 15, 20, 30, 40 pg aldosterone) together with a volume of medium 199 (10-100 µl) equal to that of the samples. Sample tubes contained 20 µl methanol plus incubation medium. 200µl aldosterone antiserum (final dilution in borate buffer containing 0.1% ɣ globulin and 300K cpm [^{125}I] aldosterone) were added to each tube. The tubes were left at room temperature for 1h then overnight at 4 C. Charcoal suspension (150µl at 4 C) was added to the tube contents, mixed well then centrifuged (1,000g, 10 min) at 4 C. Supernatants were aspirated from all tubes then the precise amount of [^{125}I] aldosterone in the free fraction assayed using the microgamma turbo spectrometer.

Cortisol assay.

Duplicate assay standards were made up in 100 µl borate buffer (containing per 100 µl; 7.8, 15.6, 31.2, 63, 125, 250, 500 and 1000 pg cortisol together with 20 µl medium 199. Sample tubes contained 20µl of sample and 100µl borate buffer. 150µl of cortisol antiserum (final diltion 1:20,000 in borate buffer containing 0.1% ɣ globulin and 21K cpm [^{125}I] cortisol) was added to all tubes, the tube contents well mixed, then

incubations and charcoal separation performed as above.

Figures 2.2 and 2.3 illustrate typical standard curves obtained for these two assays. The coefficient of variability was ±13% for the aldosterone assay and that for the cortisol assay was ±14%.

2.2.(v). Electron Microscopy.

This was carried out by the Electron Microscopy Unit, Pathology Department, Western Infirmary, Glasgow, using routine methods. Cells were fixed in 2.5% glutaraldehyde buffered with phosphate to pH 7.4 and post-fixed with osmium tetroxide. Following embedding in araldite, sections were cut employing an LKB Ultratome III, double stained with uranyl acetate and lead citrate then viewed in a Philips EM 301 G microscope.

2.2.(vi). Kinetic analysis.

The efflux of radioactivity from superfused ^{43}K-loaded cells was fitted to the sum of three exponentials by linear least squares regression using standard curve-stripping techniques (Atkins 1967). In the study of the effects of AII on superfused cells, the efflux for ^{43}K is expressed as the fractional efflux rate of counts in the eluate compared to the total number of intracellular counts at that time (i.e. the sum of counts in subsequent fractions). This compensates for the continual fall in the quantity of ^{43}K collected in successive fractions.

The efflux rate of ^{43}K in static incubations is

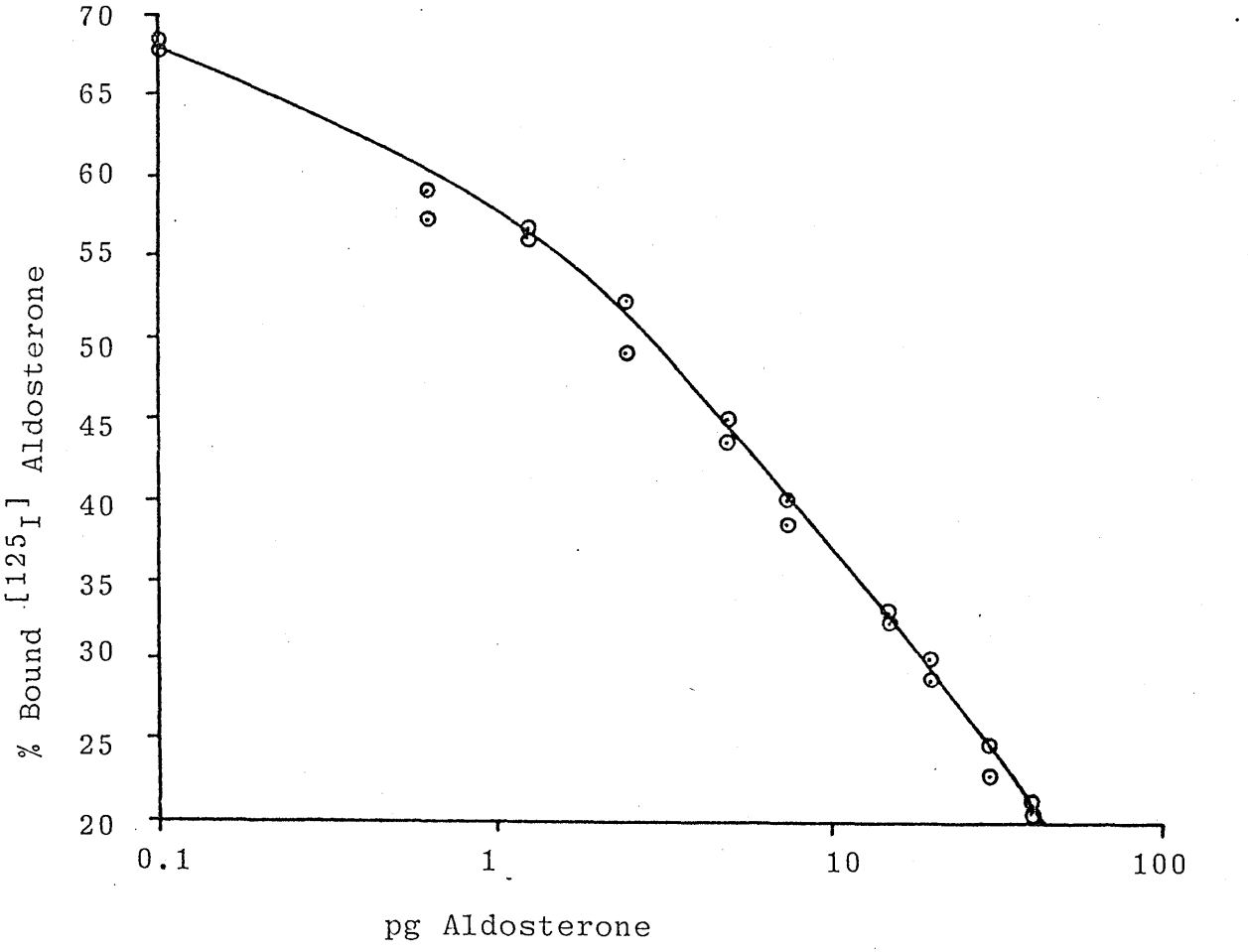


Figure 2.2. Typical calibration curve for the aldosterone assay.

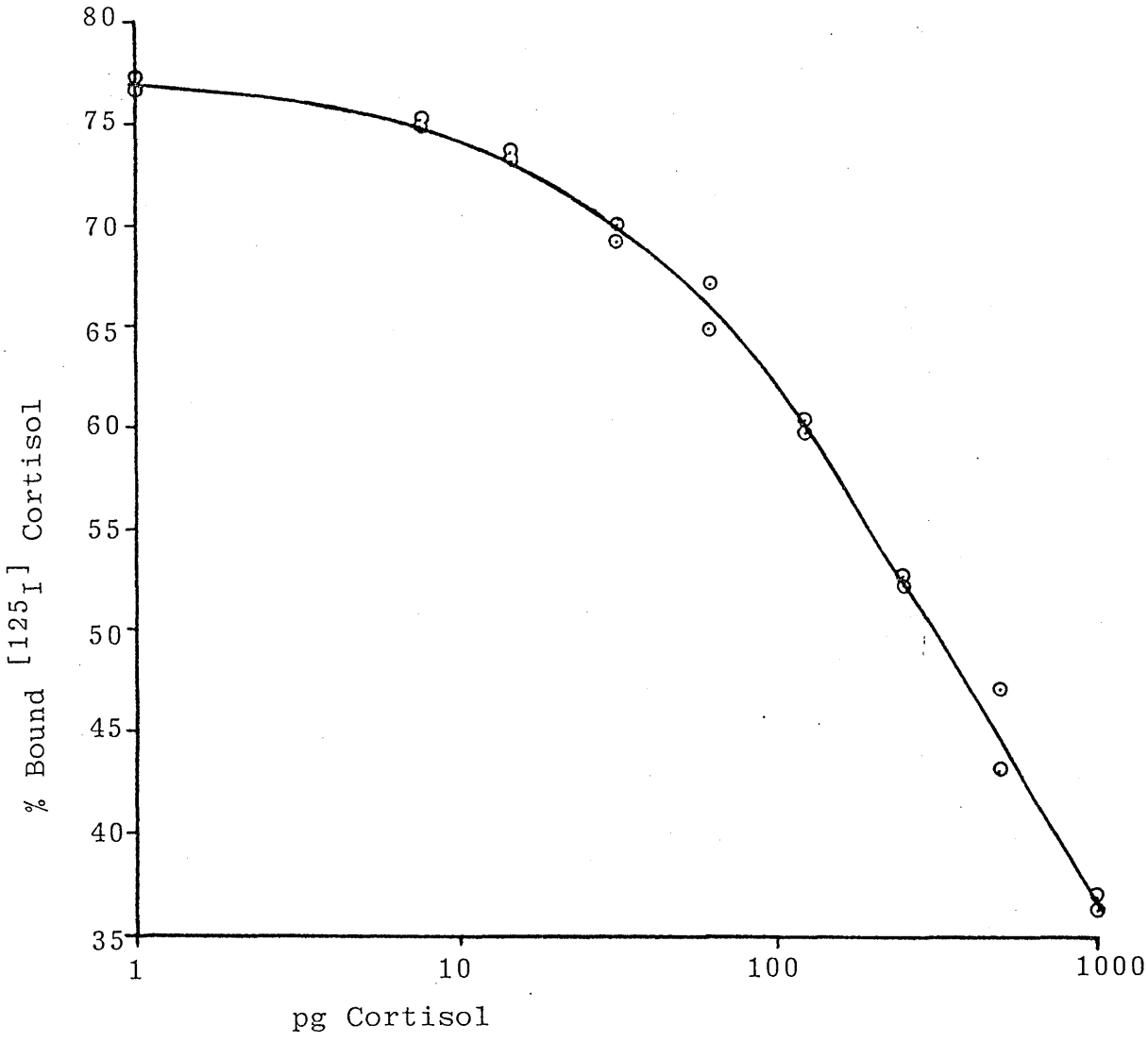


Figure 2.3. Typical calibration curve for the cortisol assay.

expressed as [log%$_1$ - log%$_2$] /time where log% is cell cpm/cell+supernatant cpm and $_1$ and $_2$ refer to different sample times. Where a linear regression analysis was performed, the $t_{\frac{1}{2}}$ was calculated by dividing log of 0.5 by the slope value. The efflux rate constants were calculated as the $\log_e$ value of the antilog of the regression slope value.

2.2.(vii). Statistics.

In most instances data were compared by analysis of variance using Newman-Keuls multiple range test where appropriate. The paired t-test or unpaired t-test was used on other occasions where indicated.

CHAPTER 3. PRELIMINARY EXPERIMENTS.

3.1. Steroid production from adrenocortical cells separated by density gradient.

Adrenocortical cells are thought to migrate from the subcapsular region through the cortical zones, (Zajicek et al 1986), i.e. ZG cells slowly evolve into ZF cells. It is quite possible that ZG cells isolated by the method described above may be contaminated with ZF cells. A discontinuous gradient of isotonic Percoll was employed to further purify the cells since in the rat, zonal function is related to cell density (reviewed Tait et al 1980). On centrifugation, the collagenase-digested cells settled out in 4 bands with the following densities >1-<1.026, >1.026-<1.039, >1.039-<1.052, >1.052-<1.097 and with a pellet >1.097. The steroidogenic profile of the cells thus separated was evaluated by determining the aldosterone and cortisol responses to ACTH (10^{-8}M), AII (10^{-6}M) and K^+ (8.6mM). These observations were correlated with appearance of the cells under the electron microscope. Cells from each band were washed in medium 199, the cell number determined by a Coulter counter and incubations (1h) of $2x10^5$ cells per ml set up for bands 2-4. The results in figure (3.1) show that band 3 synthesised the most aldosterone after stimulation with all three secretagogues. These cells also had the largest aldosterone/cortisol production ratio.
As mentioned above, ZG and ZF cells differ in their sensitivity to these stimuli in that ZG cells are stimulated by all of them but ZF cells respond most to

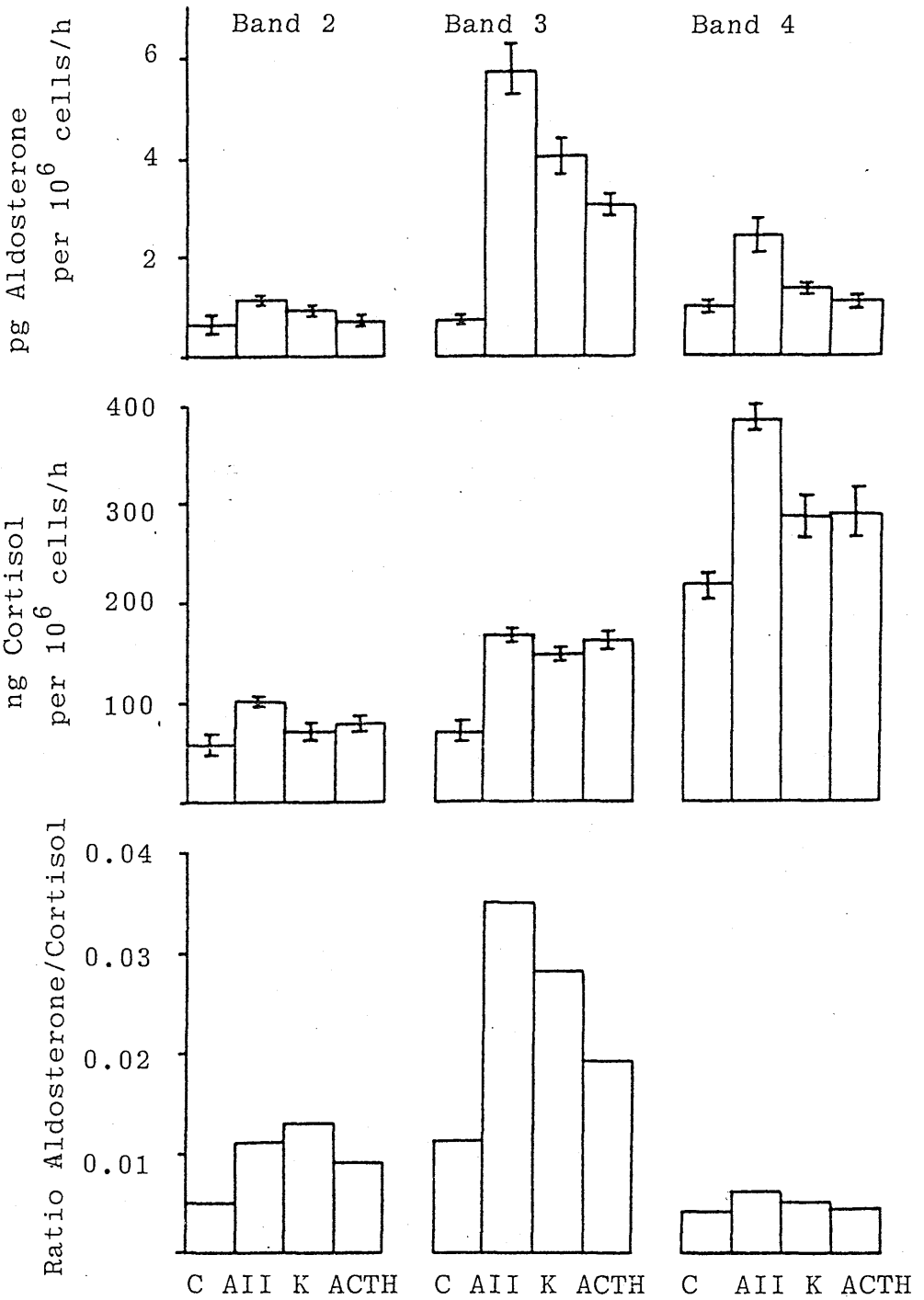


Figure 3.1. Steroid profiles of cells separated at different Percoll densities. Band numbers (top), refer to the density at which the cells separated out (see text). C: control, AII: AII 10^{-6}M, K: K^+ 8.6 mM, ACTH: ACTH 10^{-8}M. Panel A: aldosterone and panel B: cortisol produced by 1 h incubations. Panel C: ratios of mean aldosterone/mean cortisol values from A and B. (n=6 ± SEM).

ACTH and AII and not to such small increases in $[K^+]_o$. Aldosterone output in band 3 showed a 6, 4.5 and 3.5 fold increase to AII, ACTH and K^+ respectively. Bands 2 and 4 showed no effect. Cortisol production was slightly increased by AII in band 2 and it rose 2 fold in band 3 in response to all three secretagogues, basal being the same in both bands. Only band 4 showed an increased basal cortisol output but the responses to the stimulants were poor; barely a 2 fold rise in the presence of AII and smaller rises with ACTH or K^+. From the aldosterone/cortisol production rates, band 3 shows the more selective stimulation of aldosterone over cortisol production compared with bands 2 and 4. The increasing cortisol output seen in bands 2-4 may reflect an increasing proportion of ZF cells in the suspension although one would expect much greater responses to ACTH in pure ZF cells (reviewed Tait et al 1980). Possibly the ZF cells were damaged to a greater extent than the ZG cells.

3.2. Electron microscopy of Percoll-separated cells.

Many of the cells from band 1 (figure 3.2), showed disruption of the plasma membrane and myelin figures, presumably derived from damaged lipoprotein sheets, were easily identified. Isolated mitochondria were plentiful and much cellular debris was seen.

The cells depicted in figures 3.3 to 3.5 show typical fields from bands 2-4 respectively. The criteria used to classify ZF and ZG cells are the presence of rounded cristae in the mitochondria and a high lipid content in the ZF cells. ZG cells contain

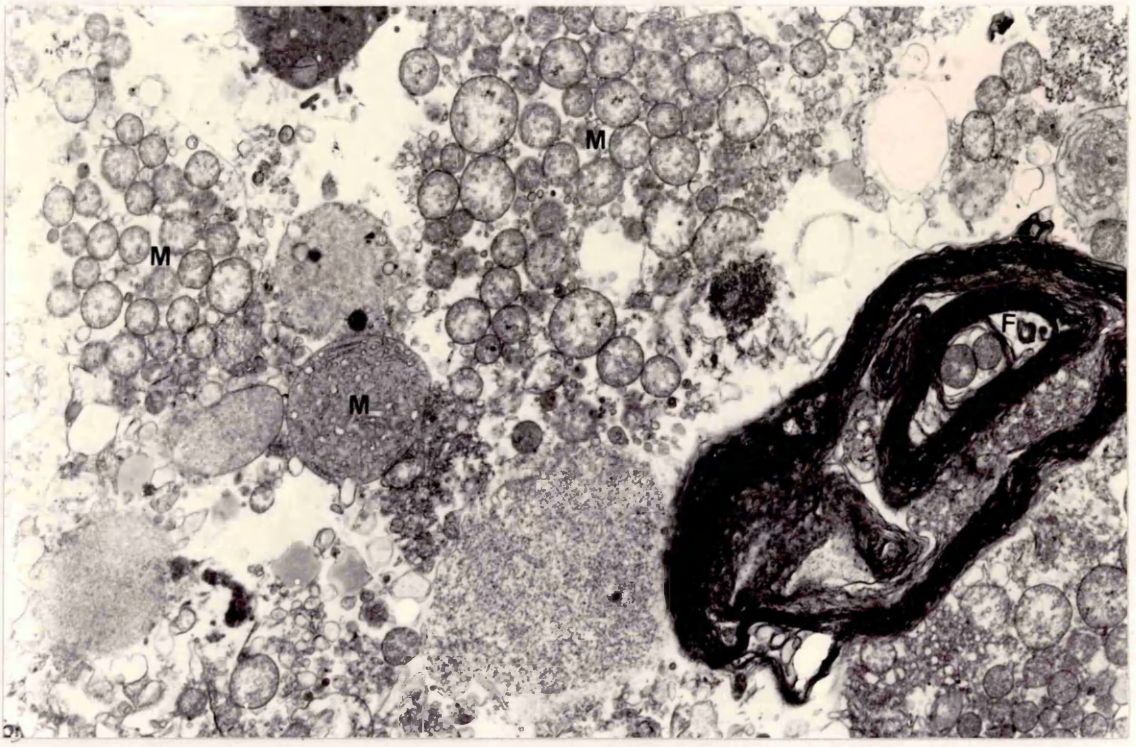


Figure 3.2. Electron micrograph of adrenocortical cells from band 1. Density > 1 < 1.026. F; myelin figure, M; mitochondria. Final magnification x8775.

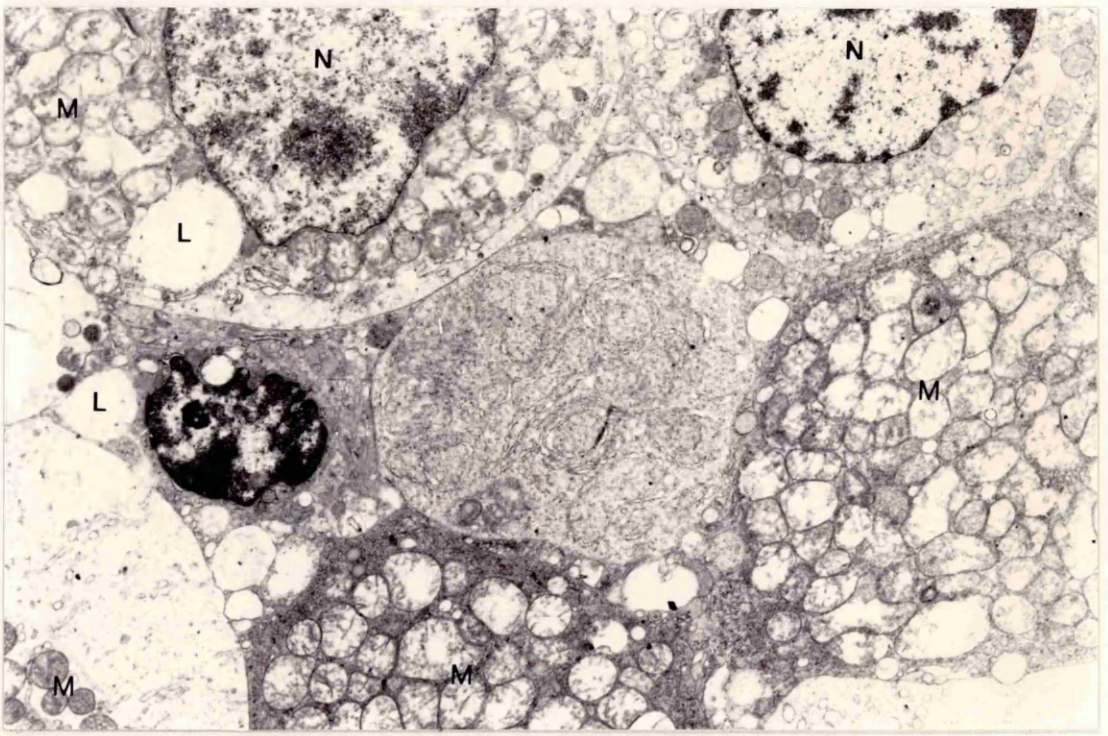


Figure 3.3. Electron micrograph of adrenocortical cells from band 2. Density > 1.026 < 1.039. L; lipid vesicles, M; mitochondria, N; nucleus. Final magnification x8775.

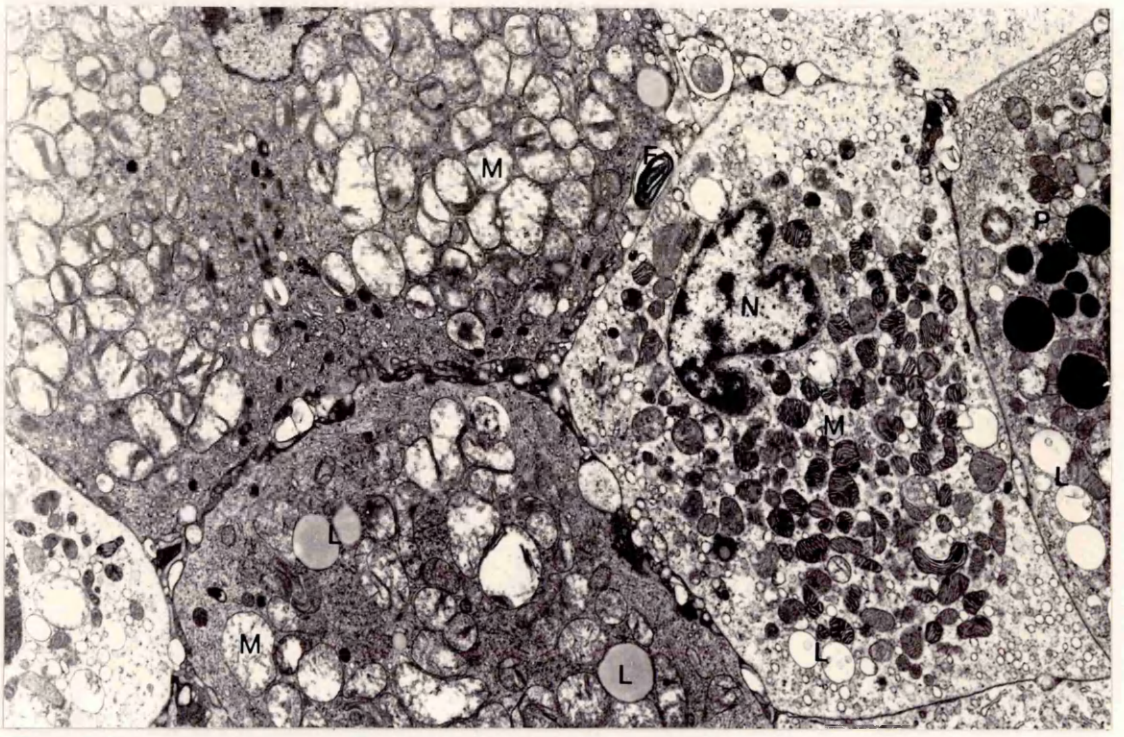


Figure 3.4. Electron micrograph of adrenocortical cells from band 3. Density > 1.039 < 1.052. F; myelin figure, L; lipid vesicles, M; mitochondria, N; nucleus, P; protein store. Final magnification x8775.

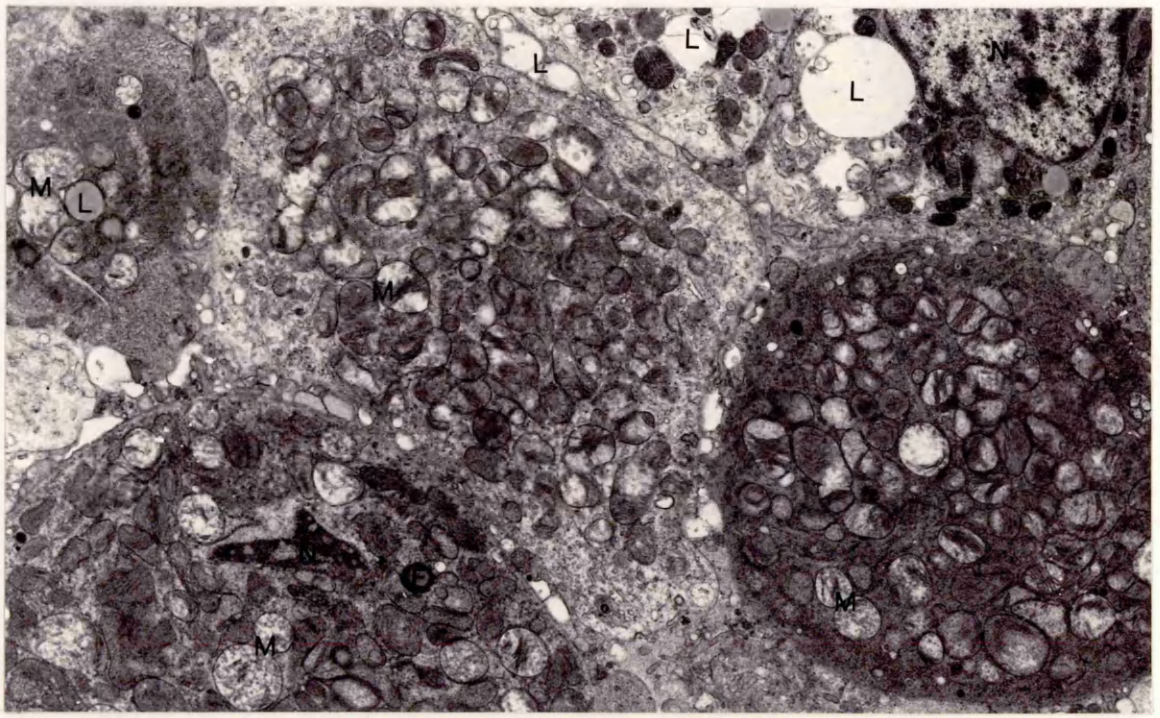


Figure 3.5. Electron micrograph of adrenocortical cells from band 4. Density > 1.052 < 1.097. F; myelin figure, L; lipid store, M; mitochondria, N; nucleus. Final magnification x8775.

