STUDIES ON THE CONTROL OF ADRENAL STEROIDOGENESIS: INTER-RELATIONSHIPS BETWEEN THE EFFECTS OF BENZODIAZEPINES, CALCIUM IONS AND LIPOPROTEINS.

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PUBLICATIONS.

Full Papers.

Thomson, I., Shepherd, R.M., Fraser, R. and Kenyon, C.J. Dantrolene inhibits adrenal steroidogenesis by a mechanism independent of effects on stored calcium release. J. Steroid Biochem. Mol. Biol. (1991) 38 703-707.

Thomson, I., Fraser, R. and Kenyon, C.J. Inhibition of bovine adrenocortical steroidogenesis by benzodiazepines: a direct effect on microsomal hydroxylation or an inhibition of calcium uptake. J. Endocrinol. (1992) 135 361-369.

Abstract.

Thomson, I., Graham, D., Caslake, M., Fraser, R. and Kenyon, C.J. Possible dual effects of high density lipoprotein subfractions on adrenocortical steroidogenesis. J. Endocrinol. (1991) 131 (Supp.) 67.

SUMMARY

Three aspects of steroidogenic control in bovine adrenocortical cells have been considered:

(i) Cholesterol supply.

(ii) Calcium metabolism.

(iii) Activity of steroidogenic enzymes.

The effects of dantrolene, high density lipoprotein cholesterol isoquinoline carboxamides and benzodiazepines, agents reported to act specifically on each control element, have been assessed.

(a) **Dantrolene**

Dantrolene is a drug which reputedly blocks specifically hormone-stimulated release of Ca^{2+} from intracellular stores.

Dantrolene abolished the aldosterone response to angiotensin-II (A-II). However, aldosterone synthesis, from added hydroxycholesterol, was more sensitive to inhibition by dantrolene (IC_{50} 0.02mmol/l) than A-II-stimulated synthesis although high concentrations of the drug (0.2mmol/l) did not totally abolish aldosterone synthesis in hydroxycholesterol-treated cells.

Aldosterone synthesis in cells incubated with hydroxycholesterol, pregnenolone, progesterone, 11-deoxycorticosterone (DOC), corticosterone and 18-hydroxycorticosterone was significantly inhibited ($p<0.05$) by 89%, 69%, 59%, 31%, 62% and 28% respectively in the presence of 0.2mmol/l dantrolene.

In zona fasiculata/reticularis cells, dantrolene (0.2mmol/l) significantly reduced cortisol production in cells treated with hydroxycholesterol ($p<0.001$) but not in those treated with pregnenolone or progesterone.

In isolated mitochondria, dantrolene significantly reduced pregnenolone production from added hydroxycholesterol (92%; $p<0.001$), aldosterone production from added corticosterone (31%; $p<0.001$) and cortisol production from added 11-deoxycortisol (27%; $p<0.01$).

Dantrolene appears not only to inhibit steroidogenesis by preventing A-II-stimulated release of intracellular calcium, but also to directly inhibit steroidogenic enzymes, in particular cholesterol side-chain cleavage activity.

(b) **High Density Lipoproteins**

Previous studies have established that HDL is readily utilised as a substrate for steroidogenesis. However, studies in platelets suggest that HDL subfractions may differentially interfere with signal-response coupling with variable consequences on platelet aggregation.

In freshly isolated cells HDL-2 and HDL-3 increased basal aldosterone (1.5-3.5 fold) and cortisol synthesis (1.3-6.5 fold) in a dose-dependent manner. Both subfractions potentiated A-II stimulated aldosterone synthesis at low concentrations of peptide (10^{-10} mol/l: 1.5 fold) but had little effect at maximal concentrations of A-II (10^{-7}mol/l). Cortisol synthesis at both low (10^{-10}mol/l: 1.8-2.3 fold) and high (10^{-7}mol/l: 2.2-2.6 fold) concentrations of A-II was further increased by HDL.

In cultured cells treated with HDL-cholesterol, basal aldosterone (3 and 3.1 fold) and cortisol synthesis (12 and 15 fold) was increased more than in cultured cells stimulated with A-II (10^{-8}mol/l). The response to A-II in the presence of HDL-cholesterol was a 10-14 fold increase in aldosterone synthesis and a 6.5-7.6 fold increase in cortisol synthesis. This compared with a 30 fold increase in aldosterone and a 42 fold increase in cortisol in cells not treated with HDL.

Neither HDL-2 nor HDL-3 (0.4mmol/l cholesterol) had any effect on acute ^{45}Ca

uptake in isolated zona glomerulosa cells. This was measured thirty seconds after addition of label. Under steady-state conditions (after thirty minutes), both subfractions significantly decreased ^{45}Ca uptake when compared with control (15%; $p<0.01$). No significant effects were observed on concentrations of basal and A-II-stimulated intracellular free calcium in zona glomerulosa cells pre-incubated with HDL-2 or HDL-3 for 15 mins.

In conclusion, HDL-2 and HDL-3 are similarly effective in providing substrate for aldosterone and cortisol synthesis. Neither subfraction has any acute effect on cell calcium. In the longer term, HDL-cholesterol may lower steady-state calcium content in adrenal cells as it does in platelets. The significance of this in signal-response coupling is not known.

(c) **Benzodiazepines**

Previous studies have suggested that benzodiazepines directly inhibit microsomal steroid hydroxylating enzymes at high concentrations. However, high affinity peripheral receptors for benzodiazepines have been identified in mitochondria of steroidogenic tissues (MBR) which have been linked to effects on calcium metabolism and/or cholesterol supply to side-chain cleavage enzyme. The effects of peripheral ligands on steroidogenesis were investigated using the benzodiazepines midazolam, diazepam and RO5-4864 (4-chlorodiazepam).

(i) Midazolam: In addition to its basic benzodiazepine structure, midazolam contains an imidazole moiety which has been demonstrated previously to inhibit steroidogenesis. The affinity of the drug for the MBR is unknown.

Midazolam inhibited basal and ACTH-stimulated cortisol synthesis (IC_{50} 22μmol/l) in zona fasiculata/reticularis cells. In the same experimental incubations, the drug (0.85-13.6μmol/l) caused an accumulation of 17-hydroxyprogesterone which

decreased at higher concentrations (>13.6μmol/l). Midazolam inhibited conversion of 11ß-hydroxyprogesterone to aldosterone in zona glomerulosa cells (IC_{50} 10μmol/l) and 21-deoxycortisol to cortisol in zona fasiculata/reticularis cells (IC_{50} 21μmol/l).

Midazolam and diazepam inhibited K^+-stimulated aldosterone synthesis (IC_{50} 1.2μmol/l for diazepam and 0.8μmol/l for midazolam) at lower concentrations of drug than that required to inhibit conversion of 11ß-hydroxyprogesterone to aldosterone, 21-deoxycortisol to cortisol or ACTH-stimulated cortisol synthesis (IC_{50} 10, 21 and 22μmol/l respectively).

Midazolam reduced overall K^+-stimulated ^{45}Ca uptake in zona glomerulosa cells by 43% and, in addition, the drug decreased basal ^{45}Ca uptake in the same cells at all concentrations between 0.85 and 55μmol/l. Zona fasiculata/reticularis cells appeared less sensitive to the drug, midazolam only causing significant inhibition of ^{45}Ca uptake at drug concentrations >13.6μmol/l.

(ii) Diazepam: Diazepam has affinity for both MBR and for well-characterised benzodiazepine receptors in the central nervous system. It is through the central receptors that the drug has its therapeutic effect as a tranquilliser and sedative.

Diazepam (10^{-9}-10^{-5}mol/l) increased basal aldosterone synthesis (2.3 fold) in cultured zona glomerulosa cells. This effect could be induced by pre-exposure to the drug.

A-II-stimulated aldosterone synthesis was only potentiated by diazepam when sub-maximal concentrations of peptide were used and when the drug was present at concentrations < 0.1mmol/l. At higher concentrations, diazepam inhibited stimulated synthesis at every A-II concentration. In cultured cells incubated with HDL-cholesterol and DOC, diazepam (10^{-9}-10^{-5}mol/l) maximally increased aldosterone synthesis 1.8 and 1.6 fold respectively.

(iii) RO5-4864: RO5-4864 binds with high affinity to MBR but not to central receptors.

RO5-4864 (10^{-9}-10^{-4}mol/l) potentiated aldosterone synthesis (maximal 1.9 fold increase) in cultured zona glomerulosa cells incubated with HDL-cholesterol. In addition, the drug (10^{-9}-10^{-7}mol/l) also increased aldosterone synthesis (maximal 1.8 fold increase) in cells incubated with added DOC.