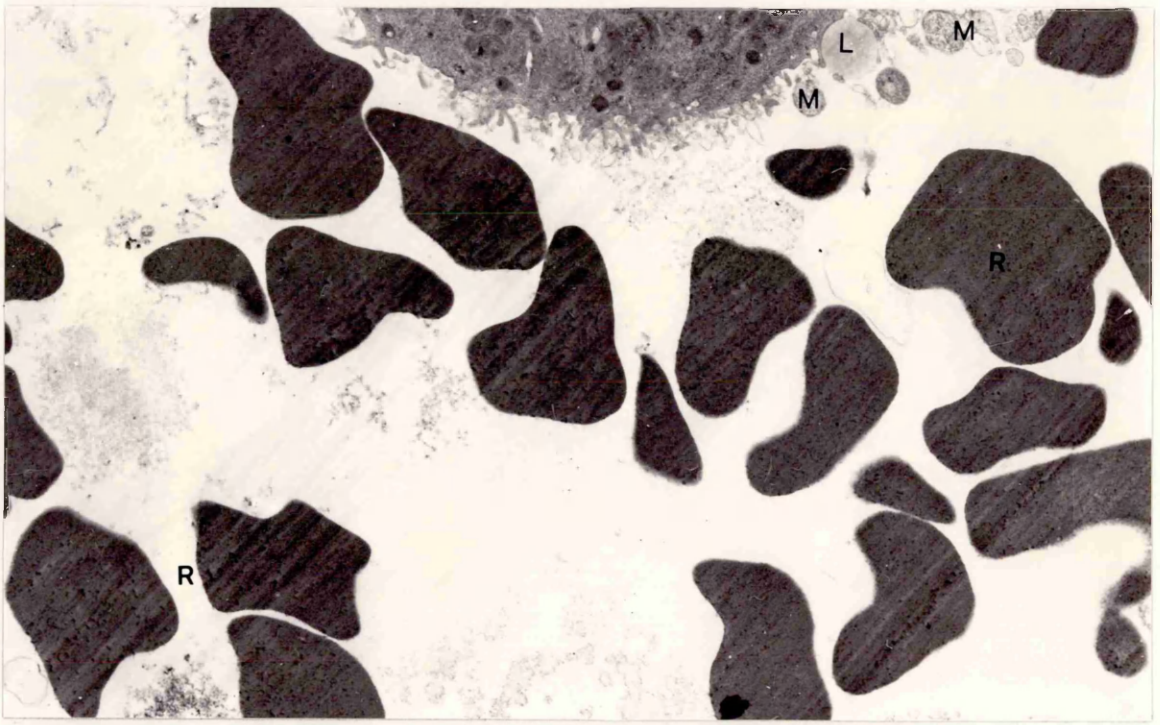


Figure 3.6. Electron micrograph of cells from band 5. Density > 1.097. L; lipid vesicle, M; mitochondria, R; red blood cell. Final magnification x8775.

more elongated mitochondria and fewer lipid vesicles (Ghadially 1980). All the mitochondria in the cells from bands 2-4 presented rounded profiles and many were damaged, being vacuolated or showing reverse staining. This latter effect is caused by the cristae becoming swollen so that they appear light and the matrix dark rather than the opposite found in normal mitochondria. Scattered, small amounts of lipid were seen in all cell groups.

It was therefore not possible to distinguish between ZF and ZG cell types by the electron microscope in cells thus isolated.

Figure 3.5 shows that red blood cells constituted the major component of the pellet at the base of the Percoll gradient.

3.3. Summary.

In conclusion, the ZF and ZG cells could not be distinguished by electron microscopy but bands 2-4 contained cells which were free of red cells and debris. From the steroid profiles, band 3 responded with the highest aldosterone/cortisol production and therefore contained the greatest proportion of ZG cells. Cells from band 3 were used in subsequent experiments (except those for the experiments in chapter 4 which were from a Percoll density band equivalent to bands 2 and 3) and these are referred to as ZG cells.

CHAPTER 4. EFFLUX OF ^{43}K FROM UNSTIMULATED AND AII-STIMULATED CELLS.

4.1. RESULTS.

4.1.1. ^{43}K Efflux from superfused cells.

Figure 4.1 shows the mean ± SEM of seven ^{43}K washout curves from adrenocortical cells superfused at 1 ml/min with isotope-free medium 199. The data could be fitted to an equation with three exponential functions $A(t) = C_1+C_2+C_3 = C_{1o}e^{-\lambda_1 t} + C_{2o}e^{-\lambda_2 t} + C_{3o}e^{-\lambda_3 t}$, where A(t) is the amount of radioactivity in a given fraction at a given time, C_{1o} , C_{2o} , C_{3o} and $\lambda_1 t$, $\lambda_2 t$, $\lambda_3 t$, are the coefficients and rate constants of the exponential functions C_1, C_2 and C_3, (figure 4.2). These functions had $t_{\frac{1}{2}}$ values of 0.2±0.06, 1.5± 0.7, and 24±3 minutes. Efflux of ^{43}K from columns loaded with radioactive medium alone fitted a profile with two exponential functions with $t_{\frac{1}{2}}$ of 0.3±0.1 and 1.68±1.2 min, which were not significantly different from those of the faster components of columns loaded with radioactive cells plus medium. Within 15 mins, ^{43}K washout of these two faster components exponents was largely complete. In subsequent experiments, the cells were therefore perfused for 30 min prior to the addition of agonist to ensure that ^{43}K derived from the slow component alone.

4.1.2. The effects of AII on superfused cells.

After perfusing cells at 0.5 ml/min, (to increase the aldosterone content in the eluate fractions) for 30

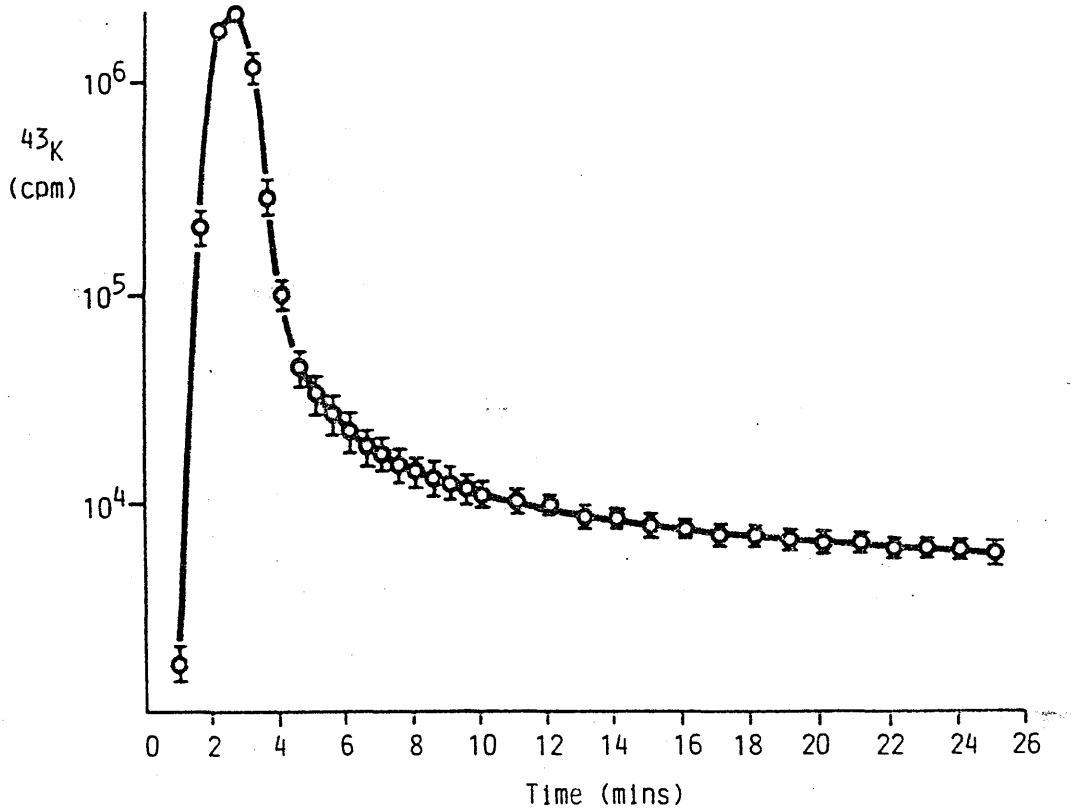


Figure 4.1. ^{43}K efflux from superfused cells. Mean counts per minute ± SEM for 7 experiments are plotted for fractions collected at 0.1 min intervals for the first 10 min and every 1 min thereafter. Not all early data points are shown for clarity.

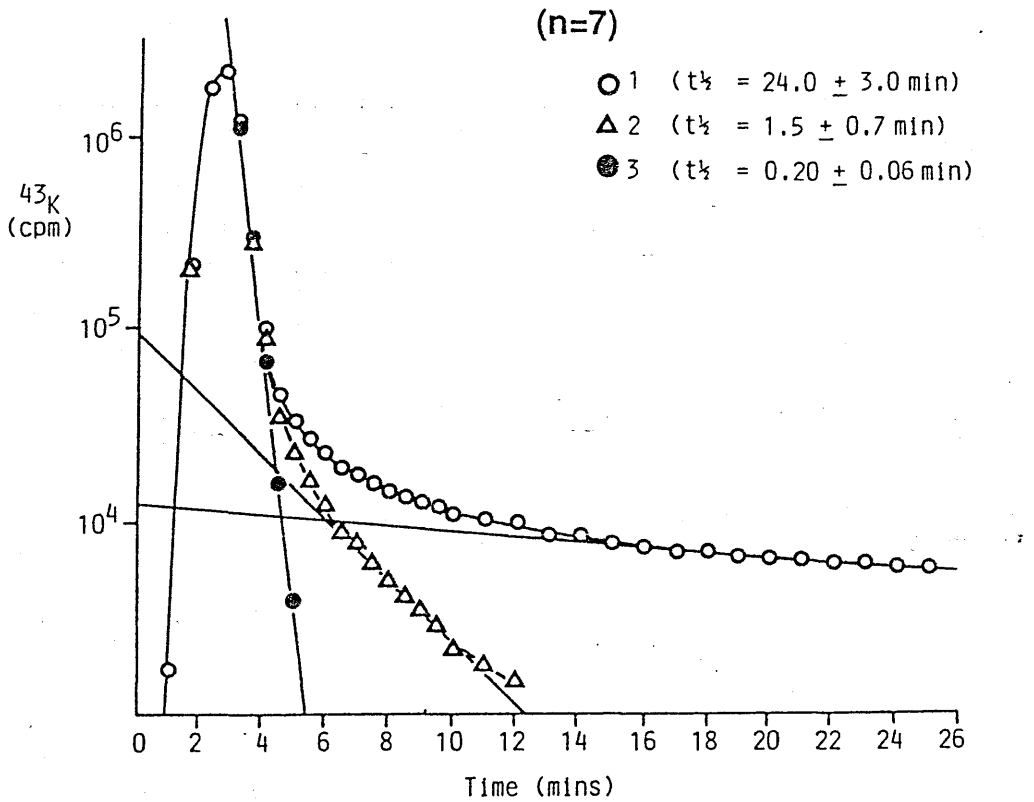


Figure 4.2. Resolution of ^{43}K efflux into three components. The data from figure separated into three components by successive curve stripping. When medium without cells is added to the column the profile resembles that of profile 2 and can be split into two components to give a profile like 3.

min, the medium was changed to one containing AII (10^{-6} M), (figure 4.3). Aldosterone secretion and fractional ^{43}K efflux rates increased simultaneously within 1 min and remained elevated for the five minutes of AII treatment and for ten min after reverting to a perfusate containing no AII. During this 15 min period, fractional ^{43}K efflux rate and aldosterone secretion were linearly correlated ($r=0.882$, $p<0.001$).

4.1.3. The effects of increasing AII concentrations in the absence of ouabain in static incubations.

During both the 20-40 min and 40-60 min period of incubation, cells treated with 10^{-8} and 10^{-10} M AII showed a significant decrease in fractional ^{43}K efflux whereas efflux was increased with 10^{-6} M AII (figure 4.4). Changes in K^+ efflux in the 40-60 min period of incubation were smaller than those for the earlier period. Aldosterone release (0-60 min) was stimulated significantly only by treatment with 10^{-8} M and 10^{-6} M AII.

4.1.4. Biphasic effect of AII on ^{43}K efflux in static incubations.

Since AII (10^{-6} M) had stimulated ^{43}K efflux the following experiment was designed to ascertain whether this dose was also able to decrease ^{43}K efflux. Samples were taken earlier and more frequently and two conditions were treated with ouabain to prevent reuptake of the isotope. In the absence and presence of $2x10^{-4}$ M ouabain, AII stimulated ^{43}K efflux during the 15-30 and 30-45 min periods of incubation (figure 4.5).

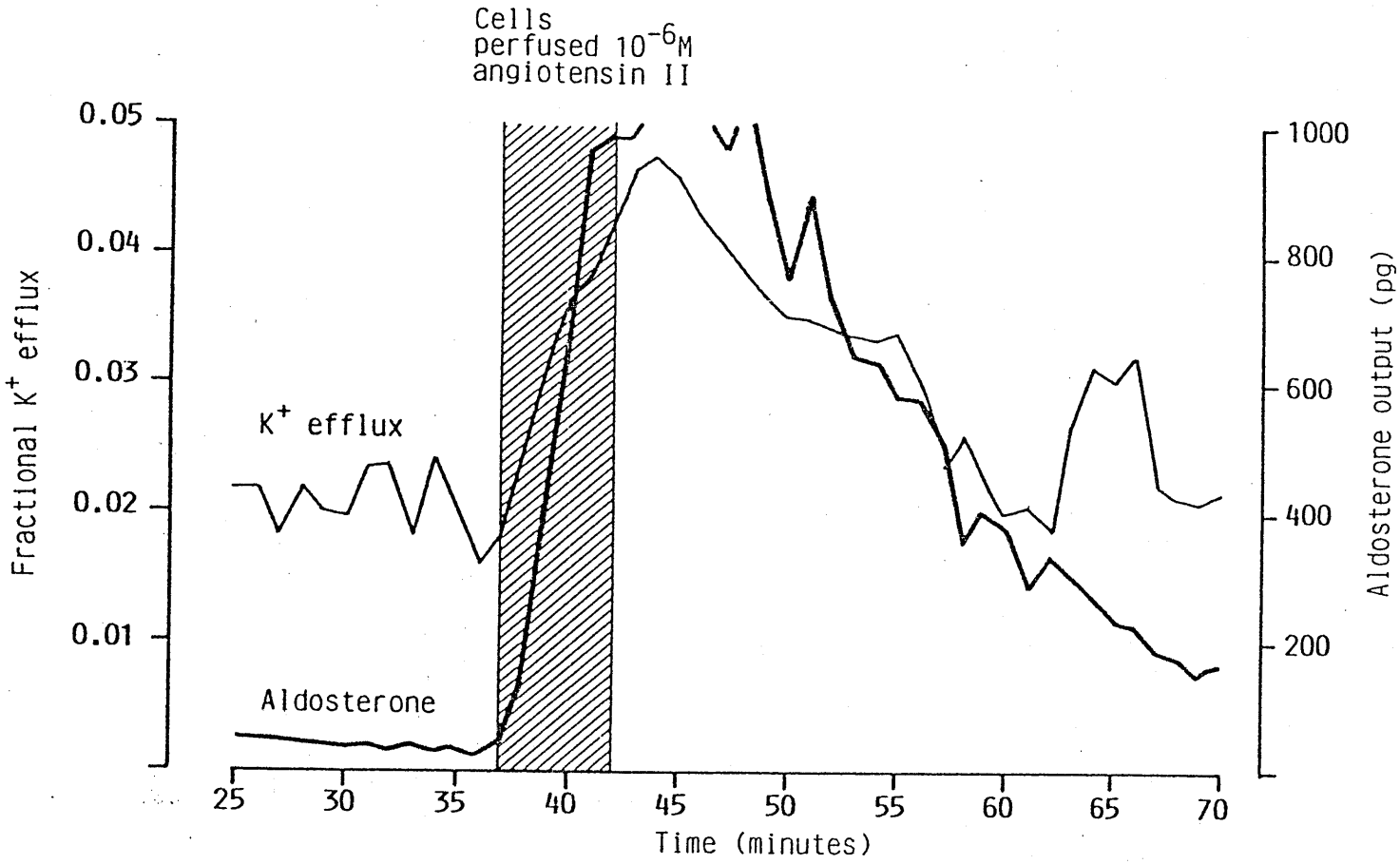


Figure 4.3. The effect of AII on fractional ^{43}K efflux and aldosterone production by superfused cells. Cells were perfused for 30 min with medium alone before switching to medium containing 10^{-6}M AII for 5 min. Data from representative experiment (one of three).

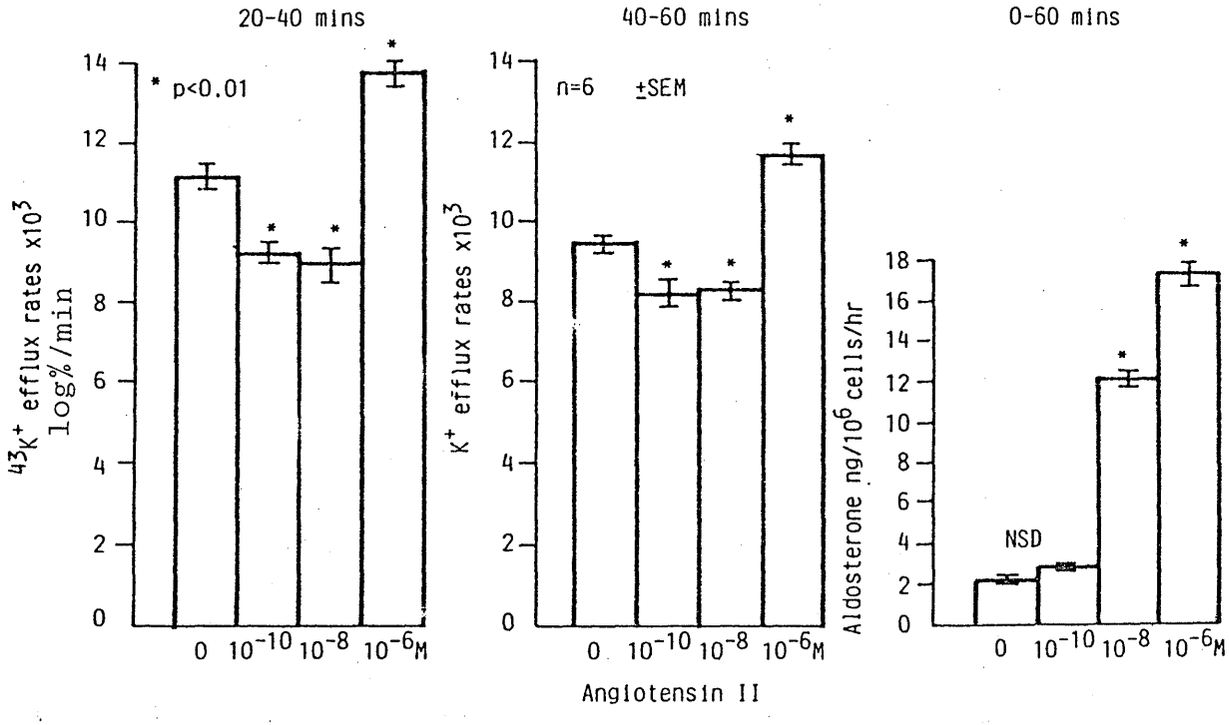


Figure 4.4. ^{43}K Efflux rates and aldosterone responses from cells incubated with various concentrations of AII. The left and centre panels show ^{43}K efflux rates corresponding to increasing concentrations of AII during 20-40 and 40-60 min periods of static incubation respectively. Aldosterone production (right panel).

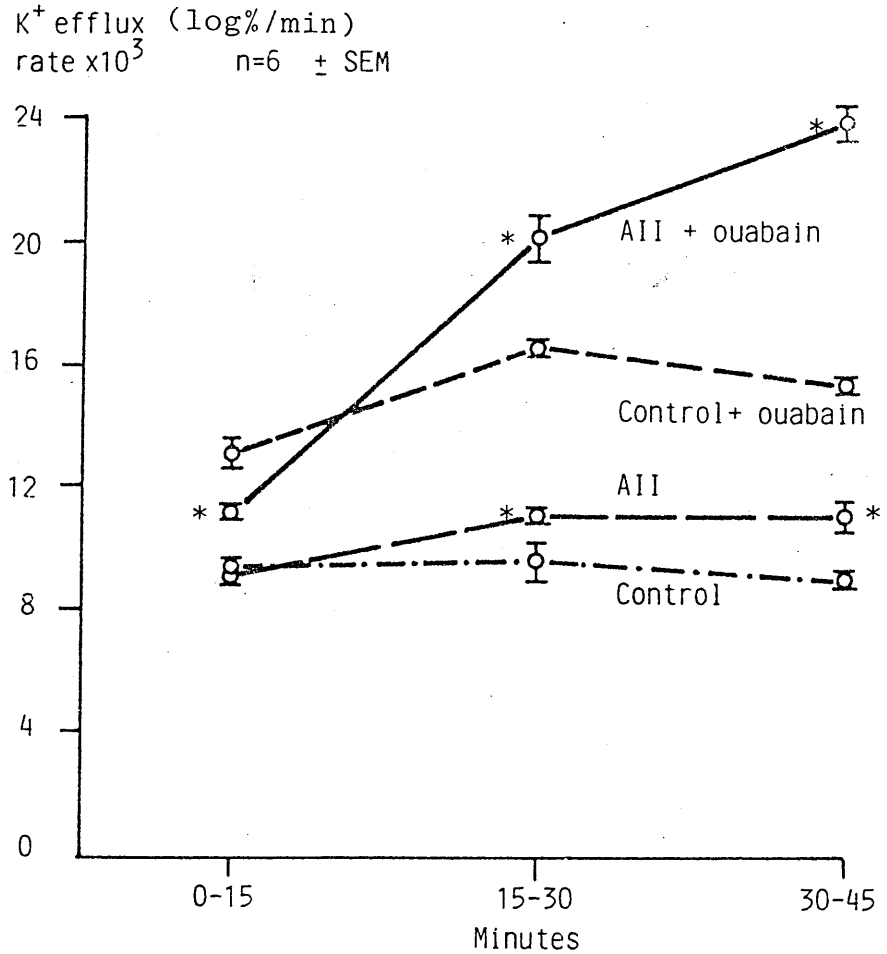


Figure 4.5. Time course of AII effects on ^{43}K efflux in cells treated with or without ouabain ($2x10^{-4}M$).
* $p<0.01$ With respect to the relevant control.

In the absence of ouabain, during the first 15 min, AII had no significant effect. However, in the presence of ouabain, ^{43}K efflux with AII was slower ($p<0.01$) than controls. Ouabain treatment in control and AII-stimulated cells increased efflux but the effects of AII, particularly during the 30-45 min period, were more marked. Basal and stimulated aldosterone release (0-45 mins) was not affected by ouabain (control 0.026±0.001 vs 0.032±0.003; AII-stimulated 0.122±0.007 vs 0.113±0.013 ng/10^6 cells/h).

A more detailed kinetic analysis of the effects of AII on static incubations of cells treated with ouabain is shown in figure 4.6a. During the 54-90 min period of incubation the fractional efflux of potassium was linear both in the presence and absence of AII. Efflux in the presence of AII was faster ($t_{\frac{1}{2}}$ values 24.2±1.7 and 28.4±1.1 min, $p<0.01$; rate constants 0.0292±0.0018 and 0.0246±0.0011 min^{-1}). The $t_{\frac{1}{2}}$ values were similar to the slowest exponential component of efflux in superfused cells (figure 4.2). When the extrapolated exponential component values for the 54-90 min period (figure 4.6a) were subtracted from the earlier time points and the data replotted (figure 4.6b), fractional ^{43}K efflux over the 0-30 min period was curvilinear for AII-treated cells but fitted a straight line for all the early times bar the 30 min point for the control cells. The $t_{\frac{1}{2}}$ for this line was 12.0±0.7 min. Presumably, in AII-treated cells K^+ efflux during this 0-30 min period of incubation is not exponential because of the combined effects of the hormone and ouabain on intracellular K^+ concentration. Ouabain will

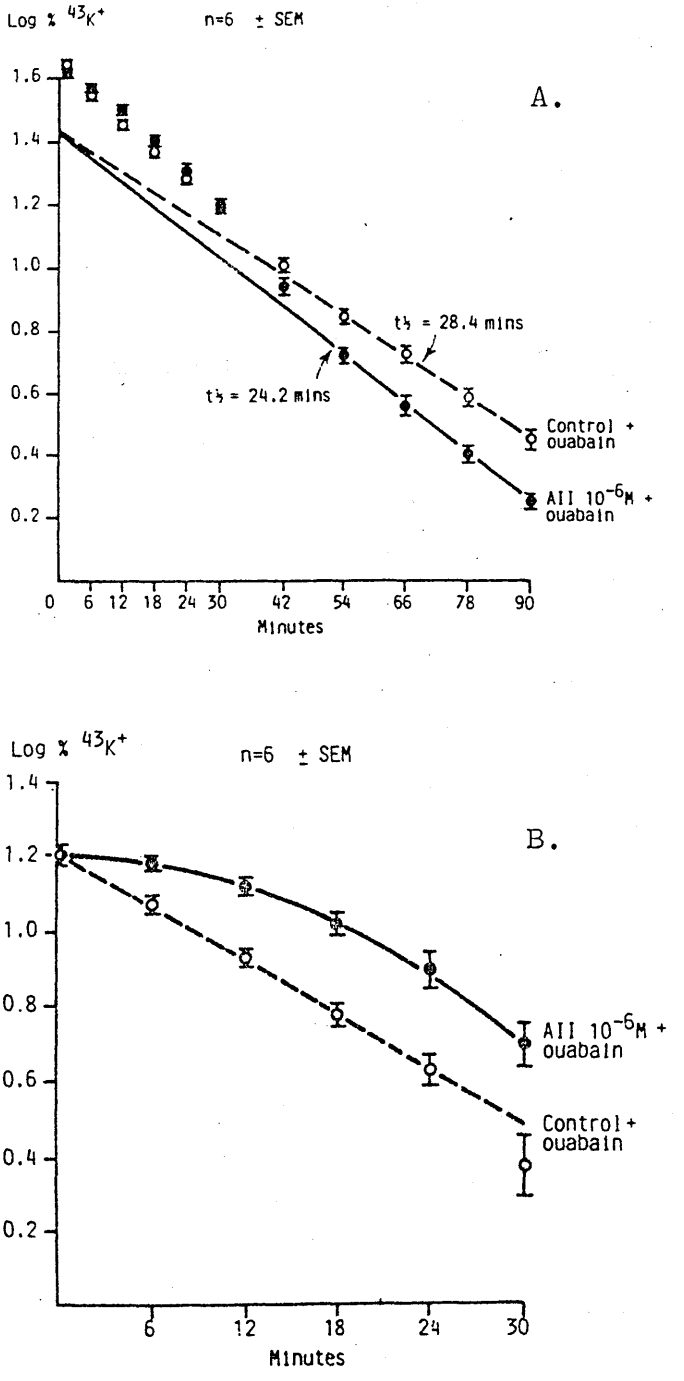


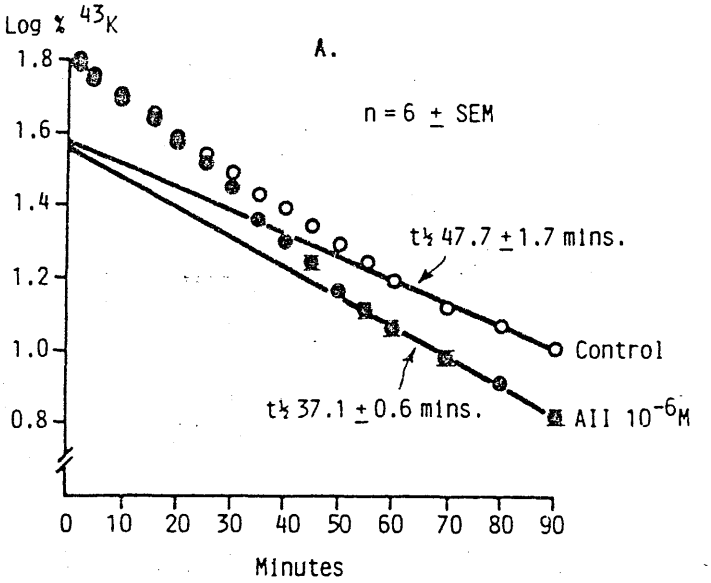
Figure 4.6. Time courses of AII effects on late (A.) and early (B.) components. Panel A shows the log of the fraction of ^{43}K remaining in the cells at various times. ^{43}K efflux for control cells between 54 and 90 min had a $t_{\frac{1}{2}}$ of 28.4 min compared with 24.2 for AII-treated cells. When this slow exchange component (calculated by extrapolation) is subtracted from earlier time points, (panel B), efflux from a faster component can be calculated. AII appears to retard ^{43}K release particularly during the first 12 min.

increase K^+ efflux and AII slows its release, particularly during the first 12 min of incubation.