In conclusion, depending upon the identity of drug, its concentration and the mode of stimulation benzodiazepines can:

(i) Increase steroidogenesis, probably by stimulating substrate delivery to the inner mitochondrial membrane.

(ii) Decrease steroidogenesis by inhibiting K^+-stimulated uptake of extracellular calcium or by inhibiting steroidogenic enzymes, in particular microsomal hydroxylases.

(d) **Isoquinoline Carboxamides (PK11195)**

PK11195, like the benzodiazepine RO5-4864, binds with high affinity to MBR but not to central receptors and has been reported as a peripheral receptor antagonist.

PK11195 (10^{-9}-10^{-7}mol/l) increased aldosterone synthesis in the presence of HDL-cholesterol (maximal 5.3 fold stimulation) but had no effect on DOC-stimulated aldosterone synthesis.

The drug stimulates rather than antagonises aldosterone synthesis at the MBR, probably by increasing cholesterol delivery to the inner mitochondrial membrane although an effect on calcium metabolism at the plasma membrane cannot be excluded.

CHAPTER 1.

LITERATURE REVIEW.

1.1 Mineralocorticoids and Glucocorticoids.

The adrenal cortex produces three types of steroid, two of which, mineralocorticoids and glucocorticoids, are important in cardiovascular regulation and renal homeostasis. In man and higher mammals cortisol is the major glucocorticoid (corticosterone in the rat) and aldosterone the major mineralocorticoid. The term mineralocortocoid has been applied to steroids that have distinct effects on ion transport by epithelial cells, resulting in sodium conservation and loss of potassium. Glucocorticoids are primarily involved in the regulation of carbohydrate metabolism but also have important effects on electrolyte metabolism.

1.2 Aldosterone

All endogenous mineralocorticoids and glucocorticoids are derived from cholesterol and therefore display marked structural similarities, with each containing a common 21 carbon backbone with a cyclopentanoperhydrophenanthrene nucleus. The angular C-18, which is a methyl group in other steroids, is oxidised to an aldehyde group in aldosterone. The proximity of this group to the 11ß-hydroxyl group means that aldosterone exists in solution as a mixture of aldehyde and hemiacetal with equilibrium favouring the latter (1) (Fig 1.1).

1.3 The Adrenal Cortex.

In mammals, the adrenals are paired glands lying anterior to the kidneys. Histologically, the gland can be divided into two parts; a central medulla which

ALDOSTERONE

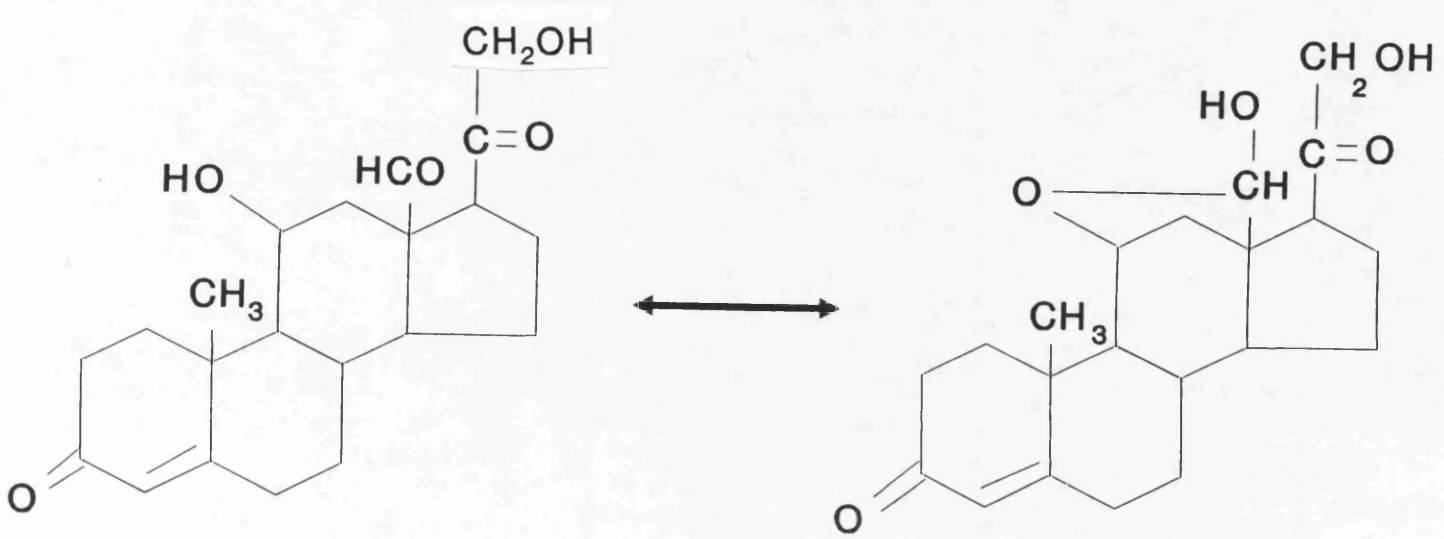


Fig.1.1

Two different forms of aldosterone which can exist in solution. Equilibrium favours the formation of the hemiacetal.

Adapted from (1), White et al, 1968.

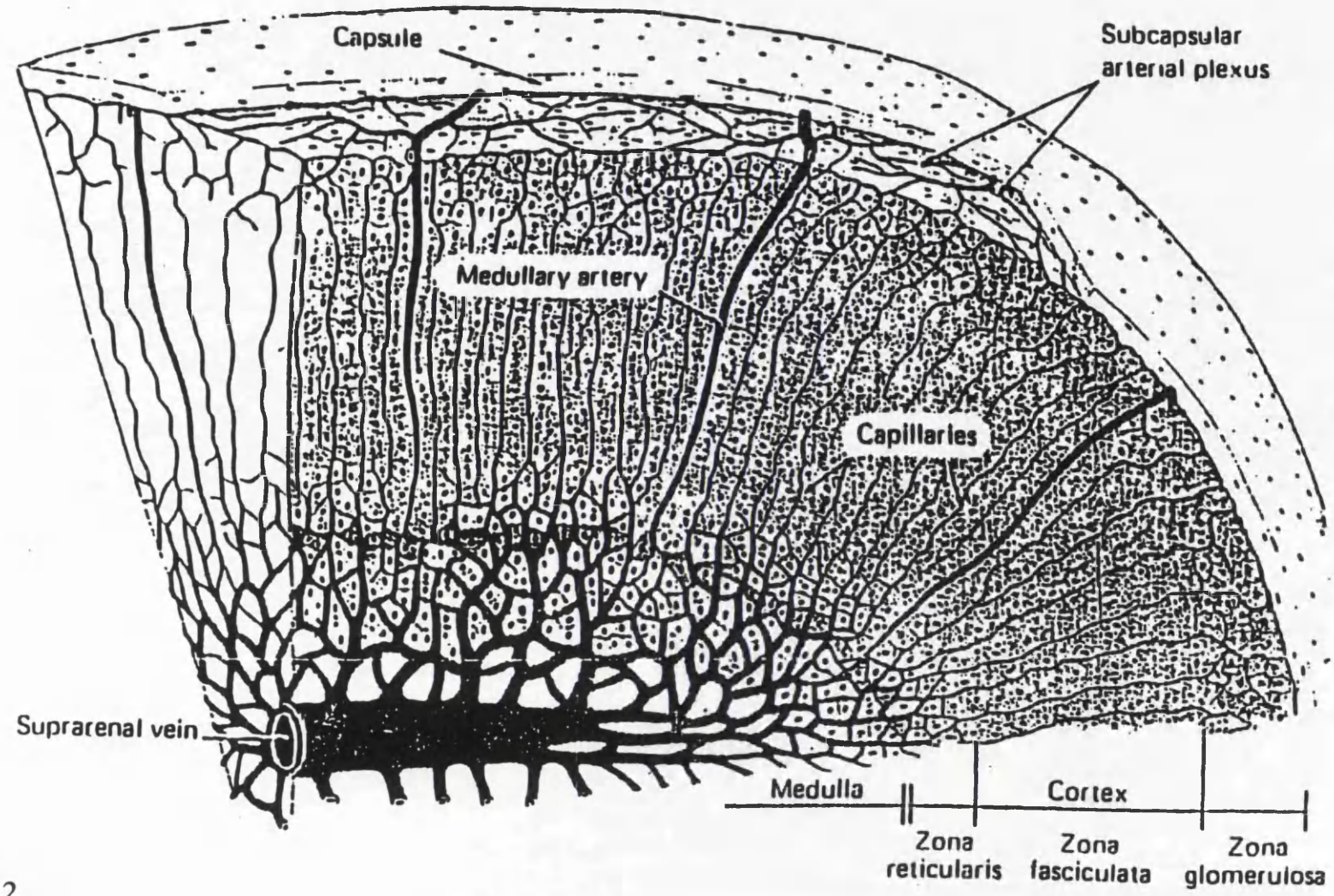


Fig.1.2

Diagram of the mammalian adrenal gland.