Figures 4.7a and b show the same kinetic analysis as in figures 4.6a and b but for cells in the absence of ouabain. During the 55-90 min period of incubation, the fractional efflux of K^+ in the presence of 10^{-6}M AII was much faster than in controls ($t_{\frac{1}{2}}$ values 37.1 ±0.6 min and 47.7±1.7 min; rate constants 0.0187±0.0003 and 0.0146±0.0005 min^{-1}). When the extrapolated exponential component values for the 55-90 min period (figure 7a) were subtracted from the earlier time points and the data replotted (figure 7b), fractional ^{43}K efflux for the controls was less linear than in ouabain-treated cells (compare figure 4.6b).

AII-treated cells, although losing ^{43}K more slowly than controls, showed a less smooth set of data points when compared to the ouabain-treated condition with AII. The $t_{\frac{1}{2}}$ value for ouabain-free controls over the 0-30 min period was 14.2±0.6 min with an efflux rate constant 0.0494±0.0020) whereas that for ouabain-treated controls was 12.0±0.7 min; efflux rate constant 0.0588±0.0034.

It would appear that the later phase of ^{43}K efflux is more sensitive to ouabain as its $t_{\frac{1}{2}}$ increases by about 50% in the presence of this agent. The early phase is less affected. However, both phases are altered by AII in the presence and absence of ouabain.



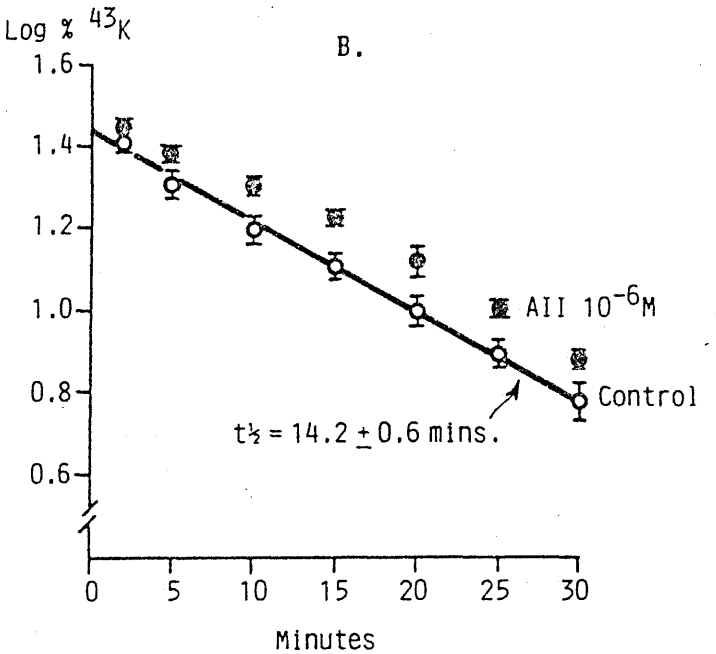


Figure 4.7. Time courses of AII effects on late (A) and early (B) components in the absence of ouabain. Panel A shows the log of the fraction of ^{43}K remaining in the cells similarly to figure 4.6 A. ^{43}K efflux for control cells between 55 and 90 min had a $t_{\frac{1}{2}}$ of 47.7 min compared with 37.1 min for AII-treated cells. When this slow exchange component is subtracted from earlier time points (panel B), efflux from the faster exchange component can be calculated. AII retards ^{43}K efflux over the first 30 min.

4.1.5. Dose-response of early and late ^{43}K efflux to AII.

Data from an experiment in which cells were incubated in the presence of ouabain and increasing doses of AII are shown in figure 4.8. Cell ^{43}K content is expressed as a percentage of initial values and plotted on a log scale against time for the 0-10 min period (left panel) and 45-85 min period (right panel). During the early phase (late phase not subtracted) control and AII 10^{-10} M-treated cells showed identical rates of isotope loss which were less than those for the higher doses of AII. However, the only significant difference was between 10^{-6} and 10^{-10} M ($p<0.05$). A reversal of these relationships was seen during the later phase where the control and 10^{-10} M AII-treated cells had significantly faster rates of isotope loss than those treated with either of the higher doses of AII. Regression lines were calculated from the data points for each treatment over the late period and efflux rate constants derived. The control and 10^{-10} M AII-treated cells had $t_{\frac{1}{2}}$ and rate constant values of 31.7 min, 0.0219 ± 0.009 min^{-1} and 30.4 min, 0.0228 ± 0.005 min^{-1} respectively which were not significantly different. Similarly, 10^{-8} and 10^{-6} M AII-treated cells had $t_{\frac{1}{2}}$ and rate constant values of 21.5 min, 0.0323 ± 0.004 min^{-1} and 20.9 min, 0.0322 ± 0.006 min^{-1} respectively, not significantly different. However, these values showed a significantly faster rate of isotope loss from the higher dose of AII compared to control or 10^{-10} M AII-treated cells ($p<0.01$).

Aldosterone production over 85 min from the same

experiment showed that only the two higher doses of AII significantly increased steroidogenesis (control; 1.26± 0.08, AII 10^{-10} M; 1.37±0.07, 10^{-8} M; 1.98±0.12 and 10^{-6} M; 2.27±0.07 ng/10^6 cells.)

4.2. DISCUSSION

From the studies of unstimulated ZG cells in static incubations (figure 4.7a and b),two components have been established for ^{43}K efflux with $t_{\frac{1}{2}}$ values of 47.7±1.7 and 14.2±0.6 min. Although the efflux profile does not follow a completely smooth curve, the line fits neither a one nor a three exponential curve but does fit a two exponential function (confirmed by D.J. Nichols using SIPHAR curve-fitting programme).

When ^{43}K efflux is followed in superfused ZG cells, three components are resolved (figure 4.2). Only component 1 with the longest $t_{\frac{1}{2}}$ is unique to the cells since components 2 and 3 are also found in columns perfused after loading with medium plus isotope alone. Component 3 reflects ^{43}K borne straight through the column and component 2 probably derives from isotope which is retained by the Biogel matrix.

A comparison of the $t_{\frac{1}{2}}$ values for the slower ^{43}K efflux component of static incubations and the component 1 of the superfused cells shows that the former is almost twice as long (47.7±1.7 and 24.0±3.0 min respectively). Such a discrepancy would be expected if these two components represent ^{43}K efflux from the same intracellular source. This is because in the static incubations both passive and active (via the

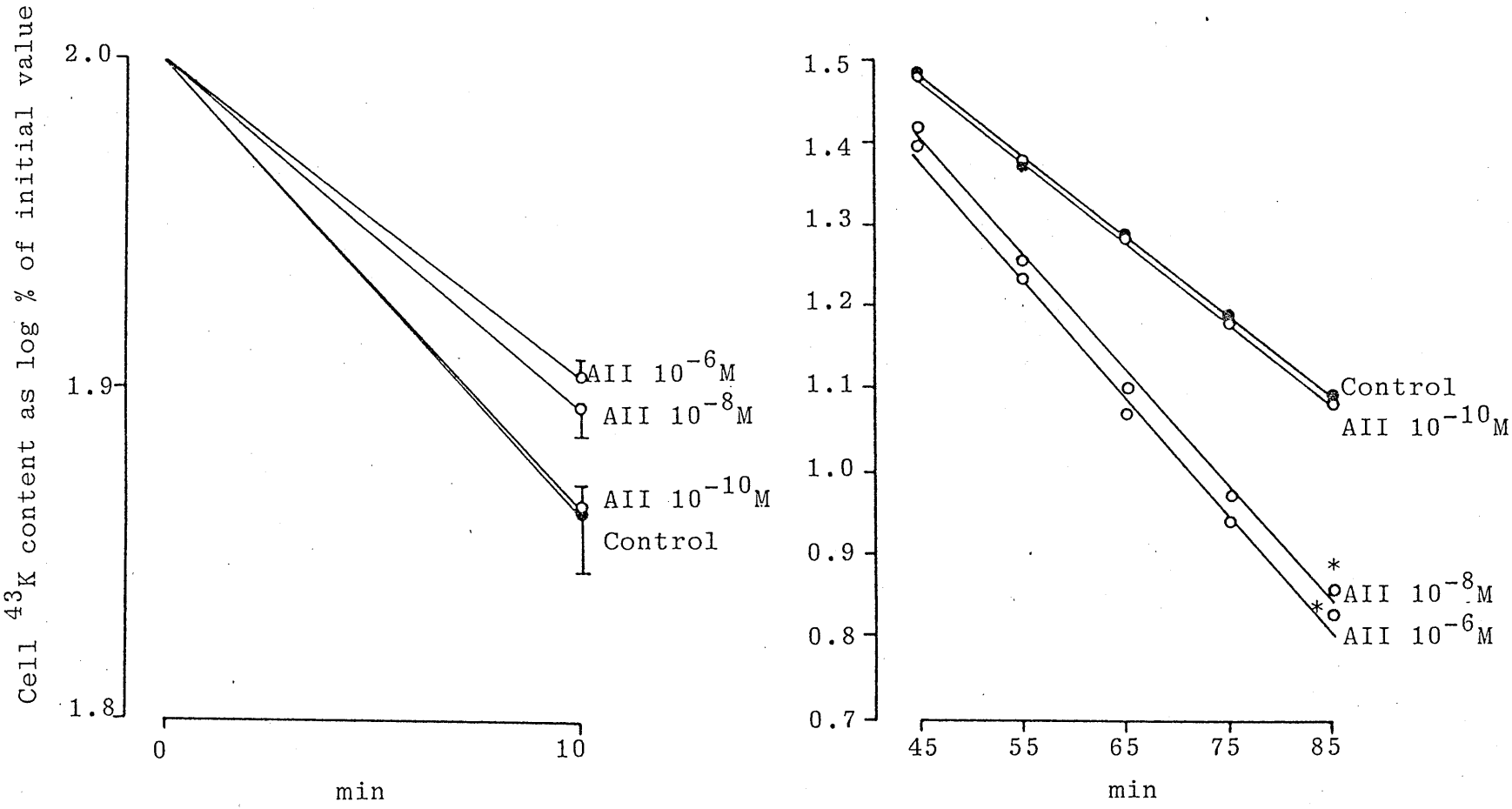


Figure 4.8. Dose-response of early and late ^{43}K efflux to AII. Cell content of ^{43}K is plotted as the % of initial values on a log scale against time for the early (left panel) and late phases (right panel).

* $p<0.01$ with respect to controls. N=5 ± SEM.

Na^+/K^+ ATPase) reuptake of the isotope from the medium is possible whereas uptake is prevented by superfusion with isotope-free medium. In static incubations where reuptake is partially prevented with ouabain, the efflux rates of the two components are both increased and one might expect even faster efflux rates if passive reuptake was also blocked. It follows that if two efflux components are also present in superfused cells that the second, as yet unidentified component, will have a $t_{\frac{1}{2}}$ of less than 12 min. In the data shown in figure 4.2 it is clear that the sizes of compartments 2 and 3 are several orders of magnitude larger than component 1. Given that the relative sizes of the fast and slow components from the static incubations are in the ratio of 2:3, it seems likely that a small component with a $t_{\frac{1}{2}}$ of 7 min or less will not be discernible in the study shown in figure 4.2 because of the larger extracellular components 2 and 3.

AII (10^{-6} M) affected the two efflux components differently. It increased the efflux rate of the slower component and decreased that of the faster one (4.7a and b). The AII effect on the slower component produced a clear-cut change in the slope of the line described by the log % ^{43}K data points. However, although the data points for the fast component were significantly different from controls, calculation of an efflux rate was not possible because efflux appeared to be non-linear. There are several reasons for this non-linearity. It appears that in unstimulated cells, the faster component of efflux becomes non-linear in ouabain-treated but not in non-ouabain-treated cells.

This would indicate that total $[K^+]_i$ may influence ^{43}K efflux rate. Since, in adrenocortical cells, AII inhibits both passive and active influx (Kenyon et al 1985) as well as stimulating the slower K^+ efflux component, it is likely that $[K^+]_i$ is much reduced. Other reasons could be that AII acts on the faster component pool for a shorter time than the slower one or that the pools are interrelated such that the slower one replenishes the faster one.

In the experiment to observe the dose-response of the ^{43}K efflux rate to AII (figure 4.4), AII 10^{-10} and 10^{-8} M significantly reduced the ^{43}K efflux rate during both the 20-40 and 40-60 min of incubation ($p<0.01$) whereas 10^{-6} M AII increased it ($p<0.01$). Unfortunately, no samples were taken at t=0 so a comparison of 0-20 min efflux rates was not possible. However, during the 0-15 min of incubation of another experiment (figure 4.5) in the absence of ouabain, 10^{-6} M AII had no significant effect on ^{43}K efflux rate but the latter became larger than control values over later periods. These results confirmed the pattern of efflux seen in figure 4.7a where AII 10^{-6} M shows an identical efflux profile to controls over the first 15 min of incubation but a greater loss of ^{43}K thereafter. In ouabain-treated conditions, (figure 4.5) AII 10^{-6} M caused a fall in ^{43}K efflux rate over the 0-15 min period.

This was further confirmed by the data in figure 4.8. At t=10 min, AII 10^{-6} M and to a lesser extent 10^{-8} M, reduced ^{43}K efflux compared with either controls or 10^{10-} M AII. Over the 45-85 min period, the higher doses increased the ^{43}K efflux rate compared

with controls or 10^{-10}M AII ($p<0.01$). These changes in ^{43}K loss were related to aldosterone synthesis in that AII 10^{-10} M produced no significant change in either measurement but both were altered by the higher doses of the hormone.

Efflux rates were not calculated for the 0-10 min period because only two time points were taken and, as discussed above, the relationship between ^{43}K content and time in the presence of ouabain and AII would be curvilinear.

Assuming that the opposing effects of AII on the two efflux components occurs simultaneously, it follows that the net effect will depend on several factors; the relative contributions that each component makes at the time of sampling; the dose-response curve for each component; the interrelationships of the two components.

In superfused cells where, presumably, the faster efflux component is exhausted, AII 10^{-6} M produces only the enhancement of ^{43}K efflux as expected from the slow component (figure 4.3). Unlike static incubations, prompt removal of the stimulus is possible and shows that the enhanced efflux starts to reverse within 2-3 min of changing the medium. It regains pre-stimulation levels within 20 min. The subsequent small rise in ^{43}K efflux may not be significant when compared with the pre-stimulus baseline. The rise and fall in ^{43}K efflux is closely associated with similar changes in aldosterone production. Both rise simultaneously but the start of the fall in ^{43}K efflux preceded that of aldosterone production by 2-3 min.

In ouabain-treated cells, in static incubations, (figure 4.6a) the two efflux components for ^{43}K were also apparent. The slower had a $t_{\frac{1}{2}}$ of 28.4±1.1, shorter than that for ouabain-free cells (4.7a) due to the inhibition of reuptake of isotope via the Na^+/K^+ ATPase. It is interesting to note the similarity between this value and that of superfused cells (24.0±3.0 min, figure 4.2) although a direct comparison is not valid since, in the presence of ouabain, net loss of total cell K^+ occurs whereas superfusion merely prohibits reuptake of isotope, and not K^+ in general, from the medium. The faster efflux component in ouabain-treated cells also obeys a straight line over the first 24 min of incubation (figure 4.6b) although the data points at 30 min fall below this. Over the 0-24 min period the ^{43}K efflux $t_{\frac{1}{2}}$ is 12.0±0.7 min, similar to that of controls in ouabain-free incubations (14.2±0.6 min).

AII 10^{-6} M decreased the $t_{\frac{1}{2}}$ of the slower efflux component in ouabain-treated cells to 24.2±1.7 min. As with AII in the absence of ouabain, the effect was significant and the data retained their adherence to a staight line. The hormone also decreased efflux from the faster component but whereas control cells lost ^{43}K at a steady rate over the first 24 min of incubation, AII reduced the loss of isotope quite markedly over the first 12 min of incubation but then the efflux rate rose so that the data points formed a curve. As with AII in the absence of ouabain (figure 4.7b), an efflux rate could not be calculated and the curvilinear relationship probably reflects the effects of AII on

passive uptake of the isotope or changes in $[K^+]_i$.

The current observations may explain some of the conflicting results published recently from groups employing different techniques for studying K^+ and Ca^{2+} movements in ZG cells. Earlier work established that the depolarisation of adrenocortical cells which accompanies the stimulation by agents such as ACTH, cAMP, AII and rises in $[K^+]_o$, (Natke and Kabela 1979, Lymangrover et al 1982), could develop from a decrease in cell membrane permeability to K^+, (Matthews 1967, Quinn et al 1987). However, in superfused ZG cells, Lobo and Marusic (1986), observed a prompt increase in ^{86}Rb efflux (substituting for K^+) within 30 seconds of starting perfusion with AII similar to that observed in the present study. This hyperpolarising effect was dependent on $[Ca^{2+}]_i$ but not $[Ca^{2+}]_o$ since rubidium efflux was increased by the Ca^{2+} ionophore A23187, and was blocked by inhibitors of Ca^{2+}-dependent K^+ channels (tetraethylammonium (TEA), quinine and apamin) but unaffected by changes in $[Ca^{2+}]_o$. Later studies showed that prolonged AII stimulation produced a dose-dependent, sustained fall in ^{86}Rb release following the earlier rise (Lobo and Marusic 1988). These opposing effects are to some extent mutually independent. It follows that the net effect of AII on K^+ efflux is not necessarily a time-dependent phenomenon but is dependent on a number of factors including the relative sensitivities of the two opposing effects of AII on the relative pool sizes, if there are two, and on the availability of other cations at the time of

stimulation. Lobo and Marusic also showed that the later decrease in permeability was not affected by apamin or TEA and was not observed in Ca^{2+}-free medium in which case a secondary, smaller rise in K^+ efflux was seen. They concluded that bovine ZG cells possess a Ca^{2+}-dependent K^+ channel which opens in response to AII-induced increases in $[Ca^{2+}]_i$. Although followed by a sustained fall in K^+ efflux it was thought that direct inactivation of K^+ channels was unlikely but that their gating properties were altered by phosphorylation of the ion channels (Lobo and Marusic 1986). The rise in rubidium efflux seen by these workers was similar to that of ^{43}K which have been observed in the current study and could arise from changes in the slower of the two efflux components as outlined above. In my experiments, superfusion was not continued for long enough to observe a second decrease in flux. However I would suggest that the two processes are not necessarily sequential and may arise simultaneously. Changes in efflux would therefore represent the net result of the two opposing processes. Even so, the early decrease in ^{43}K efflux noted in static incubations of bovine cells would not correspond to the later decrease seen by Lobo and Marusic (1988) in superfusion experiments.

Whether rubidium ions behave exactly as potassium ions in this context is not known. In unstimulated rat parotid gland slices the efflux of ^{86}Rb is significantly ($p<0.05$) slower than that of ^{43}K with $t_{\frac{1}{2}}$ values of 30±1.3 and 25.3±1.5 min respectively during the first 30 min of washout (Putney 1976). Such a

discrepancy in these time courses would invalidate the use of rubidium for quantifying efflux parameters of ^{43}K even though it is a qualitative tracer of K^+ movements.

Direct electrophysiological measurements in ZG cells have observed changes in K^+ and Ca^{2+} movements which suggest differing sequences of ionic events following secretagogue stimulation. Microelectrode studies (Quinn et al 1987b) show an initial brief hyperpolarisation of about 4mV lasting 5 s followed by a large decrease (up to 67%) in membrane conductance equivalent to a 28mV depolarisation. The depolarisation phase lasted for the duration of the AII stimulus (20 s) and took 25 s to return to control levels. Both effects were accounted for by changes in K^+ conductance rather than Ca^{2+} conductance. Neither method employed in the current thesis could have measured an event the size or duration of the preliminary hyperpolarisation reported by Quinn and co-workers, (1987b). However, since the effect was so small and not consistently observed in their experiments, it may not be a prerequisite for the cell response. Although the time scale of my experiment is very different, the fact that the depolarising phase lasted the duration of the AII application might indicate that depolarisation and inhibition of K^+ efflux are part of the same event.

Patch clamp experiments by Matsunaga et al (1987) in rat cells have shown that depolarisation of ZG cells by increases in $[K^+]_o$ from 4.7 to 10 mM would be sufficient to activate an inward Ca^{2+} current as the threshold for opening voltage-dependent Ca^{2+} channels

was reached. In contrast, bovine ZG cells appear to possess two Ca^{2+} channel types (Cohen et al 1988). One type corresponds to the T-type channels noted by Matsunaga et al and these mediate the Ca^{2+} influx evoked by small depolarisations (of a few mV), of the cell membrane. The other Ca^{2+} channels are of the L-type which are the site of action of the dihydropyridines. AII was found to be able to activate both types but since the T-type activate at -75 mV and the L-type at less negative membrane potentials, AII has its major effect on the T-type Ca^{2+} channels. It was thought by this group that AII activation of these channels was more likely to be via a second messenger than by direct interaction (Cohen et al 1988).

Similar studies by Payet et al (1987), also in rat ZG cells, characterised two outward K^+ currents, labeled "transient" and "slow" and an inward Ca^{2+} current. ACTH blocked the transient current in a dose-dependent manner as did the K^+ channel inhibitors, TEA and 4-AP. This depolarisation was followed by an increased Ca^{2+} influx. The dose-dependent inhibition by ACTH of the transient conductance is compatible with previous findings of changes in K^+ permeability in ZF/R cells (Kenyon et al 1985) and also the present demonstration of an AII-sensitive fall in early K^+ efflux. The later rise in K^+ conductance may correspond both to this slower component and to Lobo and Marusic's increased ^{86}Rb efflux.

Electrophysiological and cation flux data can be combined to suggest the following sequence of events. Binding of ACTH or AII to cell surface receptors causes

a decrease in the membrane permeability to K^+ which depolarises the cell. This in turn opens voltage-dependent Ca^{2+} channels, increasing $[Ca^{2+}]_i$ which then exerts second messenger effects to promote steroidogenesis as well as opening Ca^{2+}-dependent K^+ channels to repolarise the cell membrane. It has been mentioned already (see introduction) that AII can increase $[Ca^{2+}]_i$ by releasing calcium from intracellular stores. This series of ionic movements resembles the mechanism which has been worked out in pancreatic ß cells to explain the insulin release by glucose. Glucose depolarises these cells by the closure of ATP-sensitive K^+ channels (see chapter 1), resulting in bursts of electrical activity and the consequent opening of voltage-dependent Ca^{2+} channels which raises $[Ca^{2+}]_i$ (Herchuelz et al 1980, Rorsman et al 1984, Rorsman and Trube 1986, Arkhammar et al 1987). A Ca^{2+}-dependent K^+ channel, which is thought to control the membrane potential during its silent phases, has been described by Rosario et al (1985) but, although it can be blocked by quinine (Atwater et al 1979), it is resistant to apamin (Lebrun et al 1983). This latter observation contrasts with the findings of Lobo and Marusic (1986). Nevertheless, in both adrenocortical and pancreatic cell repolarisation of the cell membrane is almost certainly mediated by increases in K^+ permeability.

4.3. SUMMARY.

In summary, two components of K^+ efflux, possibly controlled by different channel types, appear to govern the membrane potential in ZG cells. The inhibition of one of these early on in the exposure to AII may depolarise the cell membrane to allow increased Ca^{2+} influx. Stimulation of the second efflux component which is more sensitive than the first to ouabain is likely to restore the membrane potential.

CHAPTER 5. MANIPULATION OF CELL MEMBRANE PERMEABILITY TO K^+.

5.1.INTRODUCTION

Having observed changes in cell permeability to K^+ during the stimulation of aldosterone production, experiments were carried out using agents which alter K^+ permeability to test their effects on steroidogenesis.

Valinomycin (Val) and BRL 34915 (also named cromakalim), increase the cell membrane permeability to K^+. Val is an ionophore exhibiting a high specificity for K^+ over other cations, (see review by Pressman 1976). It rapidly forms a complex with K^+ and is highly diffusible in the plasma membrane enabling K^+ to leave the cell readily. BRL 34915 (systematic name (+) 6 cyano-3, 4-dihydro-2, 2-dimethyl-trans-4 (2-oxo-1-pyrrolidyl)-2H-benzo[b] pyran-3-ol) is a new anti-hypertensive drug which is thought to prevent smooth muscle contraction by opening endogenous membrane channels (reviewed Hamilton and Weston 1989).

The agents selected to cause decreases in K^+ permeability were apamin, a bee venom neuropeptide which specifically blocks Ca^{2+}-sensitive K^+ channels (reviewed by Lazdunski 1983, Moczydlowski et al 1988), and glibenclamide which is one of the sulphonylurea drugs used as an antihyperglycaemic. These close an ATP-dependent K^+ channel in pancreatic ß cells thereby depolarising them and initiating the events leading to insulin release. Tetraethylammonium (TEA) and 4-aminopyridine (4-AP) were also used. These are much

less specific blockers of K^+ channels than apamin and glibenclamide.

5.2. RESULTS: AGENTS WHICH INCREASE K^+ PERMEABILITY

5.2.1. Effect of Val and BRL 34915 on basal and AII-stimulated aldosterone release.

Figure 5.1 shows the effect of increasing concentrations of Val on basal and AII-stimulated aldosterone release. Low concentrations of Val increased aldosterone release in all conditions by 20-70% ($p<0.01$) but higher concentrations ($>10^{-7}$ M) markedly reduced stimulated steroid synthesis so that at 10^{-6} M Val, it approached basal control values for all conditions.

Figure 5.2 plots data from a similar experiment with BRL 34915. Basal aldosterone synthesis, but not AII-stimulated synthesis, rose by 17 and 30% in the presence of 10^{-7} and $2x10^{-6}$ M BRL 34915 repectively ($p<0.05$). However, at $4x10^{-5}$ M BRL 34915, AII-stimulated aldosterone release was reduced by 75% ($p<0.01$) and basal values fell to those in the absence of BRL 34915.