From (2), Junquiera, 1986.

secretes catecholamines and an outer cortex which secretes steroid hormones. The cortex is further subdivided into three zones (2) (Fig.1.2), each of which synthesises a different pattern of hormones. The innermost zones are the zona fasiculata and zona reticularis which synthesise the major glucocorticoids cortisol and corticosterone plus adrenal androgens. Aldosterone is synthesised exclusively by the zona glomerulosa, the outermost region of the cortex. This introduction will focus mainly on the control of aldosterone secretion.

1.4 Biosynthesis of Aldosterone.

1.4.1 The Biosynthetic Pathway.

Fig.1.3 displays the pathway for the formation of aldosterone from the precursor cholesterol (3). It involves three groups of hydroxylation reactions:

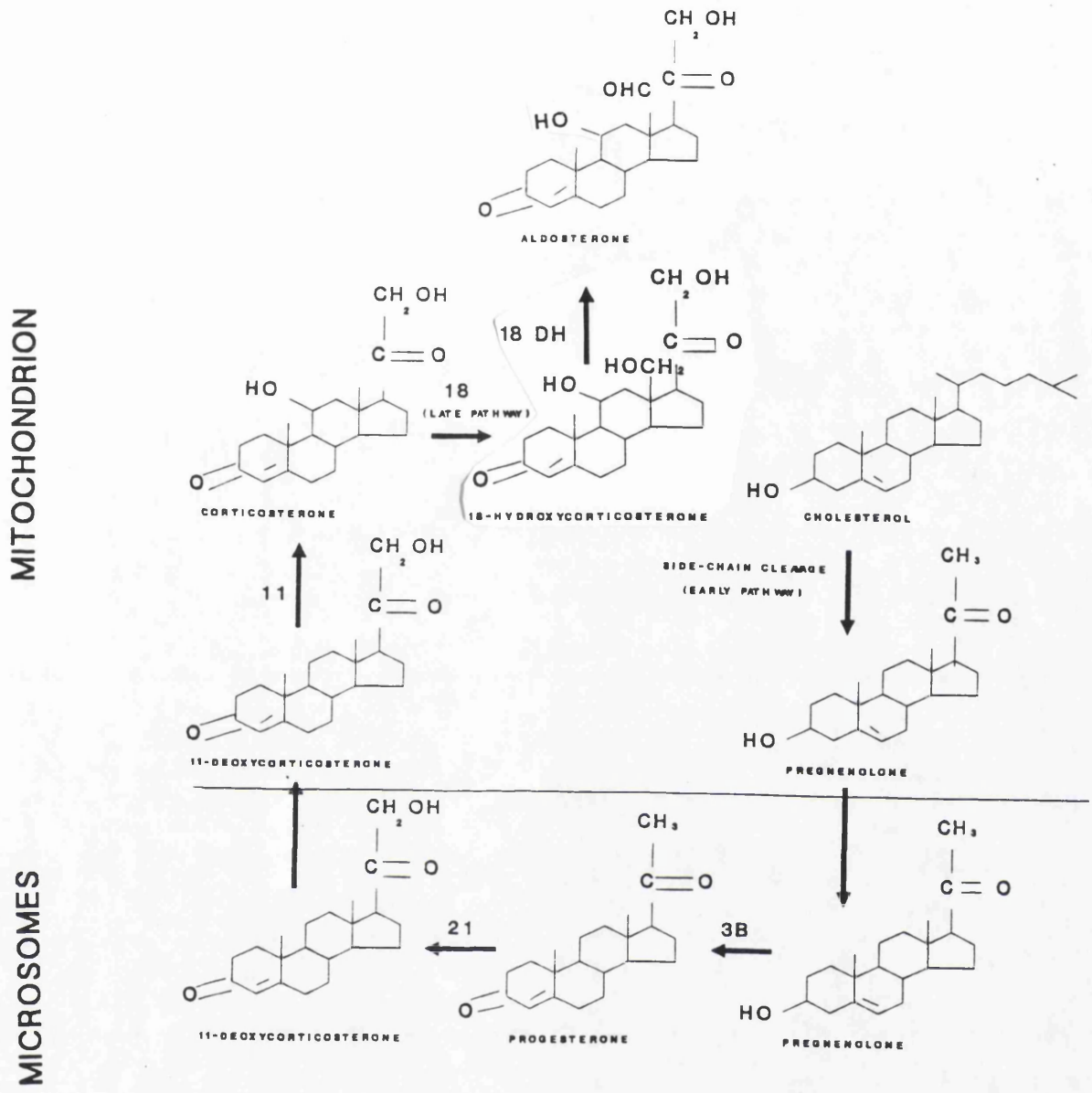
a) Cholesterol side chain cleavage.

b) C_{21} Hydroxylation.

c) C-11ß-18 Hydroxylation.

Cholesterol side chain cleavage and C-11ß-18 hydroxylation reactions take place in the mitochondria (4) whereas C_{21} hydroxylation is a microsomal reaction (5). The sequence of hydroxylations means that cholesterol and steroid intermediates shuttle between the cytoplasm, mitrochondria and microsomal compartments of the cell.

The first step, cholesterol side-chain cleavage, is an important rate-limiting step in aldosterone biosynthesis and is located on the matrix side of the inner mitochondrial membrane (6). The conversion of cholesterol to pregnenolone is a complex process which requires NADPH, H+ and 0_2 (7), for each of three consecutive mono-oxygenase reactions that result in the oxidative cleavage of a C-C bond in the side chain of cholesterol (8). Pregnenolone leaves the mitochondria and is then



3ß - 3ß-HYDROXYSTEROID DEHYDROGENASE.
21 - 21-HYDROXYLASE.
11 - 11ß-HYDROXYLASE.
18/18 DH - 18-HYDROXYLASE/DEHYDROGENASE.

Fig.1.3

The aldosterone biosynthetic pathway.

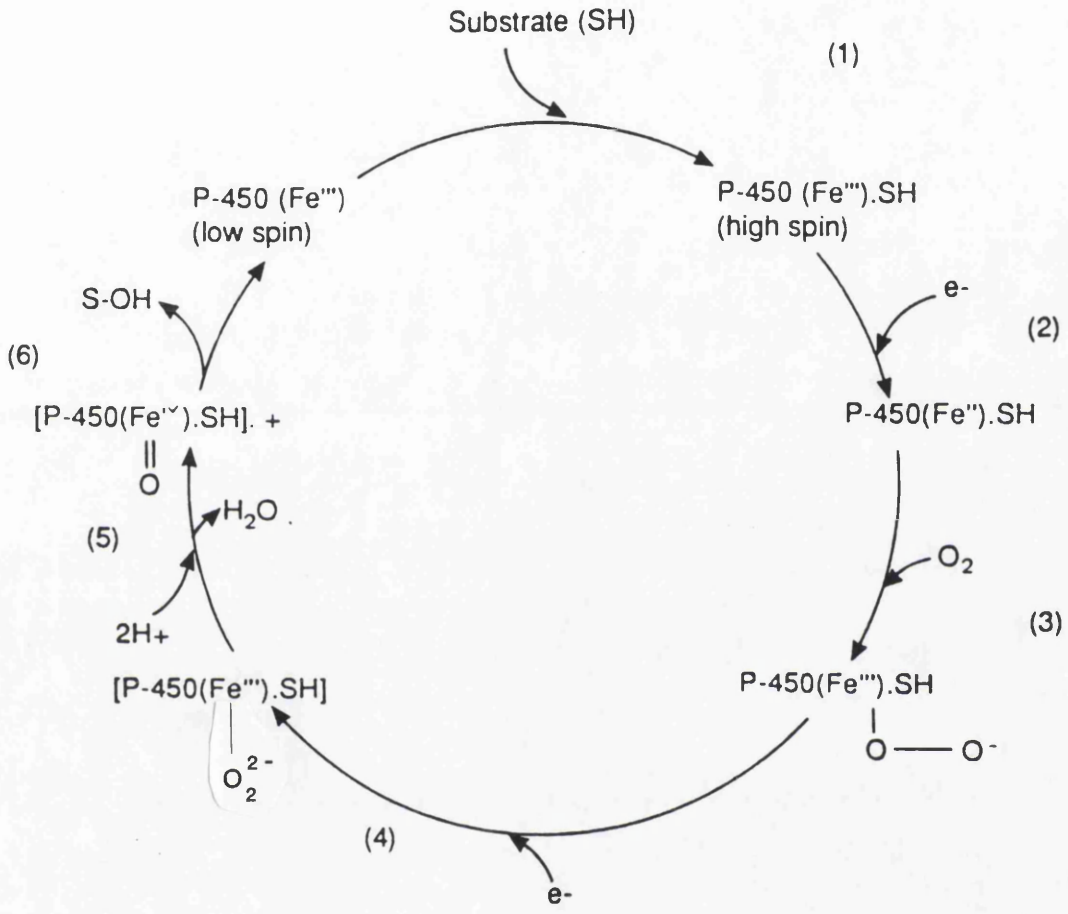
Redrawn from (3), Chattoraj, 1976.