5.2.2. Reversibility of the effects of Val and BRL 34915.

Following a 1h preincubation in the presence or absence of BRL 34915 ($4x10^{-5}$ M) or Val (10^{-6} M), the cells were washed, resuspended and incubated with or without AII in the presence or absence of BRL 34915 or Val for a further 1h as indicated in the legend to

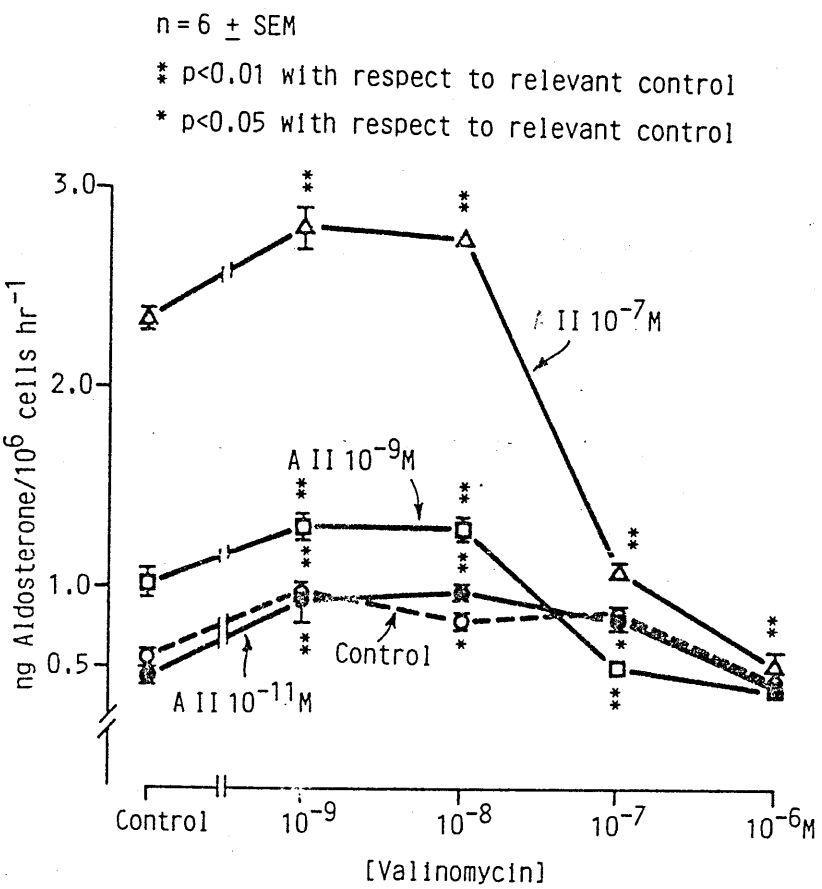


Figure 5.1. Effect of Val on basal and AII-stimulated aldosterone synthesis. Aldosterone in ng per 10^6 cells per h is plotted against increasing concentrations of Val. for different doses of AII.

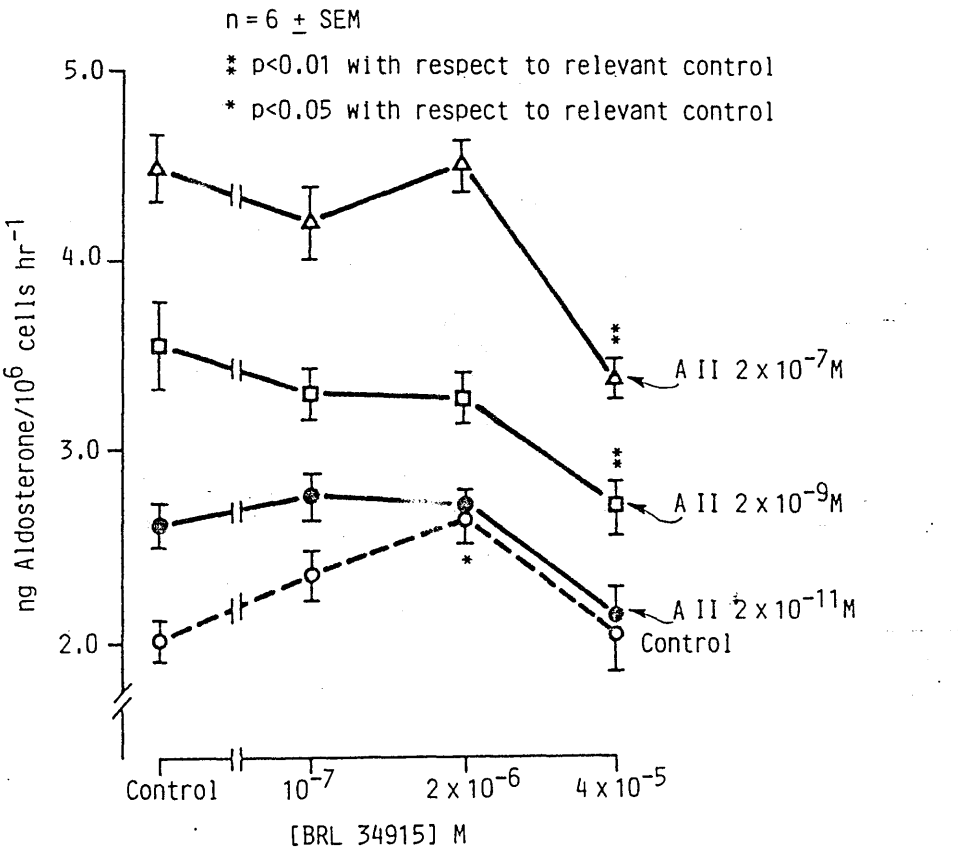


Figure 5.2. Effect of BRL 34915 on basal and AII-stimulated aldosterone synthesis. Aldosterone in ng per 10^6 cells per h is plotted against increasing concentrations of BRL 34915 for different doses of AII.

figure 5.3. The numbers in the bins refer to the percentage of live cells at the end of the second incubation as determined by trypan blue exclusion. The left hand panel shows the effect of BRL 34915. This agent did not significantly alter aldosterone production in the cells preincubated in medium alone. Cells pretreated with BRL 34915, when subsequently incubated in medium alone or with AII, had a slightly enhanced aldosterone synthesis, but those cells incubated with BRL 34915 for both periods produced less aldosterone basally or when stimulated with AII. These results confirm the inhibitory effects of BRL 34915 in figure 5.2 and demonstrate that they are reversible. In contrast, the inhibitory effects of Val were clearly irreversible although the cells were still able to exclude trypan blue to the same extent as BRL 34915-treated cells.

In basal conditions, preincubation with Val caused a significant decrease in aldosterone production ($p<0.01$), irrespective of whether Val was present in the second incubation. In cells treated with AII, aldosterone output rose to 3.5 times that of controls except in cells that had been treated at any time with Val.

5.2.3. Effects of BRL 34915 on passive ^{43}K efflux

The graphs in figure 5.4 (a-b) show the effects of BRL 34915 on ^{43}K efflux from ouabain-treated cells with time. Isotope remaining in the cells is expressed on a logarithmic scale.

Figure 5.4a shows that between 45 and 85 min after

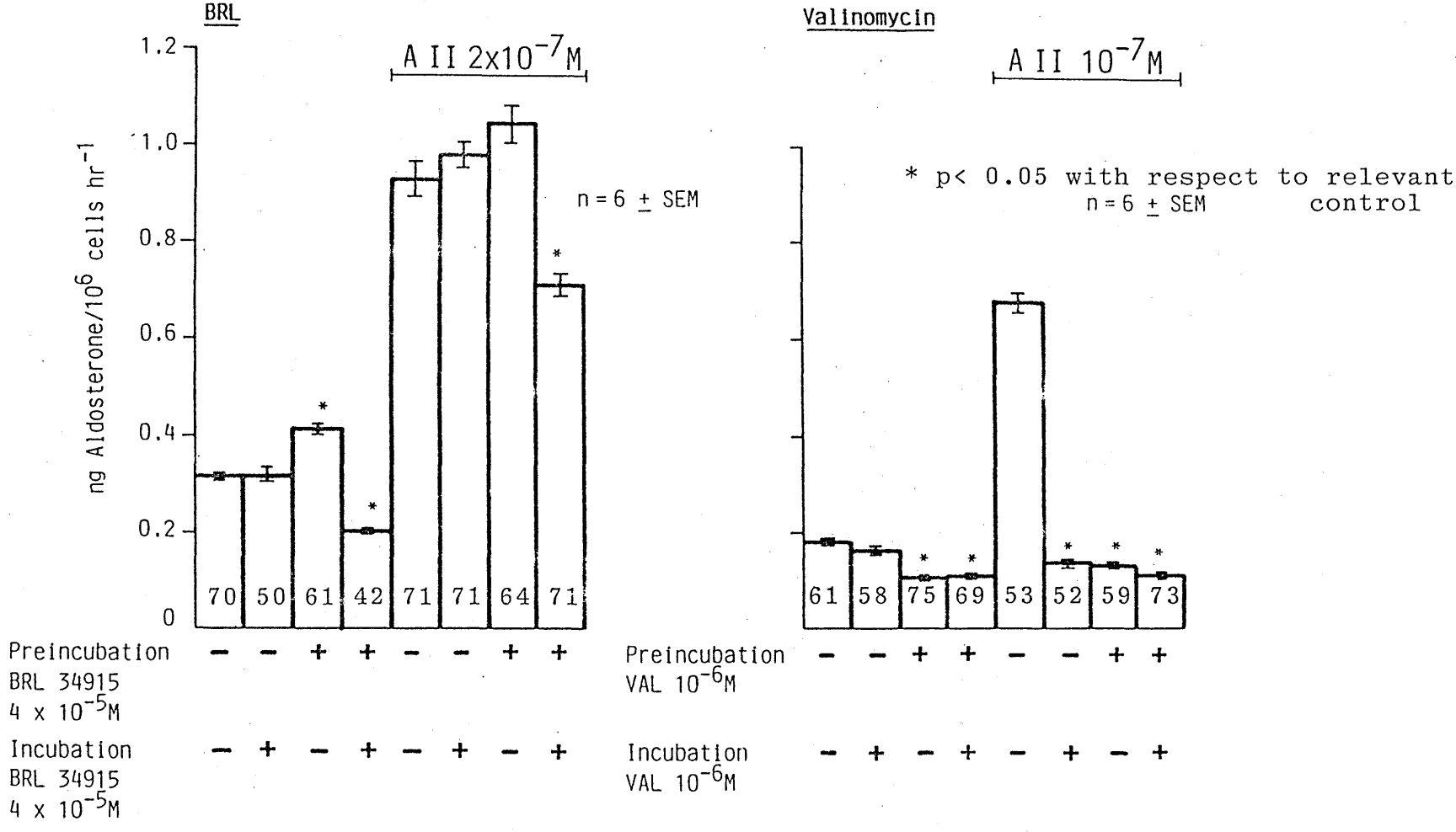


Figure 5.3. Reversibility of the effects of BRL 34915 and Val. The highest concentrations of the agents used in figures 5.1 adn 5.2 were present in the preincubation and main incubations as indicated below each panel. The cells were washed between incubations and resuspended in medium ± AII. Aldosterone, in ng per 10^6 cells/h is shown on the y axis. Figures within the bins are % live cells at the end of the experiment (Trypan Blue).

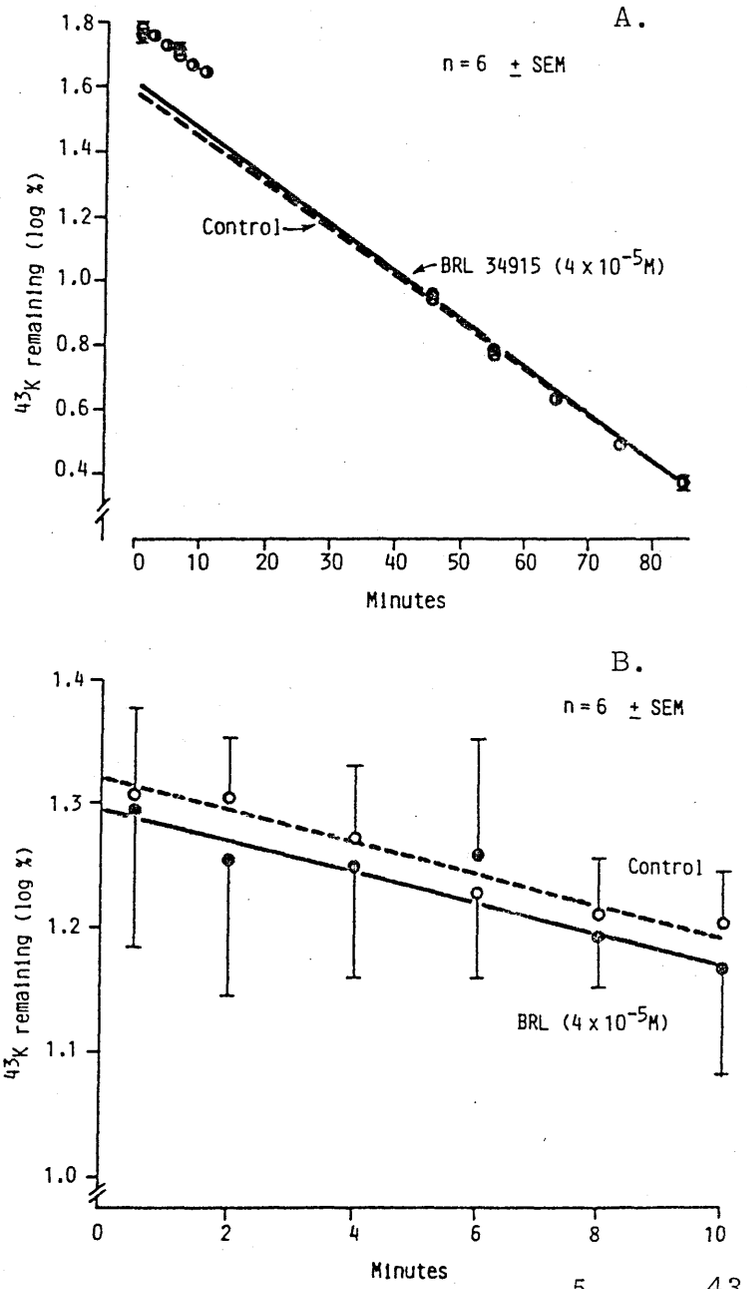


Figure 5.4. The effect of BRL 34915 ($4x10^{5}M$) on ^{43}K efflux from ouabain-treated cells. Isotope remaining in the cells is expressed as the log of the fraction of total ^{43}K against time. During the late period (A), there is no difference between control (open circles) and BRL 34915-treated cells (closed circles). Subtraction of the late phase contribution of ^{43}K efflux shows that during the early phase (B), there is no significant difference between controls and BRL 34915-treated cells.

the start of the incubation, ^{43}K efflux from BRL 34915-treated cells is not significantly different from that of controls. When these later time point values are extrapolated to t=0 and subtracted from the earlier time points, as described earlier, the data can be replotted as in figure 5.4b. Again, there is no difference in ^{43}K efflux with respect to controls.

The effects of two doses of BRL 34915 on passive ^{43}K efflux from basal and AII-stimulated cells is shown in figure 5.5. During the first 10 min of incubation, AII (10^{-7} M) alone decreased the rate of ^{43}K efflux with respect to controls ($p<0.01$). At a concentration of BRL 34915 which appeared to stimulate basal aldosterone synthesis (10^{-6} M see figure 2), ^{43}K efflux was slightly but not significantly reduced ($p<0.07$ paired t test). However, this dose of BRL 34915 in combination with AII had no effect when compared to AII alone. At a concentration ($4x10^{-5}$ M), which appeared to reduce AII-stimulated aldosterone synthesis, BRL 34915 tended to increase ^{43}K efflux, but again, this was not significant ($p<0.2$, paired t test). The AII-induced reduction in ^{43}K efflux was also unaffected.

5.2.4. Effects of Val on ^{43}K efflux.

In a similar experiment to that with BRL 34915 above, ^{43}K efflux over the first 10 min of incubation was studied at two doses of Val. Figure 5.6 shows that the pattern of response is similar in cells treated with (left panel) or without (right panel) ouabain. The lower dose of Val (10^{-9} M shown previously to enhance aldosterone release, figure 1), has no effect on ^{43}K

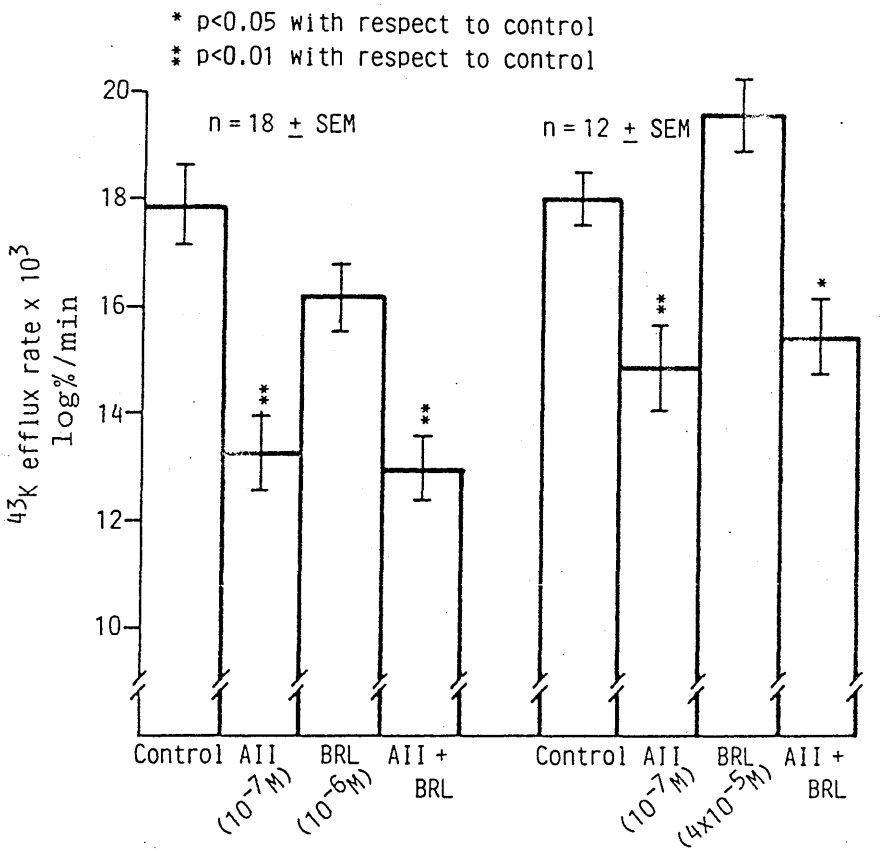


Figure 5.5. The effects of BRL 34915 at two concentrations on ^{43}K efflux from control and AII-stimulated cells. Ouabain ($2x10^{-4}$M) was present in all conditions. The left panel shows pooled data from three experiments on the effect of the stimulatory dose of BRL 34915 (10^{-6}M) and the right panel shows the pooled data from two experiments on the effect of the inhibitory dose of BRL 34915 ($4x10^{-5}$ M) on ^{43}K efflux in the presence and absence of AII. Time course of the experiments was 10 min. Significance determined by paired t-test.

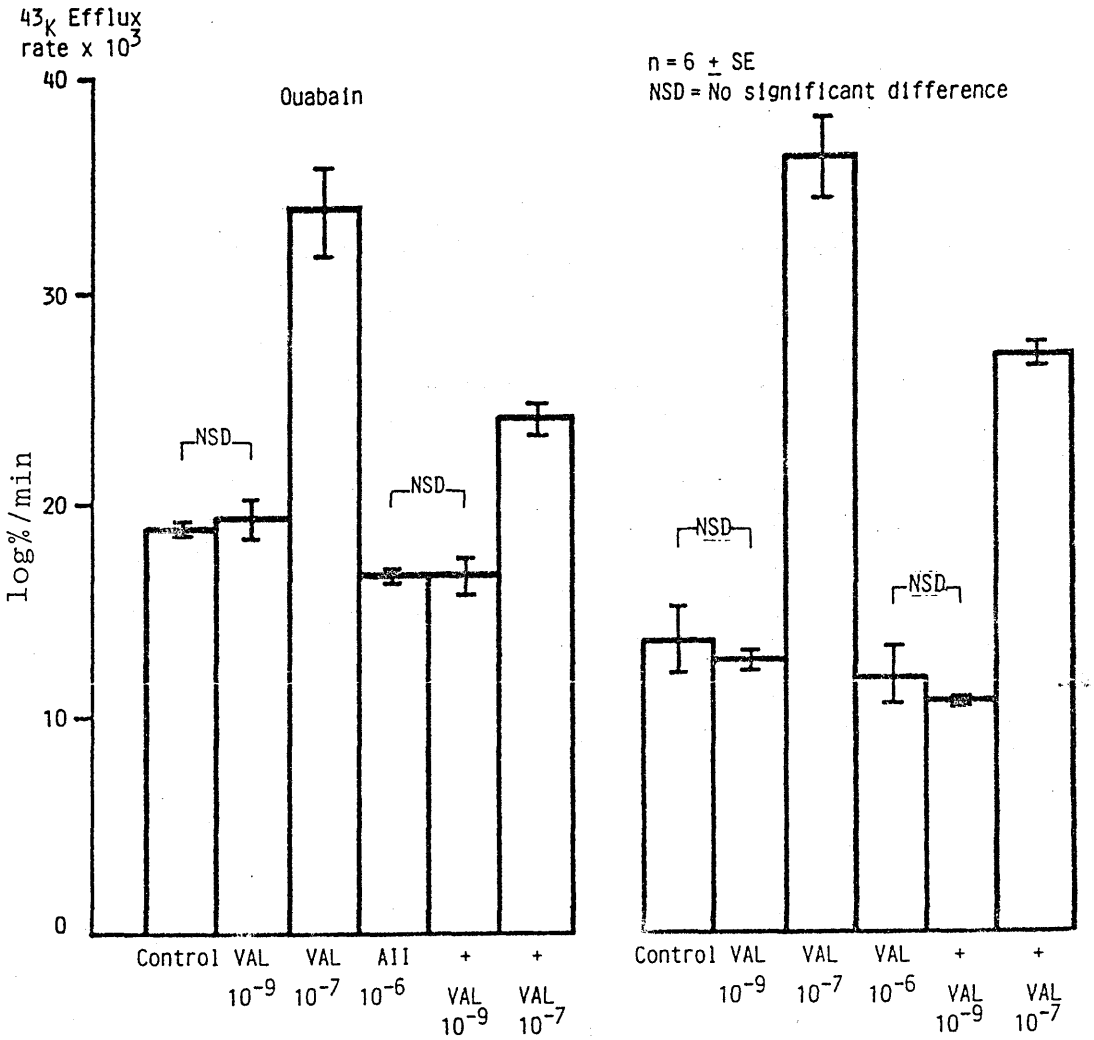


Figure 5.6. The influence of Val on ^{43}K efflux from control and AII-stimulated cells. The left panel shows ^{43}K efflux rate for cells in the presence of ouabain and the right panel that for cells in its absence against the stimulatory (10^{-9} M) and inhibitory (10^{-7} M) doses of Val ± AII. Unless otherwise shown, significance levels are <0.01.

efflux in either control or AII-treated cells. On the other hand, 10^{-7} M Val, shown previously to inhibit aldosterone production, increases ^{43}K efflux markedly in both ouabain and ouabain-free conditions, by 1.8 and 1.4 fold respectively.

5.3. RESULTS: AGENTS WHICH DECREASE K^+ PERMEABILITY.

5.3.1. The effects of apamin and glibenclamide on aldosterone production.

Figure 5.7 shows aldosterone produced in response to increasing concentrations of AII in the presence of different doses of apamin. Basal aldosterone output was significantly enhanced in an dose-related manner by apamin at 10^{-8} M and above, although the overall increase was small. Aldosterone production in AII-stimulated cells was only significantly enhanced by 10^{-7} M apamin (by 10-46%) but all doses of apamin shifted the dose-response curve to the left showing that the peptide increased the sensitivity of the cells to AII by a factor of about 100.

Figure 5.8 shows the effect of increasing concentrations of AII on aldosterone production at various doses of glibenclamide. All the concentrations of glibenclamide tested increased basal aldosterone release but only the highest dose ($4x10^{-5}$ M) shifted the dose-response curve for AII significantly to the left by a factor of about 10.

5.3.2. Comparison of the apamin and glibenclamide effects on early ^{43}K efflux.

Figure 5.9 shows the ^{43}K content as a log % of

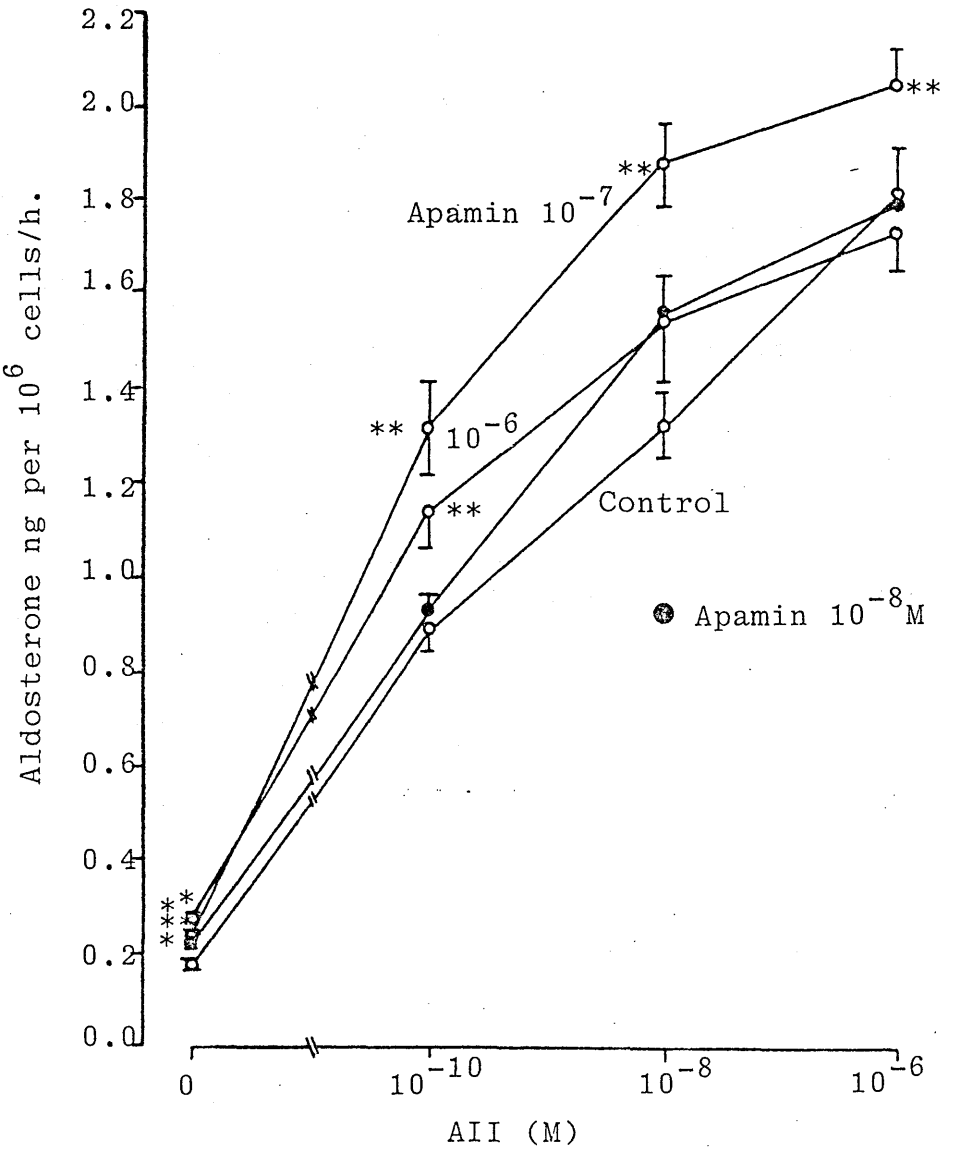


Figure 5.7. The dose-response of aldosterone production to apamin in control and AII-stimulated cells. Aldosterone in ng per 10^6 cells/h is plotted against increasing concentrations of apamin for various doses of AII.
* $p<0.05$, **$p<0.01$ with respect to controls. N=6 ± SEM.