converted to progesterone in two steps involving the oxidation of the hydroxy group at position 3 followed by the isomerisation of Δ^5 to a Δ^4 double bond. Deoxycorticosterone (DOC) is synthesised from progesterone by hydroxylation at C-21, a reaction catalysed by the microsomal enzyme, $P450_{21}$. DOC leaves the microsomal compartment and is converted to aldosterone in the mitochondrion by a series of hydroxylations reactions at C-11 and C-18 to form corticosterone (B) and 18OH corticosterone (18OHB) respectively. After a second hydroxylation at C-18 and a spontaneous loss of water, the aldehyde, aldosterone, is produced. (9).

1.4.2 Steroid Hydroxylase Enzymes.

All steroid hydroxylation reactions are catalysed by cytochrome P-450-containing mixed function oxidases. These enzymes are a family of heme-containing mono-oxygenases found in a number of tissues but perhaps most studied in the liver where they are involved in the detoxification of drugs and other substances (10). In adrenocortical cells, these enzymes are found in the inner mitochondrial membrane and in the microsomal compartment. Cytochrome P-450 itself is the terminal component of an electron transport chain whose role is hydroxylation, primarily of lipophilic substances, rather than oxidative phosphorylation (Fig.1.4). Reducing power is supplied in the form of NADPH + H^+ which transfers its high potential electrons to a flavoprotein in this chain. The electrons are then conveyed to the non-heme iron protein adrenodoxin. Microsomal hydroxylases do not contain adrenodoxin but instead a similar role for cytochrome b_5 has been suggested in electron transport to $P450_{21}$ (11). Adrenodoxin then transfers an electron to the oxidised form of cytochrome P-450. Reduced P-450 then activates molecular oxygen (O_2), one atom of which is inserted into the substrate and the other reduced to water (Fig.1.4).

$$\text{STEROID} + \text{NADPH} + H^{+} + O_2 \longrightarrow \text{STEROID-OH} + \text{NADP}^{+} + H_2O$$



Probable events in P450 catalyzed steroid hydroxylation. (1) Interaction of steroid with P450, to give the ferric complex. (2) One electron reduction. (3) Formation of P450-substrate-oxygen complex. (4) One electron reduction. (5) Addition of $2H^{+}$ and elimination of water, leaving proposed activated atomic oxygen species. [ ] = hypothetical intermediate. (6) Spatial juxtaposition of active oxygen and substrate→hydroxysteroid and ferric P450 in concerted action

Fig.1.4

Action of cytochrome P-450 steroid hydroxylases.

From (27), Vinson et al, 1992.

Hence these enzymes are also referred to as monoxygenases.