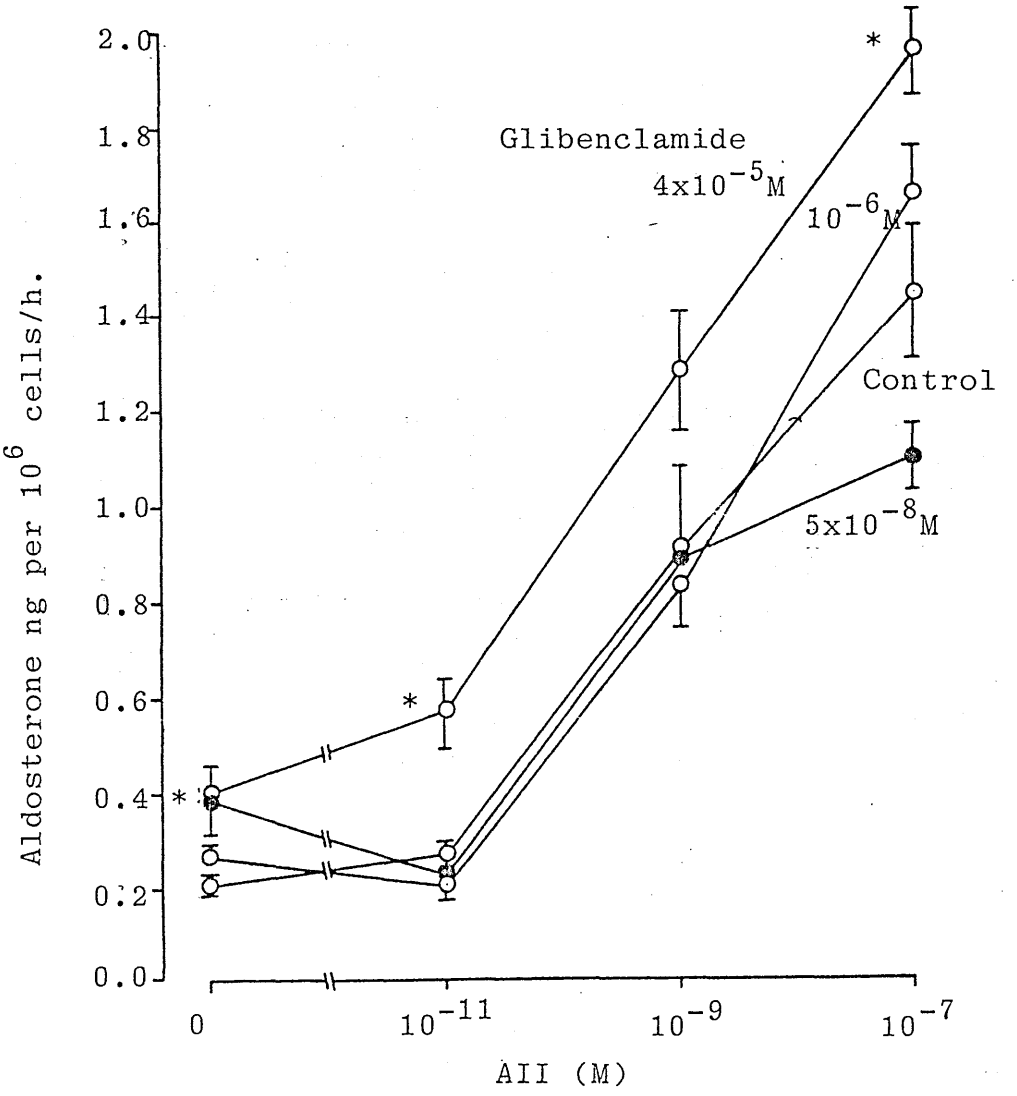


Figure 5.8. The effect of glibenclamide on basal and AII-stimulated aldosterone synthesis. Aldosterone as ng per 10^6 cells/h is shown for increasing concentrations of AII at different doses of glibenclamide.
* $p<0.05$, ** $p<0.01$ with respect to controls. N=6
± SEM.

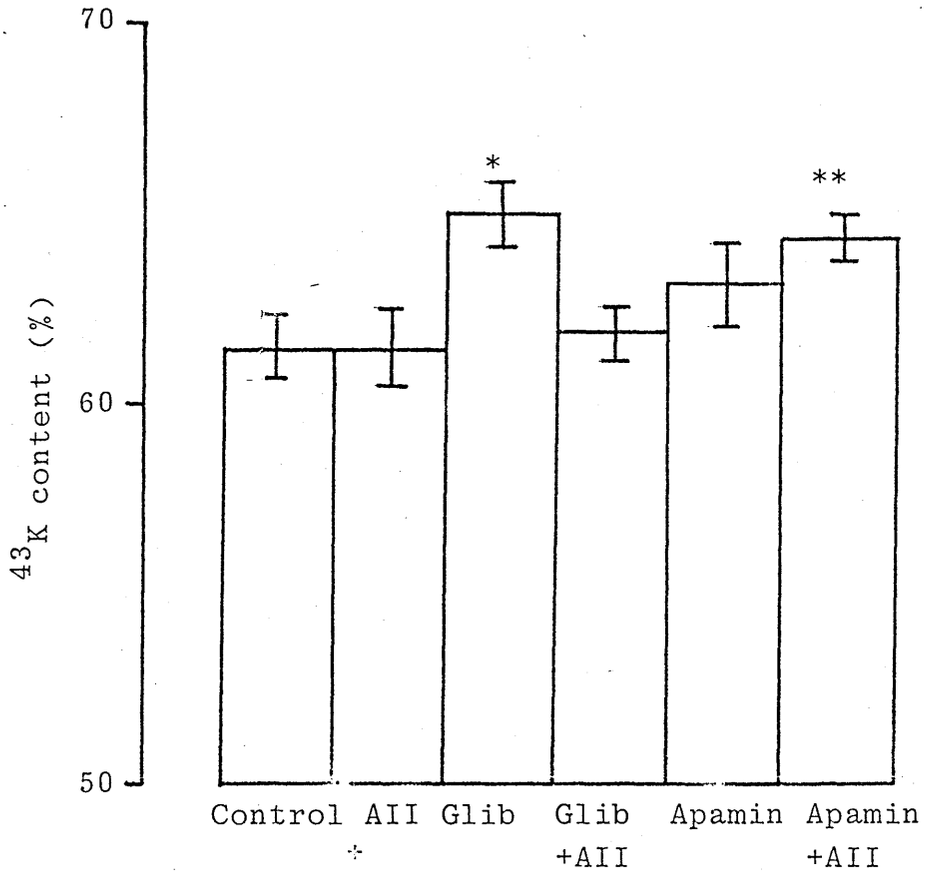


Figure 5.9. Effect of apamin and glibenclamide on the early ^{43}K efflux. Cell ^{43}K content after a 10 min incubation is expressed as a % of the initial value. Incubations contained medium with or without AII (10^{-6}M) ± apamin (10^{-7}M) or glibenclamide (4×10^{-5}M). * $p<0.05$, ** $p<0.01$ with respect to controls (paired t-test). N=6 ± SEM.

initial values for cells incubated for 10 min in the presence and absence of AII (10^{-6} M) with or without apamin (10^{-7} M) or glibenclamide ($4x10^{-5}$ M). Neither AII, apamin alone nor glibenclamide in the presence of AII altered ^{43}K loss. However, significant decreases in ^{43}K loss were seen with glibenclamide alone ($p<0.02$) and apamin in the presence of AII ($p<0.01$).

5.3.4. Effects of apamin on ^{43}K efflux over 40 min.

A lower dose of AII (10^{-8} M), than the 10^{-6} M used in the previous experiment was employed since the latter is a supramaximal dose and any additive effect produced by apamin or glibenclamide to that of AII would not therefore be apparent.

The effect of apamin (10^{-7} M) on ^{43}K efflux in control and AII-stimulated conditions was tested over 40 min. Figure 5.10 shows ^{43}K content for cells as a percentage of initial control values as 20 and 40 min. Neither AII nor apamin alone significantly reduced ^{43}K loss but together AII with apamin did ($p<0.05$). However, at 40 min ^{43}K efflux was reduced significantly by apamin ($p<0.05$) and more so by AII ($p<0.001$). Together, AII plus apamin produced a greater reduction than AII alone ($p=0.02$).

5.3.5. Effect of glibenclamide on ^{43}K efflux over 40 min.

Cells were incubated in the presence or absence of AII (10^{-8} M) with or without glibenclamide ($4x10^{-5}$ M). Figure 5.11 shows ^{43}K content as a % of initial values at 20 and 40 min. At 20 min AII alone had no

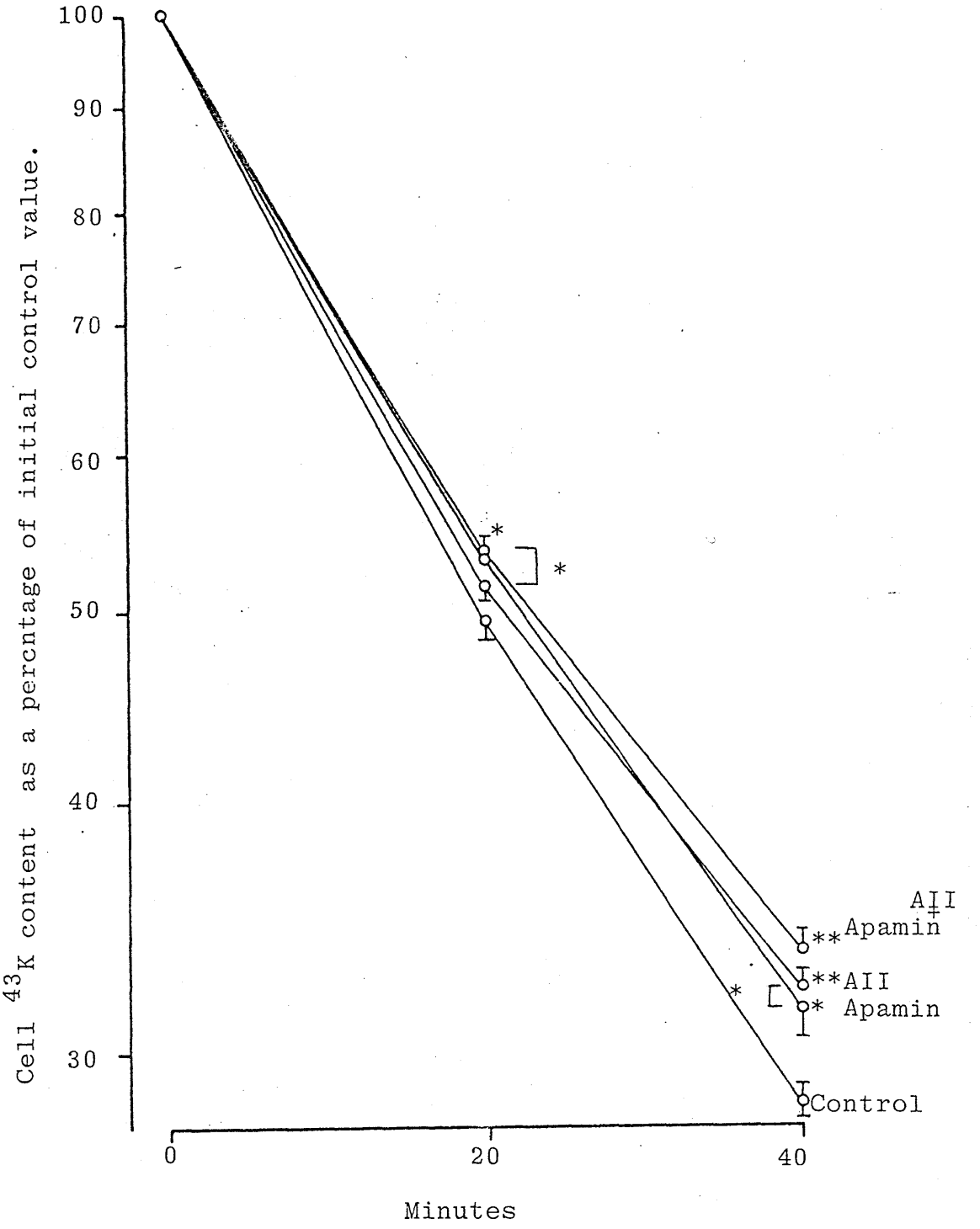


Figure 5.10. Effect of apamin on ^{43}K efflux over 40 min. Cell ^{43}K content is expressed as a % of the initial control values and plotted on a log scale aganist time. Incubations contained medium ± apamin (10^{-7}M) with or without AII (10^{-8}M).
* $p<0.05$, ** $p<0.001$ with respect to controls (paired t-test). N=6 ± SEM.

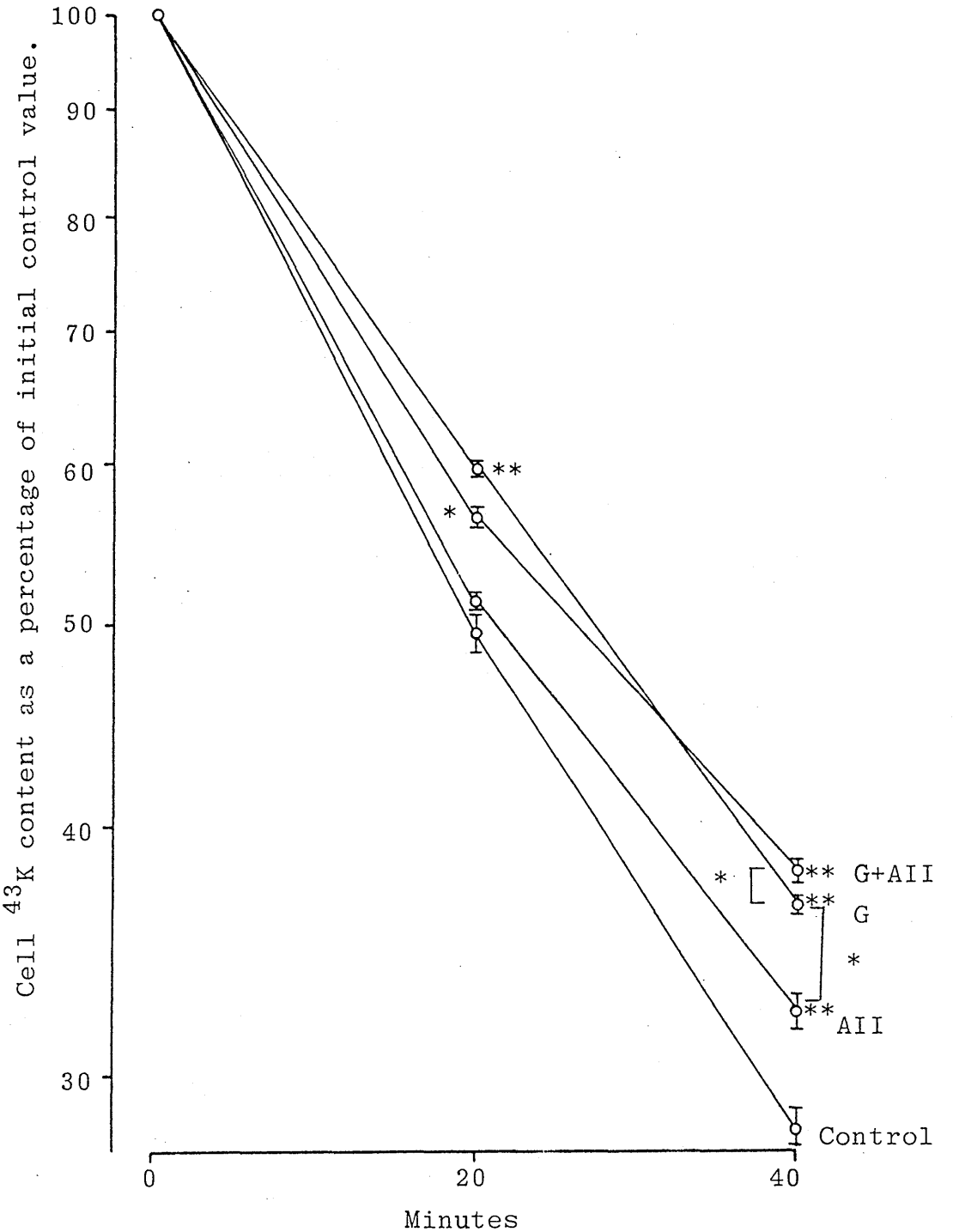


Figure 5.11. Effect of glibenclamide on ^{43}K efflux over 40 min. Cell ^{43}K content is expressed as a % of the initial control values and plotted on a log scale against time. Incubations contained medium ± glibenclamide ($4x10^{-5}M$) with or without AII ($10^{-8}M$). * $p<0.05$, ** $p<0.001$ with respect to controls (paired t-test).

significant effect but together with glibenclamide AII decreased ^{43}K loss ($p<0.005$) and glibenclamide alone did to a greater extent ($p<0.001$) although the difference between the two conditions was not significant. At 40 min AII alone did reduce ^{43}K loss ($p<0.001$). Glibenclamide alone had a greater effect which was significant compared with AII alone ($p=0.02$), but not compared with glibenclamide plus AII.

5.3.6. Effect of TEA on basal and AII-stimulated aldosterone release.

Figure 5.12 shows the dose response curves for cells treated with increasing concentrations of TEA in the absence or presence of a range of AII doses. In control cells, all concentrations of this K^+ channel blocker decreased aldosterone production. In stimulated cells, where TEA had an effect, it inhibited the steroid output. The greatest effects were seen with the intermediate concentration of $20x10^{-3}$ M TEA.

5.3.7. Effect of TEA on ^{43}K efflux.

No significant effects of TEA ($20x10^{-3}$ M) on ^{43}K efflux were seen in either basal conditions or cells treated with 10^{-8} M AII at 20 min. The efflux rates ($x10^3$) were; control: 10.8±0.2 vs. TEA: 11.4±0.2 and AII: 11.2±0.2 vs AII+TEA: 11.8±0.3 log % min^{-1}. However, TEA increased ^{43}K efflux by 20±2% in AII-treated cells at 40 min even though it had no effect on basal ^{43}K efflux. The efflux rates ($x10^3$) were; control: 7.9±0.3 vs. TEA: 8.0±0.2 and AII: 9.2±0.2 vs. AII+TEA: 11.0±0.3 log % min^{-1} ($p<0.05$).

5.3.8. 18ß Hydroxylation is affected by 4-AP.

In addition to its action on K^+ channels, 4-AP is known to have a direct inhibitory effect on steroid 11ß

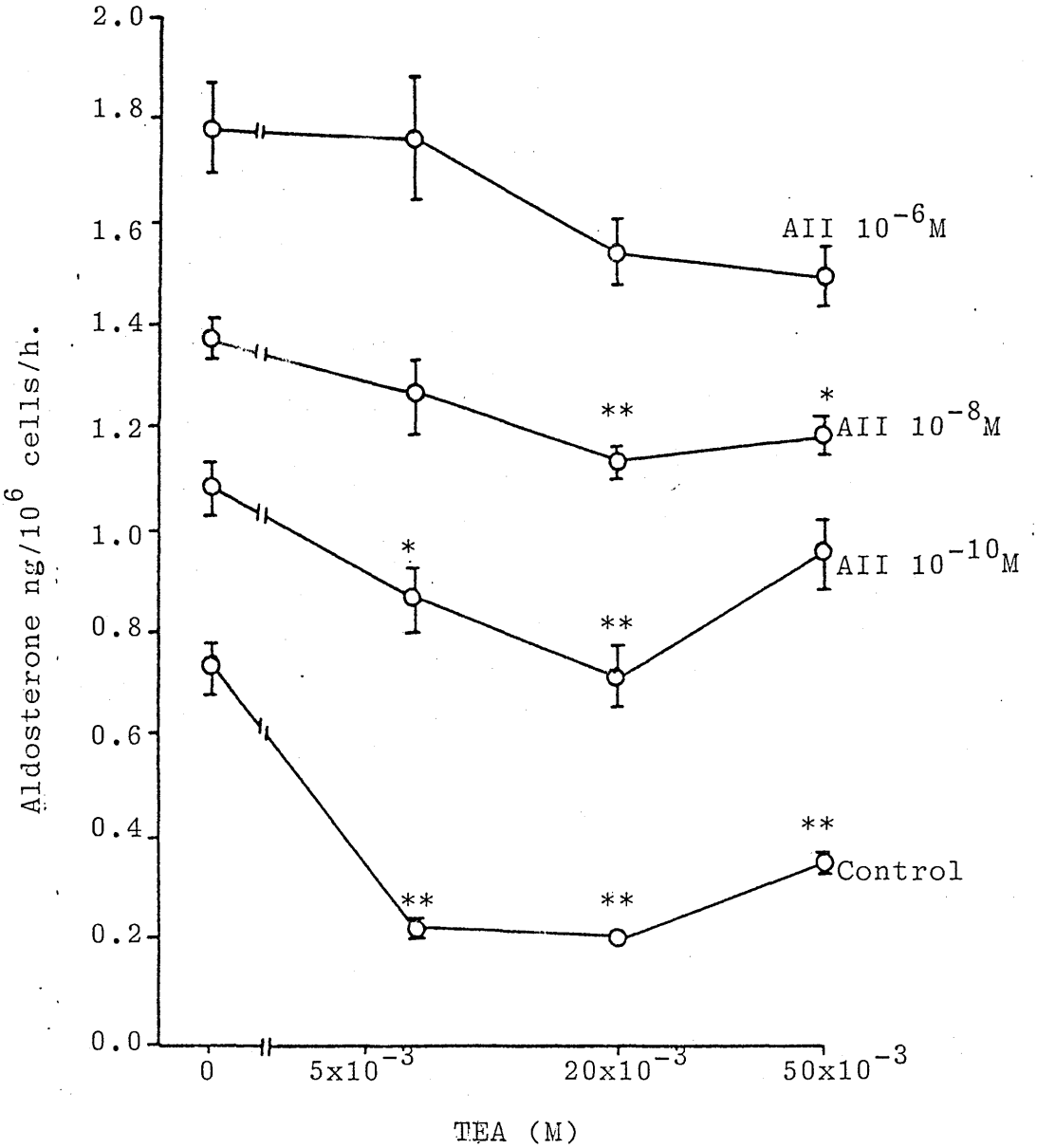


Figure 5.12. Aldosterone production dose responses to TEA in the presence and asence of AII. Aldosterone production as ng per 10^6 cells/h is shown for increasing concentrations of TEA at various doses of AII.

* $p<0.05$, ** $p<0.01$ with respect to relevant controls. N=6 ± SEM.

hydroxylation, preventing the formation of cortisol and corticosterone in bovine ZF cells (Fraser et al 1986). Since aldosterone is an 11ß hydroxy steroid, its synthesis should also be inhibited. To avoid this, cells were incubated in the presence of trilostane (cyanotrimethylandrostenolone), an inhibitor of 3ß hydroxy steroid dehydrogenase and the steroid isomerase to prevent endogenous corticosterone synthesis. The effect of 4-AP ($4x10^{-3}$ M) on the, now isolated, late aldosterone pathway was then evaluated by studying the conversion of added corticosterone (10^{-6} M) to aldosterone in the presence of AII (10^{-6} M). The 4-AP reduced aldosterone output compared to the control 1.02 ±0.06 and 1.50±0.09 ng/10^6 cells/h respectively; $p<0.01$). This shows that C_{18} hydroxylation was also inhibited by 4-AP, therefore no further experiments were performed using this agent.

5.4. DISCUSSION.

The results show that manipulations of ZG cell membrane permeability to K^+ do indeed affect steroidogenesis although the relationship between the two effects does not seem straightforward. From the results obtained in chapter 4, decreasing this permeability would be expected to increase aldosterone production and increasing it during the first minutes of incubation would be expected to decrease it.

5.4.1. Valinomycin.

Val, at 10^{-6} M reduced aldosterone release to basal control levels, irrespective of the dose of AII (figure 5.1) and at 10^{-7} M, Val greatly inhibited the aldosterone response to 10^{-7} and 10^{-9} M AII. This would be the expected response to the ionophore which raises cell membrane permeability to K^+ (figure 5.6) to cause hyperpolarisation (Pressman 1976, Dise and Goodman 1986), and thereby prevent the depolarisation associated with AII-induced steroidogenesis (Matthews 1967, Quinn et al 1987b). From figure 5.6 it can be seen that 10^{-7} M Val, although greatly increasing the ^{43}K efflux rate in control cells, does so to a lesser extent in AII-stimulated cells. This means that although the overall cell membrane permeability to K^+ is much enhanced, the AII is still able to cause a reduction in K^+ permeability presumably via some hormone/receptor activation of a specific K^+ channel. This is sufficient to mediate some increase in aldosterone production above basal levels although it is much less than that seen in conditions without Val (figure 5.1). The apparent irreversibility of Val effects at 10^{-7} M (figure 5.3) may be due to a longer period of recovery being required to re-establish the normal K^+ gradient across the cell membrane. ZG cells stored overnight at 4°C have intracellular K^+ and Na^+ values close to extracellular values due to inactivation of the Na^+/K^+ ATPase. When subsequently incubated at 37°C the cells take 3 h to raise $[K^+]_i$ to only 60 mM and decrease $[Na]_i$ to 30 mM (T. Goodfriend, personal communication).

What is surprising is that low concentrations of Val (10^{-9} and 10^{-8} M), have highly significant ($p<0.01$) stimulatory effects on aldosterone release basally and at all concentrations of AII tested. Only at 10^{-7} M Val was this reversed. Further, 10^{-9} M Val had no effect on ^{43}K efflux in either the control or AII-stimulated cells (figure 5.6). This could be explained by observations made in lymphocytes by Felber and Brand (1982). They showed that the same concentration of Val, (10^{-9} M), partially depolarised the mitochondria and that 10^{-7} M Val depolarised them further. Since the mitochondria of ZG cells are the site of several steps in the biosynthesis of aldosterone, Val at low doses may stimulate steroidogenesis by altering the mitochondrial membrane permeability to K^+ and not that of the plasma membrane.

Other effects of Val noted in cells other than the ZG could also affect steroidogenesis. Cellular $[ATP]_i$ decreases at 10^{-7} M Val in lymphocytes (Negendank and Shaller 1982) and Val also produces a dose-dependent inhibition of glucose-stimulated insulin release in pancreatic ß cells in addition to depleting $[ATP]_i$ (Henquin and Meissner 1978). These investigators suggest that the $[ATP]_i$ fall is caused by the increased mitochondrial membrane permeability to K^+ such that the K^+/H^+ exchange is increased. This in turn would decrease the H^+ gradient necessary for oxidative phosphorylation. The reduced availability of ATP would in turn also affect both K^+ and Ca^{2+} movements. On the one hand it would allow a rise in $[Ca^{2+}]_i$ due to decreased active sequestration into intracellular

stores and less active extrusion of the cation from the cell. This would lead to opening of Ca^{2+}-dependent K^+ channels. On the other hand, falls in $[ATP]_i$ would not only leave open the ATP-dependent K^+ channels (reviewed by Petersen et al 1986) as normal, if present, but also decrease the Na^+/K^+ATPase mediated influx of K^+ and efflux of Na^+. Although opening either the Ca^{2+}- or ATP-dependent K^+ channels would tend to hyperpolarise the cell membrane and presumably inhibit or attenuate aldosterone production, raised $[Ca^{2+}]_i$ would increase steroidogenesis (see introduction). It is difficult to judge the relative importance of indirect rises in K^+ efflux as opposed to enhanced $[Ca^{2+}]_i$, but since the low concentrations of Val raised aldosterone production and had no effect on ^{43}K efflux, its presumed effects on $[Ca^{2+}]_i$ seem to be a plausible explanation of the increased aldosterone output. Whether the enhanced Na^+-Na^+ exchange noted in Val-treated lymphocytes (Negendank and Shaller 1982), has any relevance to the Val-induced effects on aldosterone production (figure 5.1) is not known.

Another type of effect shown by Val is promotion of oleic acid incorporation into red cell membrane phospholipids, mainly diacylphosphatidyl ethanolamine (Dise and Goodman 1985). This effect of Val can be separated from the increases in K^+ efflux. In rat liver, Val increases the transfer of microsomal phosphatidyl-inositol to liposomes (Patumraj and Slaby 1982) although this occurred only at concentrations 1-2 orders of magnitude higher than that required to increase membrane permeability. Since phosphatidyl

inositol and phoshatidylethanloamine are, between them, involved in the production of IP3 and arachidonic acid formation, both of which are promoted in AII-stimulated cells (see introduction), alterations in the metabolism of these compounds could be another means whereby Val affects ZG cells.

5.4.2. BRL 34915.

Unlike Val, BRL 34915 had no significant effect on AII-stimulated aldosterone output (figure 5.2), except at the highest concentration of the drug tested ($4x10^{-5}$ M), where it caused significant inhibition ($p<0.01$) of the response to the higher doses of AII. However, basal aldosterone release rose slightly at 10^{-7} M BRL 34915 and this increase was significant ($p<0.05$), compared to the controls, at $2x10^{-6}$ M. Higher doses of BRL 34915 were inhibitory, returning aldosterone release to basal levels. Interestingly, the inhibition of aldosterone release by BRL 34915 was not as profound as that with Val. This may be due to the fact that, unlike Val, the effects of BRL 34915 are limited to a finite number of specific channels (Weir and Weston 1986a and b).

Using the highest concentration of BRL 34915 ($4x10^{-5}$ M), no significant change in ^{43}K efflux was observed in either the 45-85 min or the 0-10 min period (figure 5.4a and b). In the experiments described previously, AII (10^{-6} M) produced a significant increase in the ^{43}K efflux rate in the later period and decreased this parameter in the earlier period (figures 4.6a and 4.6b respectively).

Since 10^{-6} M BRL 34915 stimulated basal

aldosterone release and $4x10^{-5}$ M inhibited AII-stimulated aldosterone production, the effects of these concentrations on ^{43}K efflux were compared over 10 min incubations in the presence of ouabain (figure 5.5). AII 10^{-6} M substantially decreased ^{43}K efflux ($p<0.01$) with respect to controls. BRL 34915 10^{-6} M, produced a slight but non-significant fall in ^{43}K efflux which had no further effect on the AII-induced decrease in this parameter. These findings correspond to the aldosterone results in that this dose of BRL 34915 stimulates basal aldosterone output (even though the reduction in ^{43}K efflux is slight) and it has no effect on AII-stimulated aldosterone output and therefore produces no alteration in ^{43}K efflux. The higher dose of BRL 34915 slightly increased ^{43}K efflux from control cells but had no effect on the AII-treated cells. A rise in ^{43}K efflux would have been expected with AII present to account for the fall in aldosterone production. However, the mismatch between the rise in ^{43}K efflux and no change in basal aldosterone output may indicate that basal steroidogenesis, although sensitive to decreases in K^+ outflow is less affected by increases in K^+ permeability as noted above for basal levels in the presence of 10^{-6} M Val (figure 5.1).

These correlations of weak stimulatory and inhibitory effects of BRL 34915 on aldosterone production with reductions and increases in ^{43}K efflux rate show that this drug does affect ZG cells, albeit slightly. As with the effects of Val, the reasons for any stimulatory effects on aldosterone production are

not clear.

Despite the action of BRL 34915 being confined to specific channels (as opposed to increasing cell membrane permeability to K^+ in general), the type(s) of K^+ channel(s) it affects is (are) only now being identified. As reviewed by Hamilton and Weston (1989), electrophysiological studies have shown that BRL 34915 opens a large conductance Ca^{2+}-dependent K^+ channel in cultured rabbit aortic cells but in the portal vein cells of the same species, the drug opens a K^+ channel which is independent of changes in either voltage or $[Ca^{2+}]_i$. The K^+ efflux from this latter channel are antagonised most potently by a toxin from the scorpion Leiurus quinquestriatus (Quast and Cook 1988). A third channel type on which BRL 34915 acts is the ATP-dependent K^+ channel in cardiac myocytes (Escande et al 1988), which has also been found in isolated rat portal veins (Winquist et al 1988). Differences in responsiveness to BRL 34915 amongst tissues may therefore depend on the distribution and recruitment of different K^+ channels. So far, the properties common to channels affected by BRL 34915 are their insensitivity to apamin (see above) and the absence of the involvement of a pertussis toxin-sensitive G protein (Quast et al 1988).

Higher concentrations of BRL 34915 were required in the current experiments to inhibit aldosterone release than are required to cause 100% relaxation in guinea pig taenia caeci (Weir and Weston 1986a). This probably indicates that ZG cells, unlike smooth muscle cells, do not have significant numbers of BRL

34815-sensitive K^+ channels. A similar conclusion was reached by Williams et al (1989), who found that 10^{-5} M BRL 34915 significantly inhibited AII-stimulated aldosterone release in rat ZG cells. The ATP-dependent K^+ channel in cardiac myocytes (Escande et al 1988), seems to require a higher concentration of BRL 34915 to open it, (10^{-4} M). A higher dose of the drug should perhaps have been tested in the current study, to determine whether these channels are important in aldosterone release. However, the BRL 34915-sensitive K^+ channels all display insensitivity to apamin (Weir and Weston 1986b, Cook and Hof 1988). Studies using this venom on superfused ZG cells have shown that AII-induced rises in ^{86}Rb efflux are reduced by apamin (Lobo and Marusic 1986). Hence ZG cells are probably not sensitive to BRL 34915.

5.4.3. Apamin and Glibenclamide.

Apamin slightly increased basal and AII-stimulated aldosterone production (figure 5.7) but its effects on the latter appeared to be maximal at 10^{-7} M with higher and lower doses having relatively little effect. The highest dose of glibenclamide tested ($4x10^{-5}$ M), also shifted the dose-response curve to AII to the left significantly (figure 5.8). Hence both agents can enhance aldosterone output although ^{43}K efflux experiments suggest that they achieve this by different methods. After 10 min incubation, (figure 5.9), apamin had no effect on ^{43}K efflux but in the presence of AII (10^{-6} M), it reduced ^{43}K loss significantly. AII alone, in this instance, had no effect compared with control

^{43}K loss. However, in chapter 4, AII was shown to have opposing effects on two separate components of ^{43}K efflux during the early minutes of stimulation. Therefore, in this experiment the two opposing effects may have been balanced at 10 min. That apamin significantly reduced ^{43}K efflux only in the presence of AII implies that it closes the channels governing the slow exchange component which have been opened by AII. This results in a net reduction of ^{43}K loss since the faster-exchanging efflux is also inhibited by AII. Under unstimulated conditions apamin has no significant effect because fewer of the slow-exchange component channels are open. On the other hand, glibenclamide must therefore close channels governing the faster-exchanging ^{43}K component. In combination with AII, enhanced efflux from the slow exchange component balances the reduced fast exchange ^{43}K loss.

Over 40 min (figures 5.10 and 5.11), AII at the lower dose of 10^{-8} M is likely to exert smaller effects on both efflux components than 10^{-6} M and so greater effects with apamin and glibenclamide would be expected than with AII alone. At 20 min neither AII nor apamin (figure 5.10) altered ^{43}K loss significantly but in combination they did ($p<0.03$). At 40 min, AII and apamin alone both reduced ^{43}K loss and AII plus apamin reduced ^{43}K efflux more than AII alone. Assuming that the dose of apamin is maximally effective, the difference in the ^{43}K efflux between control and apamin treated incubations should indicate the contribution of the slow exchange component to control net efflux. Likewise, the difference between the loss of ^{43}K in

cells treated with AII plus apamin for AII-treated cells is greater in the latter case, as expected.

These observations suggest that the slower-exchange efflux component is governed by Ca^{2+}-dependent K^+ channels enhances which are open to a lesser extent under unstimulated conditions than in the presence of AII. Their closure by apamin enhances basal aldosterone release probably by slightly depolarising the cell. AII stimulation opens these channels more, presumably via rises in $[Ca^{2+}]_i$, and the enhanced K^+ efflux tends to repolarise the cell membrane. Blocking these channels during AII stimulation would prolong depolarisation and thereby raise $[Ca^{2+}]_i$ further via the voltage-dependent Ca^{2+} influx. The aldosterone production stimulated by AII in the presence of the highest dose of apamin was not as large as that seen for 10^{-7} M apamin (figure 5.7). In this case, the higher $[Ca^{2+}]_i$ presumably caused by the actions of AII plus apamin may be sufficient to compete with and partially overcome the apamin block. The resulting increase in K^+ efflux would curtail the depolarisation and reduce Ca^{2+} influx. Experiments to monitor the effects of this high (10^{-6} M) concentration of apamin on ^{43}K efflux in AII-stimulated cells would shed further light on this theory.

These results on the action of apamin are contrary to those of Lobo and Marusic (1986) who showed that 10^{-7} M apamin did not affect basal but significantly decreased 10^{-8} M AII-stimulated aldosterone release. Lobo and Marusic explain their apamin-induced fall in AII-stimulated aldosterone output in terms of it

abolishing the transient increase in K^+ permeability noted on addition of AII to superfused cells (Lobo and Marusic 1986). This agreed with their earlier findings of a brief hyperpolarisation preceding depolarisation in electro-physiological studies in AII-treated ZG cells (Foster et al 1982). A similar hyperpolarisation has also been noted by Quinn et al (1987b) but, as discussed in chapter 4, the methods used in this study are incapable of measuring such a short-lived event and further, evidence has been presented that ^{43}K efflux from superfused cells may reflect events from only one pool of K^+, a faster-exchanging one having been exhausted. The fact that a stimulation of aldosterone secretion is seen with AII means that $[Ca^{2+}]_i$ has increased; therefore it should remain elevated for some time in the presence of apamin due to the blockade of the Ca^{2+}-dependent K^+ channels which usually repolarise the cell membrane. Other routes of Ca^{2+} extrusion will then reduce $[Ca^{2+}]_i$. However, Lobo and Marusic's decrease in stimulated aldosterone output with apamin does not fit with this proposed sequence of events. No reports have been made of apamin having other effects on cell metabolism which could explain these findings.

Glibenclamide at 20 and 40 min (figure 5.11) showed a highly significant reduction in ^{43}K loss with respect to both controls and AII. The difference in cell ^{43}K content at 20 and 40 min appeared to be equal between control and glibenclamide-treated incubations. Assuming this dose of glibenclamide to be maximally effective, these differences would indicate the size of

the contribution of the fast exchange component on basal ^{43}K efflux. At 20 min, glibenclamide produced a highly significant fall in ^{43}K loss whereas AII had no significant effect alone and, in combination with glibenclamide, did not significantly alter its effect. However, at 40 min, when AII did significantly reduce ^{43}K loss, AII further reduced the greater effect of glibenclamide ($p<0.01$). The greater reduction with glibenclamide than with AII alone could be explained by its bigger reduction of fast exchange ^{43}K efflux without, presumably, the enhanced isotope loss from the slow exchange component to offset this. However, one would expect glibenclamide plus AII to have had a smaller effect on reducing ^{43}K due to this slower efflux but this was not the case; a greater reduction was seen. Perhaps, in the longer-term, depolarisation caused by glibenclamide is eventually compensated by some separate repolarisation event.

From these results, it appears that glibenclamide blocks ^{43}K efflux from the fast exchange component and hence ATP-dependent channel blockade may have a role in AII stimulation of ZG cells. However, the dose required to raise aldosterone output significantly was 10 times higher than the concentration required for maximal stimulation of insulin release from isolated rat ß cells (Grill and Cerasi 1978) and several orders of magnitude higher than the nM range over which it closes ATP-dependent channels in insulinoma cells (Schmid-Antomarchi et al 1987). Therefore ZG cells may be less sensitive than ß cells to inhibition of these channels.

The way in which glibenclamide and apamin

separately and specifically block only fast or slow efflux components respectively, contrasts with the effects of AII. It implies that the dual effects of AII are not tightly linked. Depolarisation of the cell membrane in response to AII may, via voltage-sensitive Ca^{2+} channels, cause Ca^{2+}-sensitive K^+ channels to open. In addition, AII may increase $[Ca^{2+}]_i$ through other channels. Cohen et al (1988) have reported the existence of both "T" and "L" type Ca^{2+} channels in ZG cells and suggest that AII opens the former preferentially. Glibenclamide is unlikely to mimic AII events which are not dependent on membrane potential.

5.4.4. 4-Aminopyridine.

It is known that 4-AP, in the mM range, inhibits corticosterone (Lymangrover and Martin 1981) and cortisol (Fraser et al 1986) production. Inhibition of 11ß hydroxylation has been suggested as the cause of the rises in 11-deoxycorticosterone which occur in parallel to the fall in the former steroids. It has been suggested that 4-AP may act directly as an inhibitor of cytochrome P450-containing enzymes, (Fraser et al 1986).

Experiments to measure aldosterone conversion from added corticosterone were therefore carried out. Under these conditions, 4-AP and AII together produced a marked fall in aldosterone output compared with AII alone implying an antagonism by 4-AP on the AII-mediated stimulation of the late pathway. From these results it appears that, if the inhibitory action of 4-AP on cytochrome $P\text{-}450_{11\beta}$ can be bypassed, this K^+

channel blocker also antagonises C_{18} oxidation.

Results different from those predicted for a K^+ channel blocker have been noted previously in pancreatic ß cells where ^{86}Rb fluxes and insulin release were increased and decreased respectively (Boschero et al 1987). They found that doses of 4-AP at, or below 1mM had no effect on ^{86}Rb efflux but at 10 mM 4-AP it was significantly enhanced.

Glucose-stimulated insulin release was also inhibited by this concentration of 4-AP although at low glucose levels (6 mM), 4-AP transiently raised insulin release in perfused ß cells. This concentration of 4-AP also significantly reduced glucose oxidation in cells incubated with high (16.7 mM) glucose. Since this enhancement of ^{86}Rb efflux occured at the level of 4-AP which reduces glucose metabolism and therefore ATP production, the effect is probably one of opening ATP-dependent K^+ channels and hence the membrane hyperpolarises and insulin release falls.

5.4.5. TEA.

As with 4-AP, TEA was expected to block K^+ channels, depolarise the cell membrane and increase aldosterone output. Figure 5.12 shows that all concentrations of TEA tested profoundly inhibited basal aldosterone release. This action was antagonised by increasing concentrations of AII such that at 10^{-6} M AII, the fall in aldosterone output was not significant.

TEA had no significant effect ^{43}K efflux in either basal or AII-stimulated cells over the 0-20 min period

of incubation. However, there was an increase in the ^{43}K efflux rate over 20-40 min of 20±2% in the presence of AII (10^{-8} M), (results not shown). The TEA-induced decrease in aldosterone production in AII-treated cells fits with the rise in ^{43}K efflux but no rise in ^{43}K efflux was observed to account for the fall in basal aldosterone production.

The observed rise in ^{43}K efflux in AII treated cells are in contrast to the results of Lobo and Marusic (1988), who, using superfused cells, showed increased ^{86}Rb efflux on AII stimulation could be inhibited with TEA (10 mM). Further experiments using perfusion or examining longer time course static incubations where the "later" phase K^+ efflux is enhanced in the presence of AII, would be required to study this.

From the results of Payet et al (1987), both TEA and 4-AP have been shown to reduce an outward K^+ current in ZG cells which is also blocked by ACTH. Recent experiments by this group have shown that TEA produces a dose-dependent increase in aldosterone production in rat ZG cells which reached a plateau 2.5 times higher than basal levels at concentrations of TEA above 12.5mM (Gallo-Payet and Payet 1989). This is opposite to the current findings where 5-20 mM TEA produced a progressive fall in aldosterone output.

The discrepancy between the aldosterone outputs obtained with TEA in my experiments and those of Gallo-Payet and Payet (1989) may be due to osmotic effects since these workers ensured that osmolality was kept constant and I made no such compensation.

Schneider et al (1984) showed that aldosterone production in the isolated, perfused canine adrenal could be decreased by changes in the perfusate NaCl concentration of only 3-7 mM. These effects depended on the changes in osmolality and on not the nature of the ionic species involved (Schneider et al 1985), since addition of similar doses of glucose, sucrose or mannose had the same effect. AII- or K^+- stimulated aldosterone synthesis was much more sensitive to these effects than basal or ACTH-induced aldosterone production (Taylor et al 1987). Neither cortisol nor the late path of aldosterone synthesis were affected. Obviously, these findings provide only a part explanation for the current results but may explain the reduction in aldosterone synthesis in the AII-stimulated cells. However, the inhibition of basal aldosterone production together with the slight reversal of this reduction with the highest dose of TEA (figure 5.12) remains to be accounted for. Possibly, this highest dose of TEA inhibits K^+ efflux and this partly antagonises the osmotic effects. Also, isolated cells may be more prone to basal inhibition than intact glands.

5.5. SUMMARY.

This series of experiments set out to determine whether manipulation of the ZG cell membrane permeability to K^+ affected aldosterone production. Since depolarisation is associated with stimulation of steroidogenesis (Matthews 1967), and the resting

membrane potential is dependent on K^+ (Quinn et al 1987a), various agents which alter K^+ movements across the cell membrane were tested for their effects on the release of this steroid. ^{43}K efflux rates were used as an indication of how membrane permeability to K^+ was affected.

Increasing the cell membrane permeability was attempted using an ionophore (Val) and a K^+ channel opener (BRL 34915). Val, at concentrations which raised the ^{43}K efflux rate, inhibited stimulated aldosterone release but did not affect basal steroid production. BRL 34915 inhibited aldosterone production but at concentrations higher than those needed to relax some smooth muscle preparations. It had minimal effects on ^{43}K efflux hence BRL 34915-sensitive channels may not be numerous in ZG cells.

Reductions in transmembrane K^+ flux were attempted with inhibitors of ATP-dependent channels (glibenclamide), Ca^{2+}-dependent K^+ channels (apamin) and K^+ channels in general, by TEA.

Glibenclamide reduced ^{43}K loss and shifted the aldosterone dose-response curve to the left but at a dose which was higher than that required to stimulate insulin release in pancreatic ß cells.

Apamin stimulated aldosterone release in basal and AII-stimulated cells and also decreased the ^{43}K efflux rate. At the highest dose tested, the enhanced aldosterone release in stimulated cells fell. The ^{43}K efflux rate was not tested at this dose of apamin. Closure of Ca^{2+}-dependent K^+ channels may inhibit the "late" phase of enhanced K^+ efflux from the slower-

exchanging pool characterised in chapter 4 and promote increased steroidogenesis by a combination of prolonged depolarisation and increased $[Ca^{2+}]_i$. High apamin concentrations may raise $[Ca^{2+}]_i$ enough to compete with the apamin block of these channels and so decrease the stimulation of AII-induced aldosterone compared with lower apamin levels.

4-AP inhibits 11ß hydroxlation directly but with this block bypassed, it still inhibited AII-induced conversion of corticosterone to aldosterone and therefore blocks C_{18} oxidation too.

TEA inhibited basal and AII-stimulated aldosterone release although this was overcome by increasing doses of AII. It may exert this reduction via osmotic effects and, in the case of AII stimulation by raising ^{43}K efflux.

Overall, decreases in cell membrane permeability to K^+ were associated with increased aldosterone output and increases in permeability with a decrease in aldosterone output.

CHAPTER 6. EFFECT OF VOLTAGE-DEPENDENT Ca^{2+} ENTRY BLOCKADE AND INHIBITION OF INTRACELLULAR Ca^{2+} RELEASE ON ^{43}K EFFLUX.

6.1. INTRODUCTION.

AII stimulation of aldosterone production is associated with uptake and cycling of Ca^{2+} across the ZG plasma membrane and also its release from intracellular stores (see introduction). Hence the aim of the following experiments was to investigate the effect on ^{43}K efflux of agents which inhibit these aspects of Ca^{2+} movement. Influx of Ca^{2+} from the medium can be inhibited by blockers of voltage-dependent Ca^{2+} channels such as the dihydropyridines (e.g. nitrendipine) and the phenylalkylamines (e.g. verapamil) (reviewed by Miller and Struthers 1984, Godfraind et al 1986).

The agents used most widely to inhibit the release of Ca^{2+} from intracellular stores are TMB-8 (8-(N,N-diethylamino) octyl-3,4,5-trimethoxybenzoate) and dantrolene (1-(5-(p-nitrophenyl)-furfurylidene-amino) hydantoin sodium hydrate).

In the following experiments, verapamil, TMB-8 and dantrolene were used to investigate their effects on ^{43}K efflux and aldosterone release.

6.2. RESULTS.

6.2.2. Dose-related effects of verapamil on aldosterone release.

Concentrations of verapamil $2x10^{-5}$ M and above profoundly inhibited aldosterone release both in untreated and AII-stimulated cells ($p<0.01$, figure 6.1). However, the the highest dose of verapamil tested did not reduce stimulated aldosterone synthesis to basal levels.

6.2.2. Effects of verapamil on ^{43}K efflux in control and AII-stimulated cells.

Figure 6.2 shows the effect of verapamil ($2x10^{-5}$ M) on ^{43}K efflux over the first 10 min of incubation in the absence and presence of ouabain. In the absence of ouabain, AII 10^{-6} M, decreased the ^{43}K efflux rate ($p<0.05$) and verapamil caused a significantly greater decrease in ^{43}K efflux ($p<0.05$), which was further reduced when both AII and verapamil were present ($p<0.05$). A similar pattern was seen in the presence of ouabain with AII and verapamil together producing an additive effect.The effect of AII alone was significantly less than that of verapamil alone in both the presence and absence of ouabain.

6.2.3. Effects of verapamil on the late phase of ^{43}K efflux and aldosterone synthesis.

To try to isolate changes induced by verapamil on the slow exchange pool, incubations were set up in the absence or presence of AII (10^{-6} M). After 54 min,

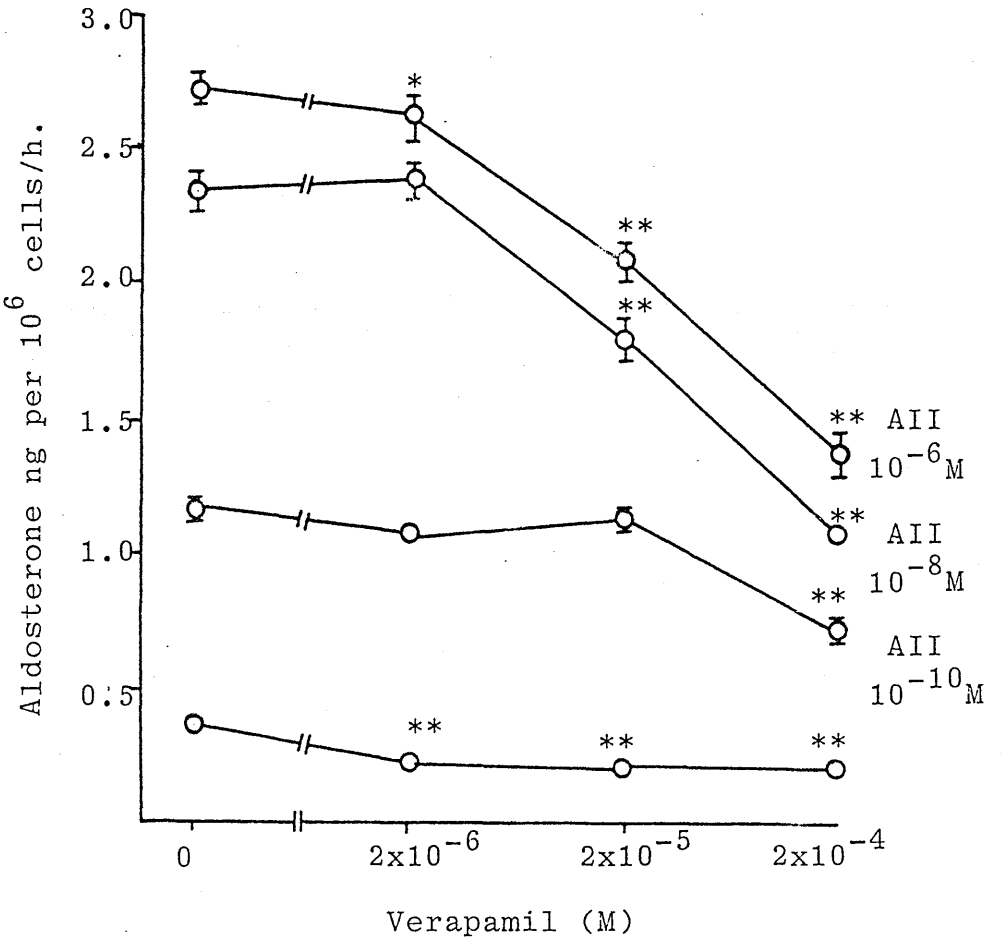


Figure 6.1. Dose response of aldosterone production to verapamil. Aldosterone production as ng per 10^6 cells/h is plotted against increasing concentrations of verapamil for different doses of AII.
* $p<.05$, ** $p<0.01$ with respect to relevant controls.
N=6 ± SEM.

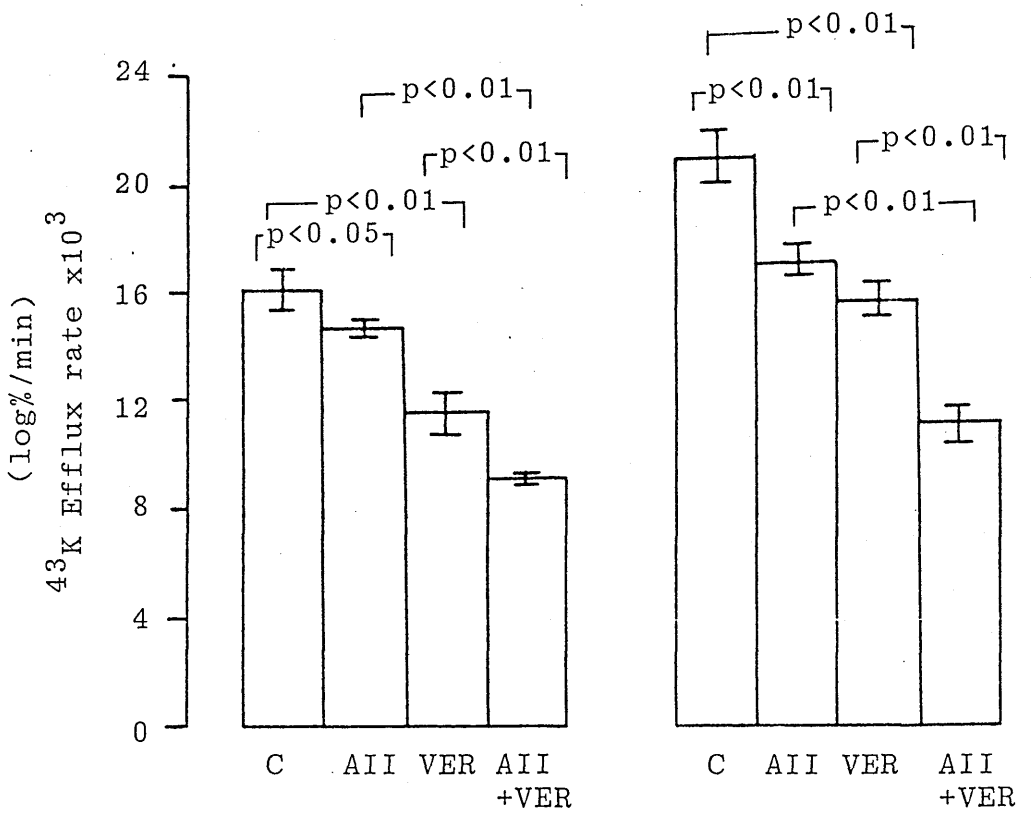


Figure 6.2. Effect of verapamil on the early ^{43}K efflux rate. ^{43}K Efflux rates are shown for cells incubated in the absence (left panel) and presence (right panel) of ouabain. Incubations were carried out in the presence or absence of AII (10^{-6}M) with or without verapamil (VER $2x10^{-5}$M). Efflux rates are for 10 min incubations. N=6 ± SEM.

identical volumes of medium ± AII and/or verapamil were then added to give final concentrations of 10^{-6} M and $2x10^{-5}$ M respectively. Table 6.1 shows the ^{43}K remaining in the cells as a % of the initial control value at t=0, 15, 55 and 85 min as well as the aldosterone produced over 85 min. Over the 0-15 min period AII significantly reduced ^{43}K efflux ($p<0.05$) but at 55 min the cell content of the isotope was identical for untreated and AII treated cells. The effects of AII when added at 54 min were different from the 10 min experiment shown in figure 6.2. Whereas AII inhibited efflux over 10 min, it had no effect when added alone but stimulated efflux when added with verapamil (as compared with verapamil alone; $p<0.02$). Efflux in the presence of verapamil, either with or without AII, was less than basal.

In cells treated throughout with AII, ^{43}K efflux was greater between 55 and 85 min compared with basal values and again verapamil tended to decrease efflux although the effect was not statistically significant.

Aldosterone synthesis was partly inhibited by verapamil only when AII and verapamil were added in combination at 54 min and not in cells stimulated by AII from t=0.

6.2.4. Time course of verapamil action on ^{43}K efflux.

Because of differences in the relative effects of AII and verapamil in short (10 min) and longer (30 min i.e. 55-85 min) incubations, a more detailed study of the time course of events was undertaken. As in the 10 min study, the effects of AII (10^{-6} M) and verapamil

TREATMENT 0-54 min		% ^{43}K		TREATMENT 54-85 min	% ^{43}K	ALDOSTERONE 0-85 min ng/10^6 cells/h
	t=0	t=15	t=55		t=85	
CONTROL	100%	73.1±1.2	38.7±0.5	BASAL	24.3±0.2	1.98±0.06
				AII	24.4±0.4	4.56±0.13 **
				VERAPAMIL	27.7±0.4 **	2.31±0.18 *
				AII + VERAPAMIL	26.0±0.5 *	3.27±0.43 *
AII	100%	76.5±0.7*	38.6±0.8	AII	22.7±0.4 *	7.90±0.46 **
				AII + VERAPAMIL	23.8±0.3	9.19±0.38 **

p<0.02 (AII vs AII + VERAPAMIL, basal period)

p<0.01 (AII vs VERAPAMIL, ALDOSTERONE 0-85 min)

p<0.08 (AII vs AII + VERAPAMIL, basal period)

p<0.06 (AII vs AII + VERAPAMIL, ALDOSTERONE 0-85 min)

Table 6.1. Effects of verapamil on the late phase of ^{43}K efflux and aldosterone synthesis. Cell ^{43}K content is shown as % of initial values at t=0 for samples taken at t=15, 55 and 85 min. Cells were treated with or without AII (10^{-6}M) in the absence or presence of verapamil ($2x10^{-5}$M) over the different periods as shown. Also shown is the aldosterone produced.
* p<0.05, ** p<0.001 with respect to basal conditions (unpaired t-test). N=6 ± SEM.

$2x10^{-5}$ M) were considered either alone or in combination. Cell ^{43}K content was measured at 3 min intervals over 21 min of incubation. Figure 6.3 shows that by 9 min, AII significantly decreases the loss of ^{43}K which was generally highly significant compared with AII alone, and together AII plus verapamil exhibited an additive effect by 3 min. Table 6.2 shows the corresponding significance values for figure 6.3.

Earlier experiments using ouabain-free conditions showed that a lower dose of AII (10^{-8} M), produced a more profound, but slower developing, inhibition of ^{43}K efflux (see figure 4.4). Therefore, in another experiment, cells were treated with or without verapamil in the absence or presence of AII 10^{-8} M. Samples were removed immediately prior to commencement of the incubations and at 20 and 40 min. Figure 6.4 shows that at 20 min AII produced no significant decrease in ^{43}K loss but verapamil did ($p<0.001$). There was a slightly greater effect of AII in combination with verapamil at this time. At 40 min, AII reduction of ^{43}K loss was highly significant ($p<0.001$) and the marked decrease in isotope loss by verapamil was further exaggerated. The greater effect of AII in combination with verapamil was also more pronounced but still not significant and therefore not additive.

6.2.5. Effects of TMB-8 on basal and AII-stimulated aldosterone production.

As shown in figure 6.5, increasing concentrations of TMB-8 had no effect on basal aldosterone release but at 10^{-5} M, significantly decreased stimulated output at

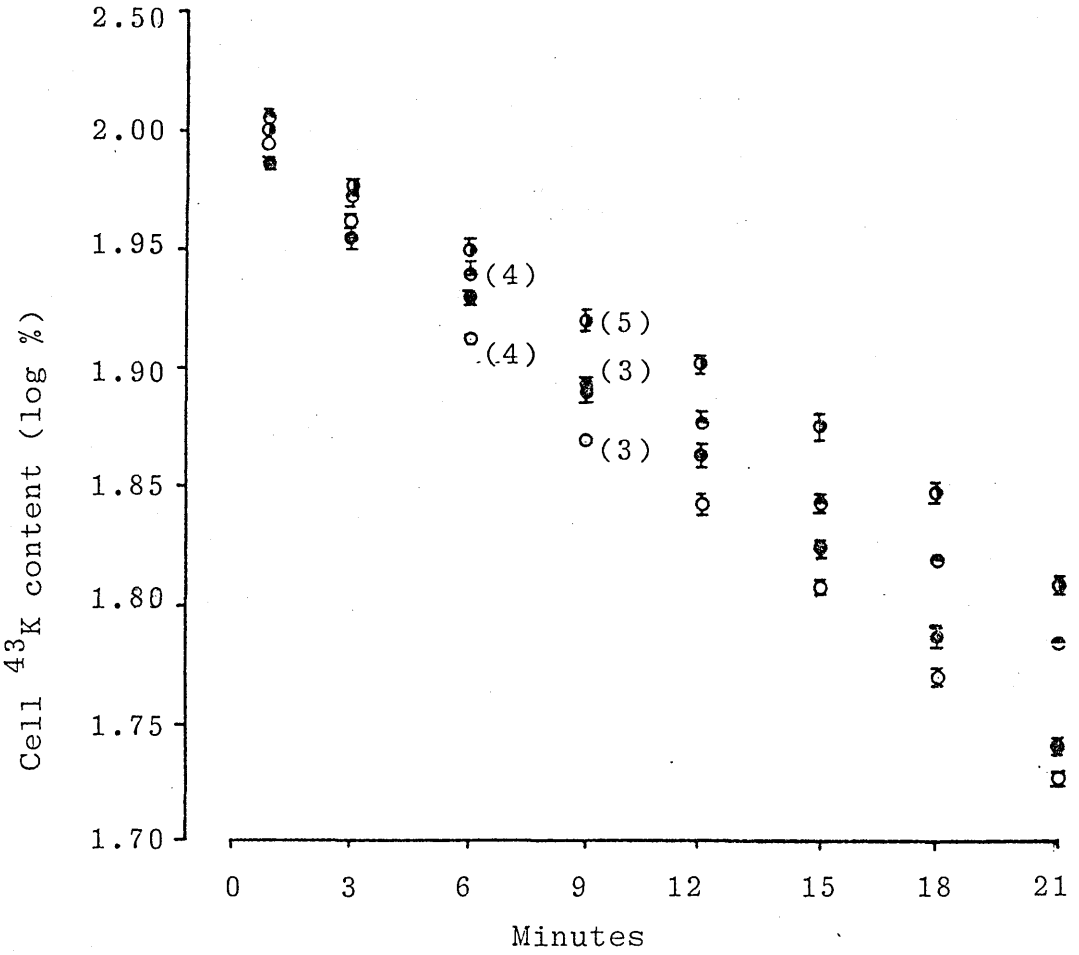


Figure 6.3. Effect of verapamil on ^{43}K efflux over 21 min. Cell ^{43}K content is plotted on a log scale with respect to initial values against time. (O) Control, (●) AII (10^{-6}M), (◓) verapamil ($2x10^{-5}$M) and (◑) AII plus verapamil.
N=6 ± SEM except where shown otherwise in parentheses.

SIGNIFICANCE VALUES

TIME	C v. AII	C v. V	C v. AIIV	AII v. V	AII v. AIIV	V v. AIIV
50'	NSD	NSD	NSD	0.01	0.05	NSD
3	NSD	0.05	0.01	0.01	0.01	NSD
6	NSD	0.05	0.01	NSD	0.05	NSD
9	0.05	0.05	0.01	NSD	0.01	0.01
12	0.01	0.01	0.01	NSD	0.01	0.01
15	0.05	0.01	0.01	0.05	0.01	0.01
18	0.01	0.01	0.01	0.01	0.01	0.01
21	0.05	0.01	0.01	0.01	0.01	0.01

Table 6.2. Signifcance values for data in figure 6.3.

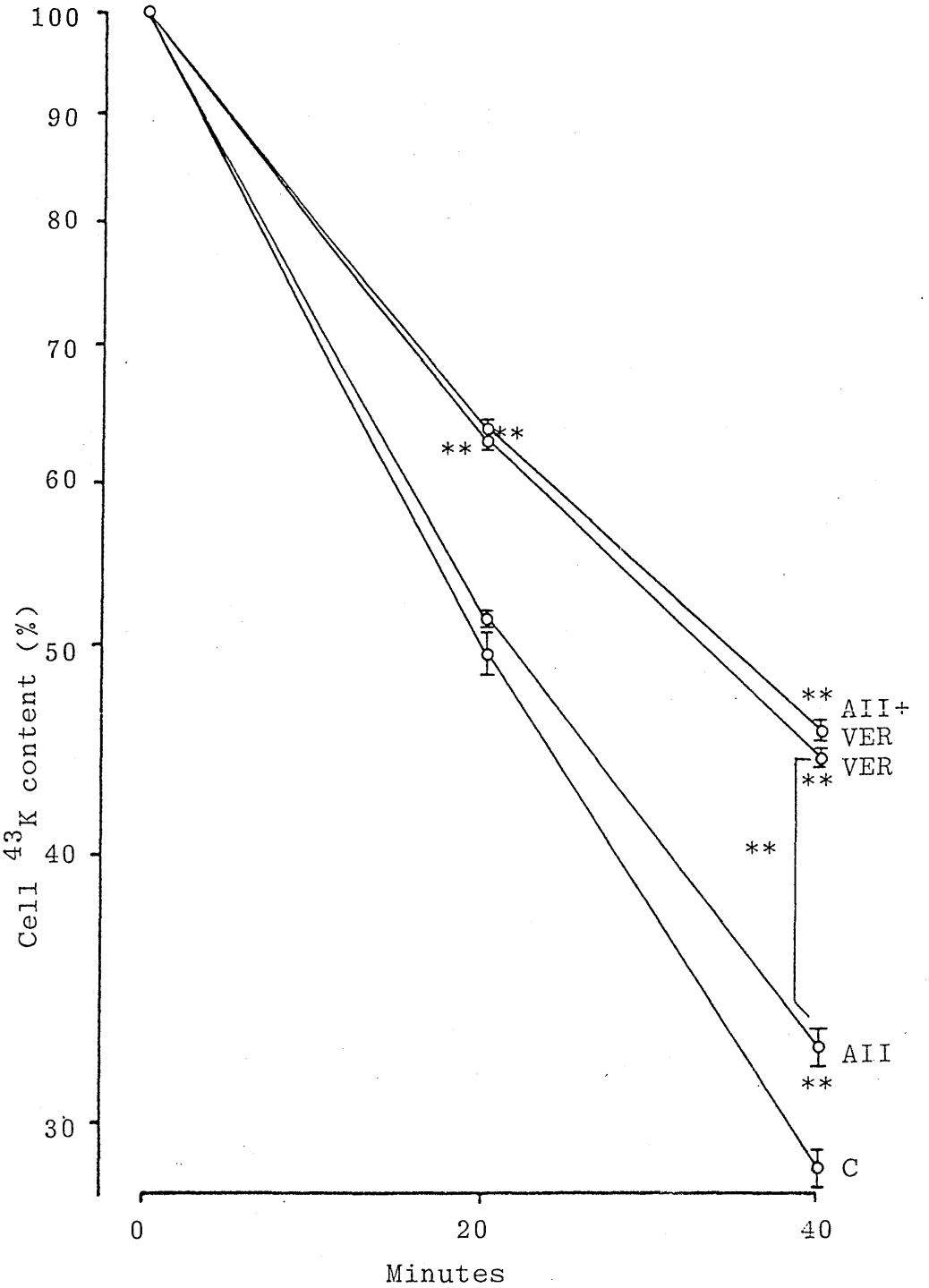


Figure 6.4. The effect of verapamil on ^{43}K efflux over 40 min. Cell ^{43}K content as a % of initial control values is plotted on a log scale against time. Incubations were with or without AII 10^{-8}M ± verapamil 2×10^{-5}M.

* $p<0.05$, ** $p<0.001$ with respect to controls (paired t-test). N=6 ± SEM.

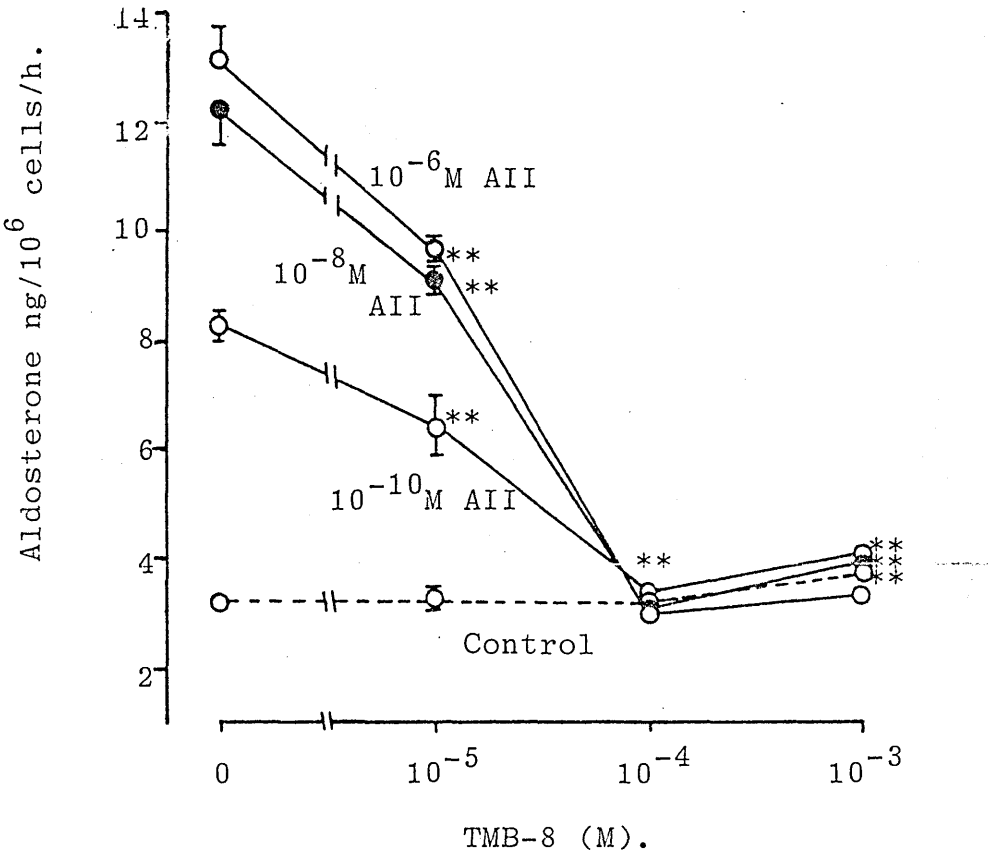


Figure 6.5. Dose response of aldosterone production to TMB-8. Aldosterone production as ng per 10^6 cells /h. is plotted against increasing concentrations of TMB-8 for different doses of AII.
* $p<0.05$, **$p<0.01$ with respect to the relevant controls. N=6 ± SEM.

all concentrations of AII tested. Higher doses of TMB-8 reduced aldosterone release to basal levels in all conditions.

6.2.6. Comparison of the short and longer-term influence of TMB-8 on ^{43}K efflux.

Figure 6.6 shows the pooled results for experiments conducted over a 10 min period in the absence (three experiments) and presence (two experiments) of ouabain. The ^{43}K efflux rate is expressed as a percentage of that of the controls. In the absence of ouabain, AII (10^{-6} M) produced a non-significant fall in efflux rate ($p<0.1$) but TMB-8 (10^{-5} M) significantly decreased ^{43}K efflux ($p<0.05$). Together with AII, TMB-8 decreased ^{43}K efflux still further ($p<0.001$) compared with controls although differences between AII alone, TMB-8 alone and TMB-8 plus AII were not significant. In the presence of ouabain, a similar pattern was observed.

Figure 6.7 shows the cell ^{43}K content after 20 and 40 min of incubation in the presence and absence of AII 10^{-8} M with and without TMB-8. At 20 min, AII had produced a non-significant fall in ^{43}K efflux ($p<0.3$) whereas TMB-8 had markedly reduced ^{43}K loss ($p<0.001$). Together, AII plus TMB-8 reduced the isotope loss with respect to TMB-8 alone ($p<0.05$). By 40 min, the AII-induced fall in ^{43}K loss was highly significant ($p<0.001$), that of TMB-8 more marked than at 20 min and TMB-8 plus AII showed an additive effect. Control incubations showed no marked change in the rate of isotope loss over the 0-20 to 20-40 time points whereas

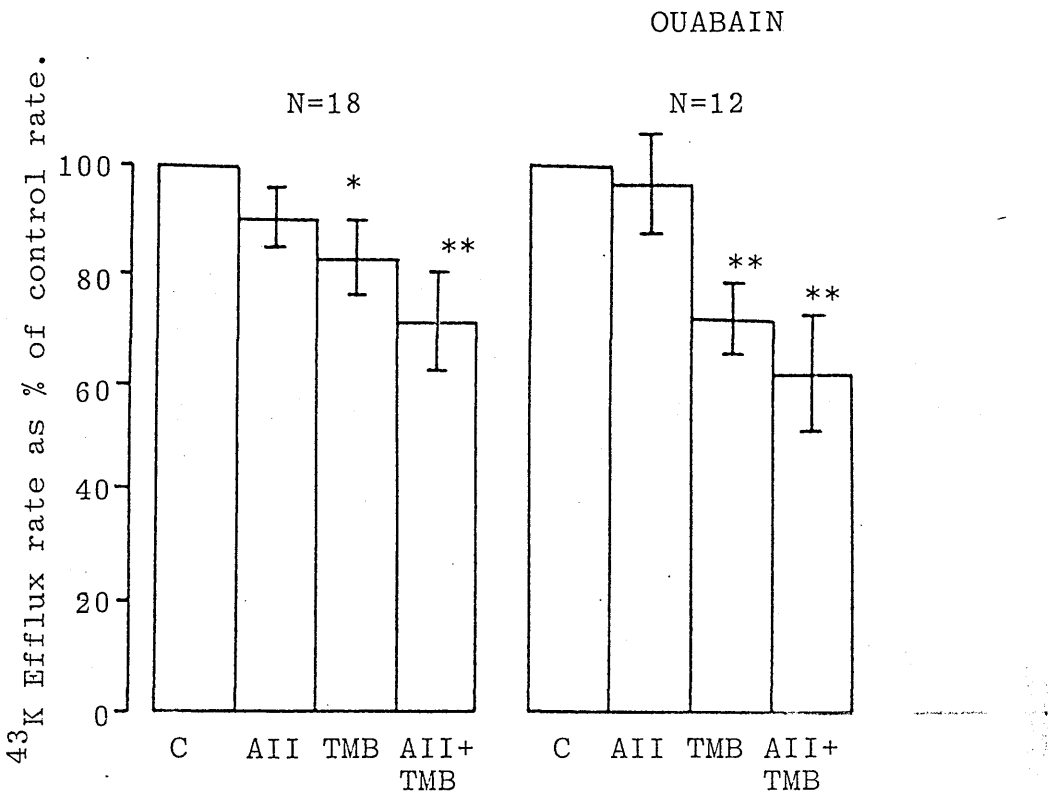


Figure 6.6. Effect of TMB-8 (10^{-4}M) on the early ^{43}K efflux. Pooled data from three experiments in the absence of ouabain (left panel) and two experiments in the presence of ouabain (right panel) are shown for incubations over 10 min. Cells were treated with or without AII (10^{-6}M) ± TMB-8 (TMB, 10^{-4}M).
* $p<0.05$, ** $p<0.001$ with respect to controls (paired t-test).

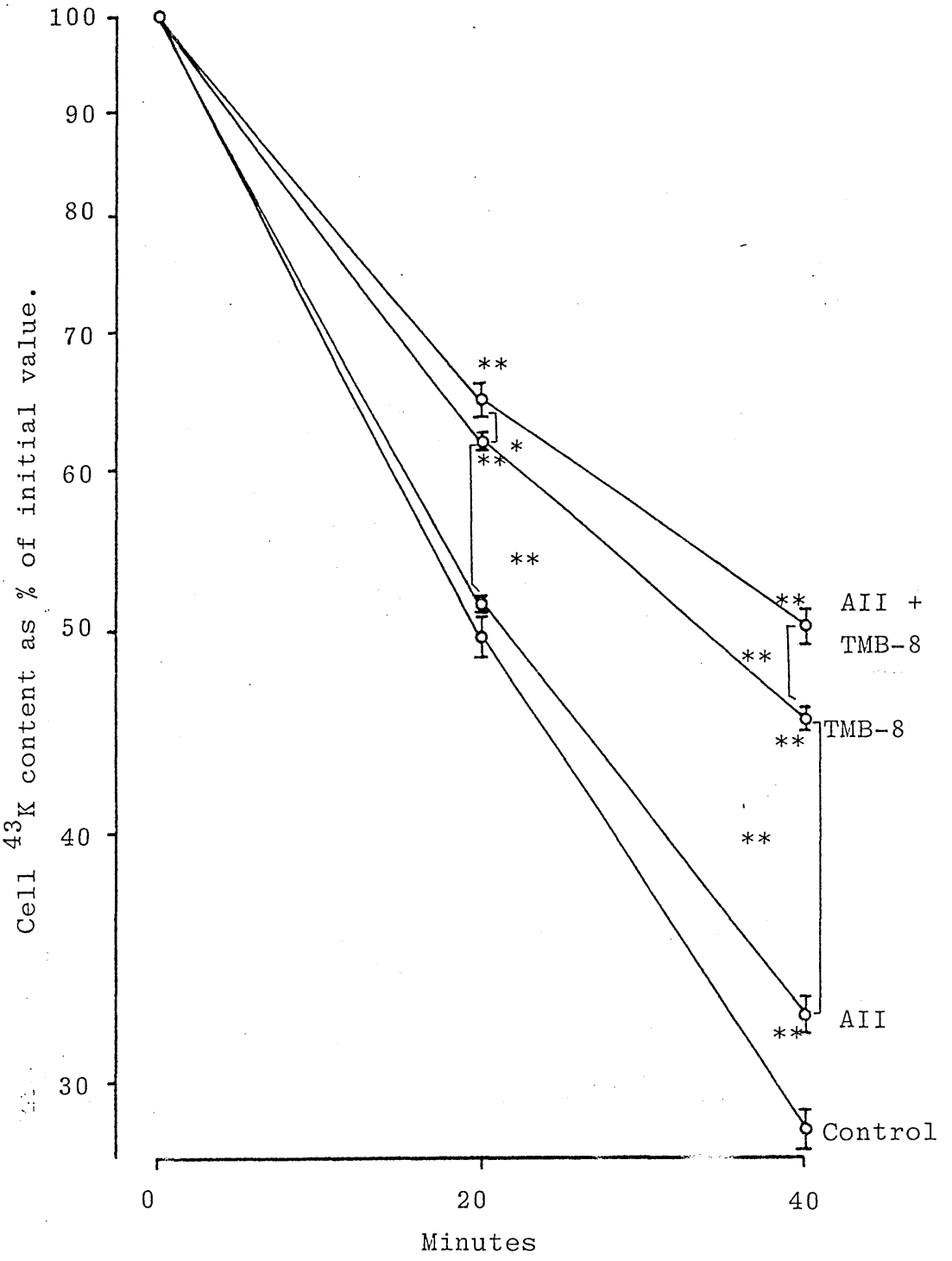


Figure 6.7. Effect of TMB-8 on ^{43}K efflux over 40 min. Cell ^{43}K content as a % of the initial content is plotted on a log scale against time. Incubations contained AII (10^{-8}M) or medium alone ± TMB-8 (10^{-4}M). * $p<0.05$, ** $p<0.01$ with respect to controls (paired t-test). N=6 ± SEM.

TMB-8 in particular, showed a much slower rate of loss during the later period than in the earlier one.

6.2.7. The influence of dantrolene on aldosterone production in stimulated and unstimulated cells.

Depending on concentration, dantrolene either stimulated or inhibited aldosterone synthesis (figure 6.8). At $2x10^{-6}$ M dantrolene, basal and AII-stimulated (10^{-10}, 10^{-8} M) aldosterone synthesis was increased. At $2x10^{-5}$ M, dantrolene partially inhibited aldosterone synthesis stimulated by 10^{-6} M AII whereas at $2x10^{-4}$ M, aldosterone synthesis for all concnetrations of AII were reduced to basal levels.

6.2.8. Evaluation of the short and longer-term effects of dantrolene on ^{43}K efflux.

The effects of the intermediate ($2x10^{-5}$ M) dose of dantrolene on ^{43}K efflux rate over a 10 min incubation are shown in figure 6.9. This dose was chosen because it is reported to maximally affect ^{45}Ca efflux from ZG cells (Kojima et al 1985a). In the absence of ouabain, no significant effects were seen. Changes in the efflux rates in the presence of ouabain were also insignificant with the exception of a rise in the efflux rate caused by dantrolene, with respect to controls ($p<0.05$).

Figure 6.10 shows the ^{43}K content for cells incubated with or without AII (10^{-8} M) in the presence and absence of dantrolene over 40 in. At 20 min, the loss of ^{43}K was not significantly inhibited by AII but that by dantrolene was ($p<0.05$). Together, AII and

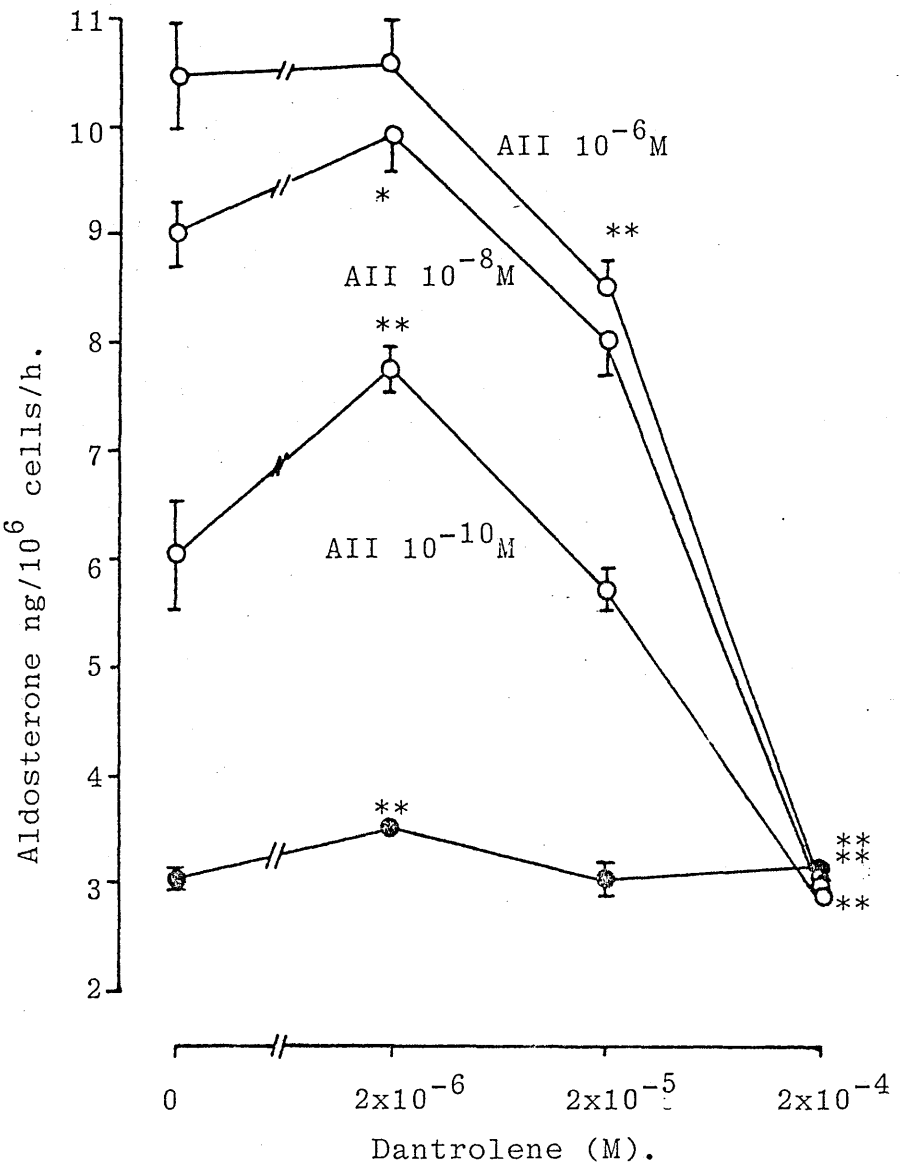


Figure 6.8. Dose response of aldosterone production to dantrolene. Aldosterone prodution as ng/10^6 cells /h is plotted against increasing concentrations of dantrolene for different doses of AII.
* p0.05, ** p<0.01 with respect to the relevant controls. N=6 ± SEM.

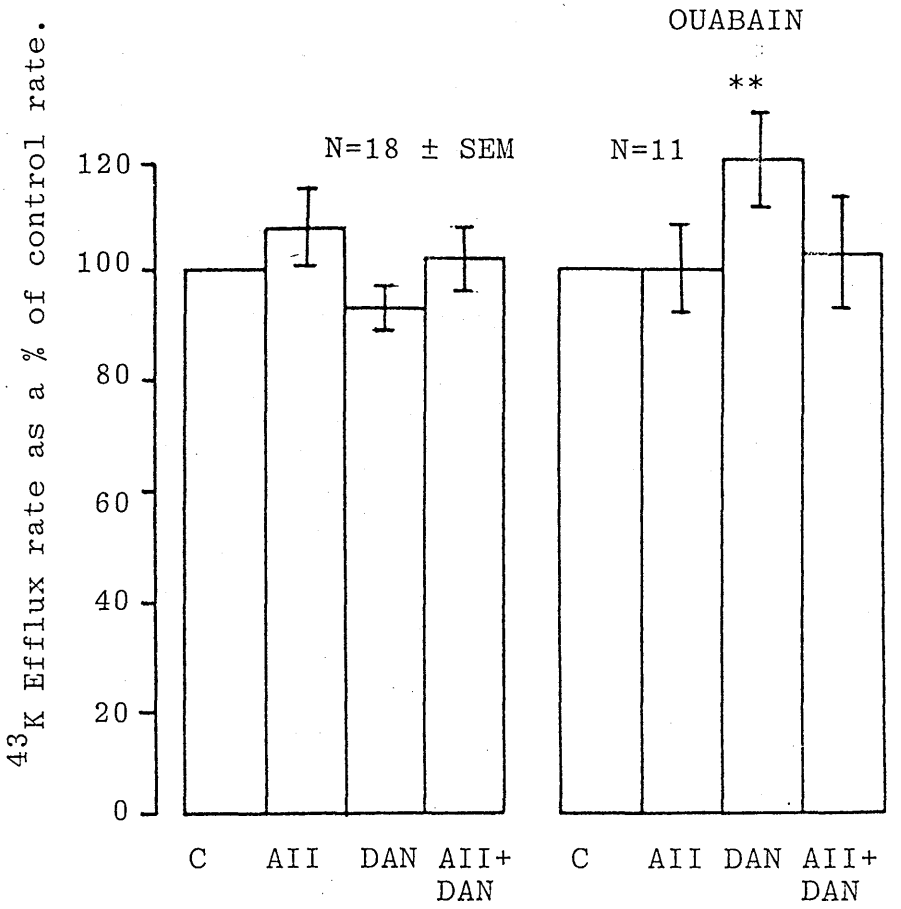


Figure 6.9. Effect of dantrolene ($2x10^{-5}$M) on the early ^{43}K efflux. Pooled data from three experiments in the absence of ouabain (left panel) and two experiments in the presence of ouabain (right panel). Cell ^{43}K efflux is expressed as a % of the control efflux rate over 10 min. Incubations contained medium with or without AII (10^{-6}M) ± dantrolene (DAN $2x10^{-5}$M).

** $p<0.001$ with respect to control (paired t-test).

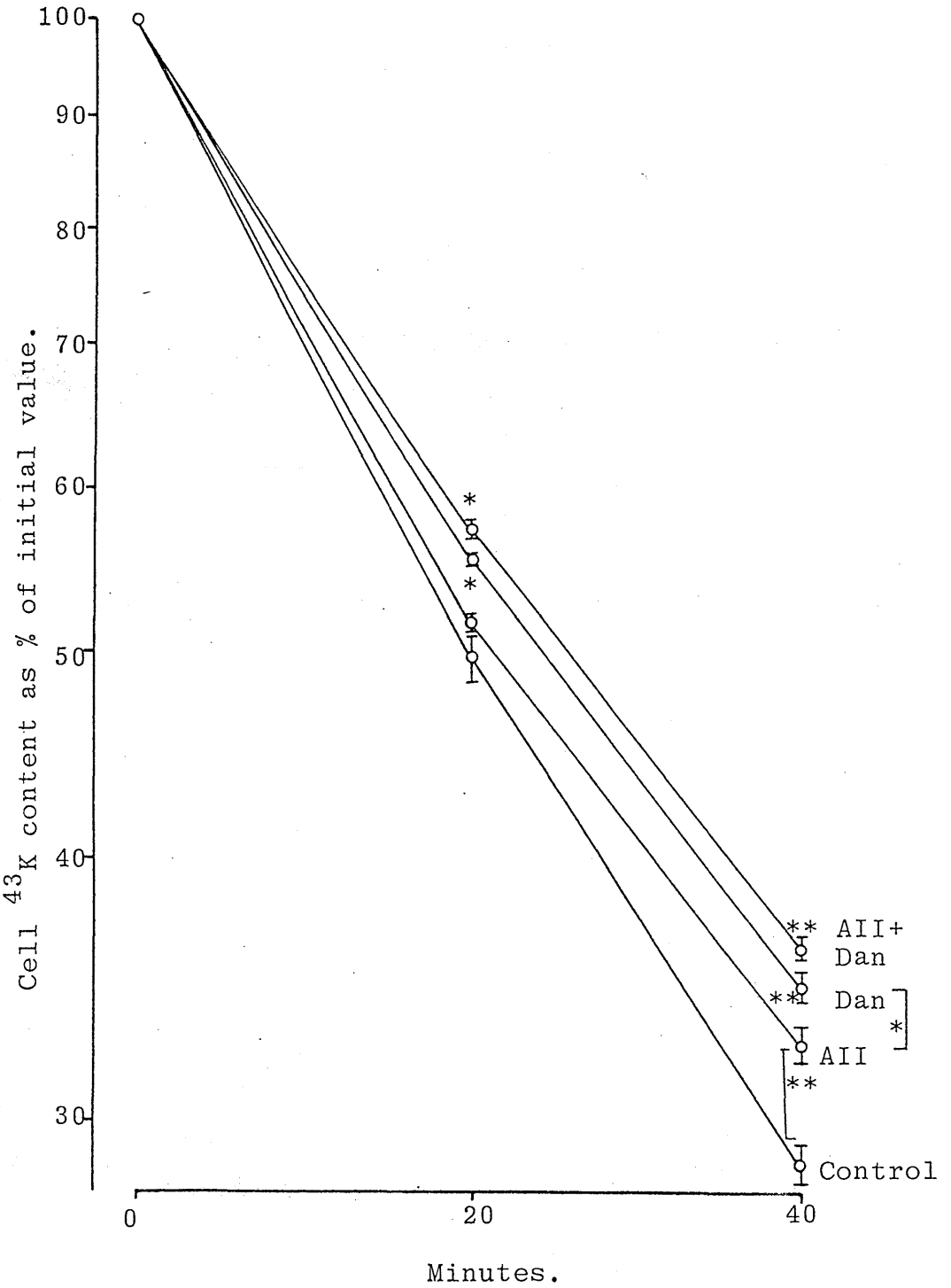


Figure 6.10. Effect of dantrolene on ^{43}K efflux over 40 min. Cell ^{43}K content as a % of the initial ^{43}K content is plotted on a log scale against time. Incubations contained medium with or without AII (10^{-8}M) ± dantrolene (Dan $2x10^{-5}$M).

* $p<0.05$, ** $p<0.001$ with respect to controls (paired t-test). N=6 ± SEM.

dantrolene produced a fall greater than dantrolene alone. At 40 min, AII significantly reduced ^{43}K loss ($p<0.02$) and dantrolene caused a greater reduction. In combination, dantrolene plus AII inhibited the isotope loss to an even greater extent but not significantly more than dantrolene alone.

6.3. DISCUSSION.

6.3.1. Verapamil.

Although verapamil at concentrations of $2x10^{-5}$ M and above inhibited basal and AII-stimulated aldosterone production, the latter was not reduced to basal levels. A similar partial anti-steroidogenic effect was noted with verapamil but not with nitrendipine in phorbol ester-stimulated ZF cells (Kenyon et al 1988). It was suggested that nitrendipine may have better access to additional, possibly intracellular, sites of action as compared with verapamil. In cardiac mitochondria, for example, nitrendipine inhibits Ca^{2+} release whereas verapamil does not (Buss et al 1988). However, higher doses of verapamil than those used in the current study might have inhibited stimulated aldosterone output more completely.

Verapamil greatly reduced ^{43}K efflux during the first 40 min of incubation (figures 6.2, 6.3 and 6.4). This effect is more likely to be due to an inhibition of Ca^{2+}-dependent K^+ efflux following voltage-sensitive Ca^{2+} channel blockade rather than a depolarisation event. However, verapamil has been shown to slow ATP

and creatine phosphate breakdown in the heart (Lange et al 1984). Such a conservation of energy-rich compounds, by increasing their availability for ATP-dependent K^+ channels and by supplying substrate for Na^+/K^+ ATPase could result in the retention of intracellular K^+. It is interesting that the reduction of ^{43}K loss by verapamil becomes greater with time (figure 6.4).

During the first 10 min of incubation, verapamil and AII produced additive inhibitory effects on ^{43}K. This indicates that any depolarisation caused by AII cannot be attributed to the transmembrane movement of Ca^{2+} as suggested by Lymangrover et al (1982). In their experiments on superfused mouse ZF tissue, when $CoCl_2$ was used to block the Ca^{2+} channels, much of the depolarisation caused by ACTH was inhibited and steroidogenesis was reduced by 60% over 2 h.

The fact that the early effects of verapamil and AII are additive also strengthens the hypothesis that K^+ efflux is controlled by two opposing and independent forces which are simultaneously regulated by AII. Verapamil is not considered to be a depolarising agent and yet it inhibits K^+ efflux. The depolarising effects of AII, on the other hand, are clearly associated with decreased K^+ conductance (Quinn et al 1987b) i.e. verapamil and AII appear to inhibit K^+ efflux by two different mechanisms. The present observation that verapamil and AII effects are additive suggests that the two mechanisms can be evoked simulataneously even though steroidogenically their actions are opposed. It follows that simultaneous and opposite effects of AII on two different efflux processes is not implausible.

Verapamil inhibited both aldosterone production and ^{43}K efflux only when added at the same time as AII (table 6.1). In cells pretreated with AII, ^{43}K content at 85 min was less than controls (table 6.1) and the addition of verapamil had no significant effects. If, as suggested, AII simultaneously affects two efflux components with different $t_{\frac{1}{2}}$ values, then the overall effect of AII will depend on the relative dose-response relationships and the initial sizes of the two components. Assuming that the two components are physically separate, it follows that cell ^{43}K content in control cells incubated for 54 min (table 6.1), may be relatively depleted of the early efflux component. Over the first 10 min (figure 6.2) or 15 min (table 6.1), the predominant effect of AII is to inhibit ^{43}K efflux whereas the effects of AII on the two components are equal when added at 54 min so that no decrease in ^{43}K content at 85 min is apparent.

Where AII has been present throughout, ^{43}K content was less than controls at 85 min, showing efflux to be enhanced i.e. Ca^{2+}-dependent K^+ efflux predominates. One would expect therefore, that verapamil should block efflux even more effectively. However, efflux with AII and verapamil under these circumstances was not significantly different from that of AII alone (table 6.1) and may indicate that Ca^{2+}-dependent efflux is regulated by a different mechanism. AII, by releasing Ca^{2+} from intracellular stores, may promote ^{43}K efflux via a mechanism which is independent of any AII-induced depolarisation and is therefore insensitive to Ca^{2+} channel blockade. Initially, Kojima et al (1984a)

suggested that IP_3-induced Ca^{2+} release from intracellular stores occurred only during the early minutes of AII stimulation, but more recent studies suggest that it does have a role in sustained aldosterone production (Balla et al 1989).

6.3.2. TMB-8.

In keeping with its reputed inhibition of intracellular Ca^{2+} release, TMB-8 inhibited AII-stimulated aldosterone production (figure 6.5). It did not affect basal steroidogenesis, perhaps because this is not dependent on release of stored Ca^{2+} whereas that induced by AII is.

The marked decreases in ^{43}K efflux rate observed with TMB-8 (10^{-4} M) in unstimulated cells is presumably caused by reducing $[Ca^{2+}]_i$ followed by closure of Ca^{2+}-dependent K^+ channels. Since in unstimulated cells, IP_3-induced Ca^{2+} release would be minimal and its contribution to $[Ca^{2+}]_i$ small, it is likely that TMB-8 affects other ion channels in addition to the inhibition of Ca^{2+} mobilisation. Indeed, Koijma et al (1985d) have shown 10^{-4} M TMB-8 to inhibit ^{45}Ca uptake in AII-stimulated bovine ZG cells. The site of this addtional TMB-8 block is not known but may involve voltage-sensitive Ca^{2+} channels. Kojima et al (1985d), also claimed that TMB-8 did not inhibit IP_3-induced Ca^{2+} release from permeabilised cells but that it did directly inhibit protein kinase C activity.
The effect of TMB-8 on ^{43}K efflux appears, like that of verapamil, to increase with time (figure 6.7).

6.3.3. Dantrolene.

Dantrolene inhibits cellular effects requiring raised $[Ca^{2+}]_i$ such as muscle contraction (Hainaut and Desmedt 1974, Ally et al 1978 and Ward et al 1986) and neurotransmitter (Duran et al 1980) and catecholamine release (Cohen and Gutman 1979). The unexpected increase in aldosterone output elicited by dantrolene ($2x10^{-6}$ M), could be explained in terms of a Ca^{2+} set-point hypothesis proposed by Wiedenkeller and Sharp (1984) and Pian-Smith et al (1986) to account for dantrolene's potentiation of stimulated insulin release from ß cells. They suggested that, even with no change in Ca^{2+} influx, prevention of Ca^{2+} release from intracellular stores would saturate them making them unable to regulate $[Ca^{2+}]_i$ to its normal resting value of 0.1-0.2 µm. Since mitochondria sequester Ca^{2+} only once $[Ca^{2+}]_i$ reaches 0.7 µm, the raised $[Ca^{2+}]_i$ enhances insulin release.

The total inhibition of stimulated aldosterone output at higher concentrations of dantrolene may be caused by its effects on inhibiting cytochrome P450-containing enzymes (Francis and Hamrick 1979). In vivo studies in rats showed that dantrolene lowered serum corticosterone after 5 days administration (Francis and Hamrick 1980). The suggested site of inhibition was the 11ß hydroxylation of 11-deoxy-corticosterone, which, if correct, may account for the inhibition of stimulated aldosterone synthesis. Dantrolene had no significant effect on ^{43}K efflux over the first 10 min (figure 6.9) but produced a significant reduction in ^{43}K loss at 20 min (figure

6.10). This may reflect a time lag between its application and action. Pian-Smith et al (1988) showed that the maximum effect a dose of dantrolene produced in ß cells on Ca^{2+} movements occurred 7 min after its application. Also, in previous studies with steroidogenic tissue, dantrolene was added prior to testing with AII (Kojima et al 1984b, 1985a, Rossier et al 1987) although the authors do not say why.

Even though the effect of dantrolene on reducing ^{43}K loss was persistent, it did not increase as much with time as had that of TMB-8 or verapamil (compare figure 6.10 with figures 6.4 and 6.7). Presumably, dantrolene has a less potent effect than TMB-8 or verapamil. The dose of dantrolene used in this experiment had had little effect on aldosterone output (figure 6.8) perhaps reflecting a conflict between inhibition by cytochrome P450 interactions and stimulation, possibly by the paradoxical raising of $[Ca^{2+}]_i$ discussed earlier. Lihrman et al (1987) noted that TMB-8 markedly reduced basal, ACTH and AII-stimulated aldosterone and corticosterone secretion in the frog interenal gland whereas dantrolene inhibited only basal and AII-induced release. This group inferred that TMB-8 blocked the raising of $[Ca^{2+}]_i$ to a much greater extent than dantrolene since ACTH-invoked aldosterone output is much less dependent on $[Ca^{2+}]_i$ than that of AII.

6.4. SUMMARY.

Blockage of voltage-dependent Ca^{2+} entry by verapamil decreased both basal and AII-stimulated aldosterone production, suggesting that these channels are involved in aldosterone synthesis even under basal conditions. ^{43}K efflux was decreased by verapamil ($2x10^{-5}$ M) to a greater extent than by a maximal dose of AII over 10 min and by AII 10^{-8} M over 40 min, showing a dependency of ^{43}K efflux on Ca^{2+} entry. This has been interpreted to show that Ca^{2+}-dependent K^+ channels are inhibited as $[Ca^{2+}]_i$ falls due to Ca^{2+} uptake being reduced by verapamil. Verapamil added during the "late" period of AII-stimulated cells tended to decrease ^{43}K efflux and had no significant effect on aldosterone output. When added with AII to cells pretreated with medium alone, verapamil decreased aldosterone production and increased ^{43}K efflux. These results suggest that voltage-dependent Ca^{2+} entry is not required once increased steroidogenesis has been maintained for almost an hour.

TMB-8 did not affect basal aldosterone release but at 10^{-4} M and above, it reduced all AII-stimulated aldosterone production to basal levels. TMB-8 (10^{-4} M) greatly reduced the ^{43}K efflux rate after 20 and 40 min of incubation which may reflect falls in Ca^{2+}-dependent K^+ channel opening caused by a fall in $[Ca^{2+}]_i$. Dantrolene at a low dose slightly augmented basal aldosterone output and that stimulated by low doses of AII. Higher doses of dantrolene inhibited AII-stimulated aldosterone synthesis to basal levels. The

low-dose effects of dantrolene on steroidogenesis may relate to an effective raising of $[Ca^{2+}]_i$ but the high-dose effects probably relate to inhibition of cytochrome $P450_{11B}$ hydroxylation. Dantrolene ($2x10^{-5}$ M) decreased the ^{43}K efflux rate over a 40 min incubation to a greater extent than AII.

These data suggest that ^{43}K efflux is inhibited by TMB-8, dantrolene and verapamil by mechanisms involving limitation of Ca^{2+} availability which in turn prevents Ca^{2+}-dependent K^+ channels from opening. In comparing the effects of these drugs with apamin, an agent which affects K^+ channels directly rather than through Ca^{2+}, it is interesting that apamin potentiates the action of AII whereas verapamil, dantrolene and TMB-8 are inhibitory. It is inferred that relative concentrations of intra- and extracellular K^+ and Ca^{2+} are steroidogenically more important than the activity of different channels when the cells are under conditions in which channel activation/inhibition is uncoupled from movements of these ions.

CHAPTER 7. GENERAL DISCUSSION.

The experiments undertaken for this thesis have provided evidence that K^+ efflux from ZG cells reflects K^+ loss from two sources. It seems reasonable to regard these sources as separate pools because isotope can be depleted from one before the other and also because AII affects them in opposite ways. If the different $t_{\frac{1}{2}}$ values for each efflux component merely reflected the ^{43}K efflux from the cytosol in general via two independently operated channel types, then depletion of isotope from the faster-exchanging component in unstimulated cells would not occur. How these pools might be arranged is a matter of conjecture. Much is known about the release of Ca^{2+} from, and its sequestration into, organelles but similar studies on K^+ are lacking. Possibly, the faster-exchanging pool represents K^+ closely associated with the cell membrane because of the ease with which it is depleted by superfusion.

^{43}K efflux from the pools is governed by different channel types. The faster-exchanging pool efflux is blocked by glibenclamide. This implies the involvement of ATP-dependent channels, since alone it decreases ^{43}K efflux but in combination with AII its effect is cancelled out by the additional action of AII to raise ^{43}K efflux from the slower-exchanging pool. The slower-exchanging efflux is blocked by the Ca^{2+}-dependent K^+ channel inhibitor apamin in that on its own it has little effect on decreasing ^{43}K efflux but in combination with AII it does because more

Ca^{2+}-dependent K^+ channels are open in the presence of AII. Since the concentration of glibenclamide required to affect steroidogenesis was an order of magnitude higher than that associated with a maximal effect on insulin release in rat pancreatic ß cells (Grill and Cerasi 1978), ZG cells may be less sensitive to ATP-dependent K^+ channel blockade. A better evaluation of the role of these channels in ZG cells would require binding studies with glibenclamide, dose-response curves of ^{43}K efflux with the drug and alterations in $[ATP]_i$.

Ca^{2+} movements are influenced by those of K^+ and vice versa. It is unfortunate that the evidence presented here is not backed by data on ^{45}Ca uptake and efflux but relies on the known pharmacology of verapamil, TMB-8 and dantrolene. As discussed earlier, all three have effects in addition to their actions on voltage-sensitive Ca^{2+} entry (verapamil) or release of Ca^{2+} from intracellular stores (TMB-8 and dantrolene). However, these agents have marked effects, reducing ^{43}K efflux and aldosterone production, presumably via reductions in $[Ca^{2+}]_i$ which close the Ca^{2+}-dependent K^+ channels. Confirmation of this mode of action could be obtained by seeing whether ^{43}K efflux in cells treated with these agents could be restored by the Ca^{2+} ionophore A23187 or whether they correspond to $[Ca^{2+}]_i$.

Measurements of ^{45}Ca uptake attempted during the course of the current work (results not shown), were unable to show consistent, statistically significant changes during treatment with AII and/or verapamil despite the use of two techniques. These were firstly

vacuum filtration of cells washed with ice-cold 5mM $CaCl_2$ TRIS buffer (Mauger et al 1979) or lanthanum/ HEPES buffer (Wermelskirchen et al 1986) and secondly centrifugation through oil with or without a HEPES/EGTA wash. Although some workers have reported clear results for ^{45}Ca in ZG cells using the method of Mauger et al (or refinements of it) these have given indications of both increased (Kojima et al 1985) and decreased (Elliott et al 1982, T. Goodfriend, personal communication), uptake in the presence of AII. Failure to observe any definite trend during the current work probably indicates that both uptake and efflux of Ca^{2+} are stimulated by AII. The only way to monitor both movements of this ion simultaneously would involve the use of ^{45}Ca and ^{47}Ca. This technique seems to be used only as a clinical tool for measuring the gastro-intestinal absorption of Ca^{2+}. There are some technical problems in calculating the activity arising from each isotope but these can be overcome (Ogg et al 1968, Greissen et al 1985).

Experiments combining uptake or efflux of both ^{43}K and ^{45}Ca (or the uptake of one and efflux of the other) would enable a better understanding of how the agents used herein modulate the movement of these two ionic species. Assessment of the possible exchange of K^+ with Ca^{2+} released from intracellular stores could be achieved by electropermeabilising the cells. This method of accessing the cytosol enables manipulation of intracellular composition whilst retaining the cells' ability to produce aldosterone, albeit to a lesser extent than when intact (Capponi et al 1988a and b).

The role of Na^+ would also be of interest to study, particularly with respect to establishing the mode of Na^+/Ca^{2+} exchange in these cells. Is it an extrusion mechanism for Ca^{2+}, as in the majority of cells in which it is found, rather than a method of Ca^{2+} entry as proposed by Hunyady et al (1988) and Spät et al (1989)? If so, does this involve K^+ as suggested by Cervetto et al (1989)? This group established that extracellular Na^+ exchange for one Ca^{2+} in retinal rods, although usually regarded as a $3Na^+:Ca^{2+}$ exchange, also involves K^+ with a stoichiometric exchange of $4Na^+$ for 1 Ca^{2+} and 1 K^+. This not only accounts for the observed net influx of one positive charge but the coupling of both the inward Na^+ and outward K^+ concentration gradients, would also explain how the $[Ca^{2+}]_i$ is maintained at a lower level than could be achieved if the exchange were coupled to the Na^+ gradient alone.

Figure 7.1 sets out a model of events in ZG cells following AII stimulation to which the results from this study fit. Interaction of AII with its receptor closes K^+ channels to depolarise the cell membrane and promote Ca^{2+} entry. Glibenclamide is also able to close these K^+ channels and verapamil can reduce Ca^{2+} entry. Ca^{2+} entry by a means not sensitive to verapamil may also be increased. Activation of the AII receptor also induces IP_3-mediated release of Ca^{2+} from intracellular stores which can be inhibited by TMB-8 and dantrolene. The raised $[Ca^{2+}]_i$ increases aldosterone synthesis and also opens Ca^{2+}-sensitive K^+ channels to repolarise the cell. These channels can be blocked by apamin.

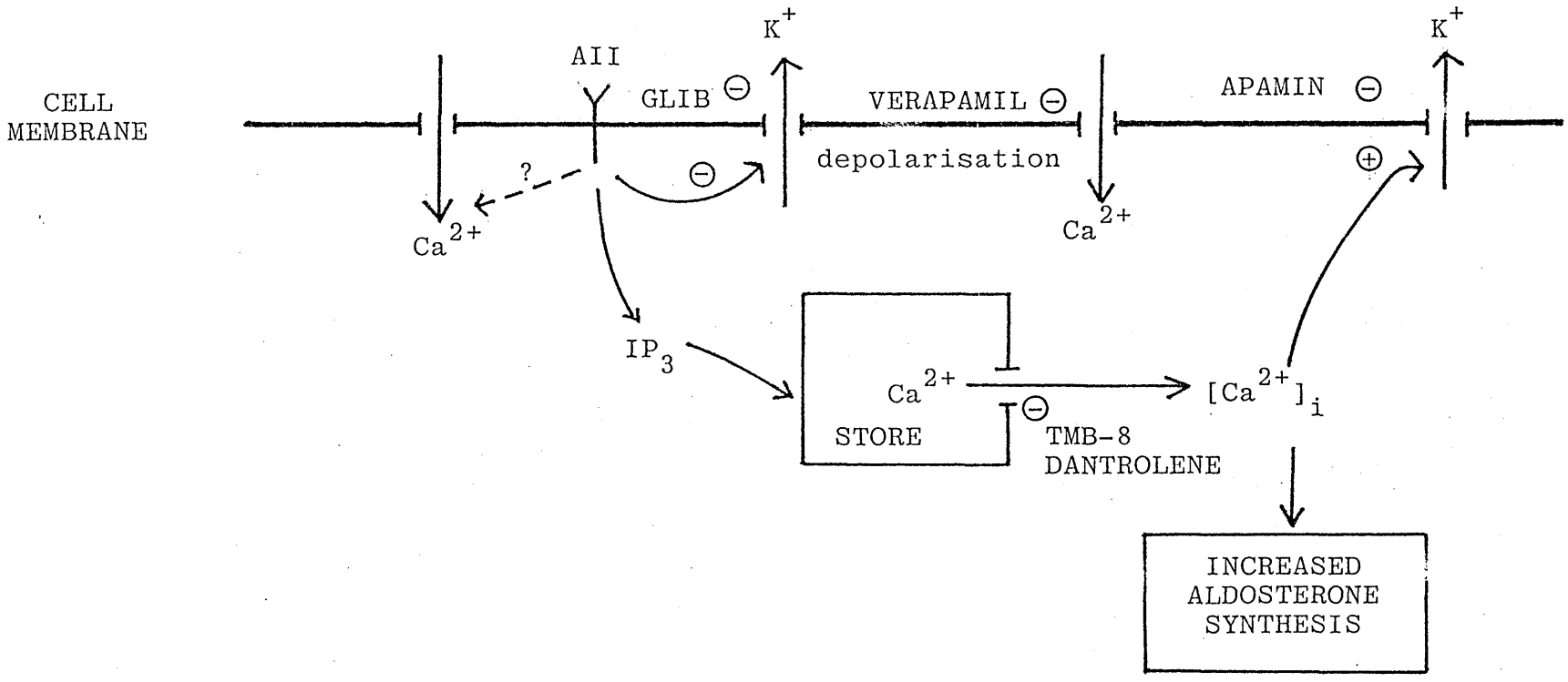


Figure 7.1. Model of ionic movements following AII stimulation of ZG cells. The above scheme incorporates the effects of the agents studied during the course of this project. ⊖ Denotes inhibition and ⊕ stimulation.

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