1.4.3 Zone-Specific Synthesis of Aldosterone.

Aldosterone is synthesised only in the zona glomerulosa and evidence originally suggested the existence of a specific enzyme, corticosterone methyl oxidase catalysing the final two steps of aldosterone synthesis (12). In a "two enzyme" model (13) this would catalyse only the conversion of corticosterone to aldosterone and be found exclusively in the zona glomerulosa. The preceeding step, conversion of DOC to B would be catalysed by $P450_{11\beta}$ found in all zones of the adrenal cortex. This appears to be the case in the rat and in man where two forms of $P450_{11\beta}$ (henceforth designated $P450_{11\beta}$ and $P450_{aldo}$) with molecular weights 51Kd and 49Kd have been identified (14,15). $P450_{aldo}$ differs from the major form of the enzyme ($P450_{11\beta}$) in several important ways. Firstly, it is localised strictly to the two or three outermost cell layers in the zona glomerulosa (16) and secondly its expression is dependent upon dietary sodium and potassium intake: the enzyme can be induced in rats by sodium restriction (13,17) or by potassium repletion following a potassium deficient diet (13). Finally, and most importantly, it has the ability to synthesise aldosterone. However, in other species, most notably in the bovine adrenal gland, there appears to be one multi-functional enzyme $P\text{-}450_{11\beta}$ throughout the cortex which is capable of catalysing 11ß hydroxylation of DOC and deoxycortisol to corticosterone and cortisol respectively and of the conversion of corticosterone to aldosterone (18,19). It follows that if aldosterone is synthesised exclusively in the zona glomerulosa, then this enzyme complex must be under zone specific negative control in the ZF/R. A number of hypotheses have been put forward to explain this regulation.

(i) **Substrate inhibition**: Hornsby (20) proposed that accumulated ZF/R steroid products could act either as pseudosubstrates for 11ß hydroxylase, a process resulting in superoxide production and enzyme inactivation or as real substrates (the most likely candidates being cortisol (21) or androstendione) for 18 hydroxylation.

(ii) **Calmodulin-mediated inhibition**: Calcium-activated calmodulin (see later) selectively inhibits the aldosterone-synthesising activity of purified bovine 11ß hydroxylase (22) and a calmodulin-like protein factor may be present in ZF/R cells which mediates this effect.

(iii) **Membrane phospholipids**: Enzyme configuration and catalytic activity can be determined by membrane phospholipid composition (23). Subtle differences in the phospholipid content of ZG and ZF/R mitochondria may account for differences in steroid output.

(iv) **Availability of reducing power**: ZG mitochondria contain higher concentrations of the enzyme semidehydroascorbate reductase than those of the ZF/R (24). Yanagabashi et al (24) have suggested that this enzyme provides increased reducing power to drive the additional hydroxylation reactions involved in aldosterone biosynthesis.

(v) **Oxygen sensitivity**: It has been demonstrated in bovine cells that aldosterone synthesis is particularly sensitive to pO_2 whereas corticosterone synthesis from DOC is not (25). Given the centripetal flow of blood in the cortex this means that the ZG on the outside is exposed to higher concentrations of O_2 which will allow 18 hydroxylation whereas the inner zones with lower concentrations of O_2, can only carry out 11ß hydroxylations (26).

1.5 Regulation of Aldosterone Secretion (an overview).

Aldosterone synthesis, in vivo, is under both positive and negative control by a number of factors (Fig.1.5). Those exerting a positive influence include angiotensin-II (A-II) and adrenocorticotropin hormone (ACTH) while atrial natriuretic factor (ANF) and perhaps dopamine are inhibitory. Cholesterol supply to the gland may also be rate-limiting under certain circumstances. Sodium and potassium can affect both stimulatory and inhibitory pathways depending upon their relative dietary intake. For example, sodium restriction increases aldosterone synthesis, while sodium loading inhibits synthesis. The opposite is true of potassium. There are also a number of other factors (see later) which may influence secretion but whose exact physiological role remains uncertain.

1.6 The Renin-Angiotensin System.

The circulating renin-angiotensin system (RAS) plays a crucial role in the regulation of arterial blood pressure, renal haemodynamics and fluid and electrolyte balance. The proteolytic enzyme renin is synthesised and secreted from juxtaglomerular cells (located in the afferent arteriole of the kidney) in response to a number of factors including reduced extracellular volume, low concentrations of sodium in the glomerular filtrate, high dietary protein and sympathetic stimulation of juxtaglomerular cells (27,28). Within plasma, renin cleaves a decapeptide, angiotensin I, from the amino terminus of angiotensinogen, a 452 amino acid single polypeptide (29), which is released from the liver under the control of a number of factors including glucocortocoids, estrogens and thyroid hormones (30). When the two carboxy-terminal amino acids of angiotensin I are subsequently removed by angiotensin converting enzyme (ACE) on passage through capillary beds,

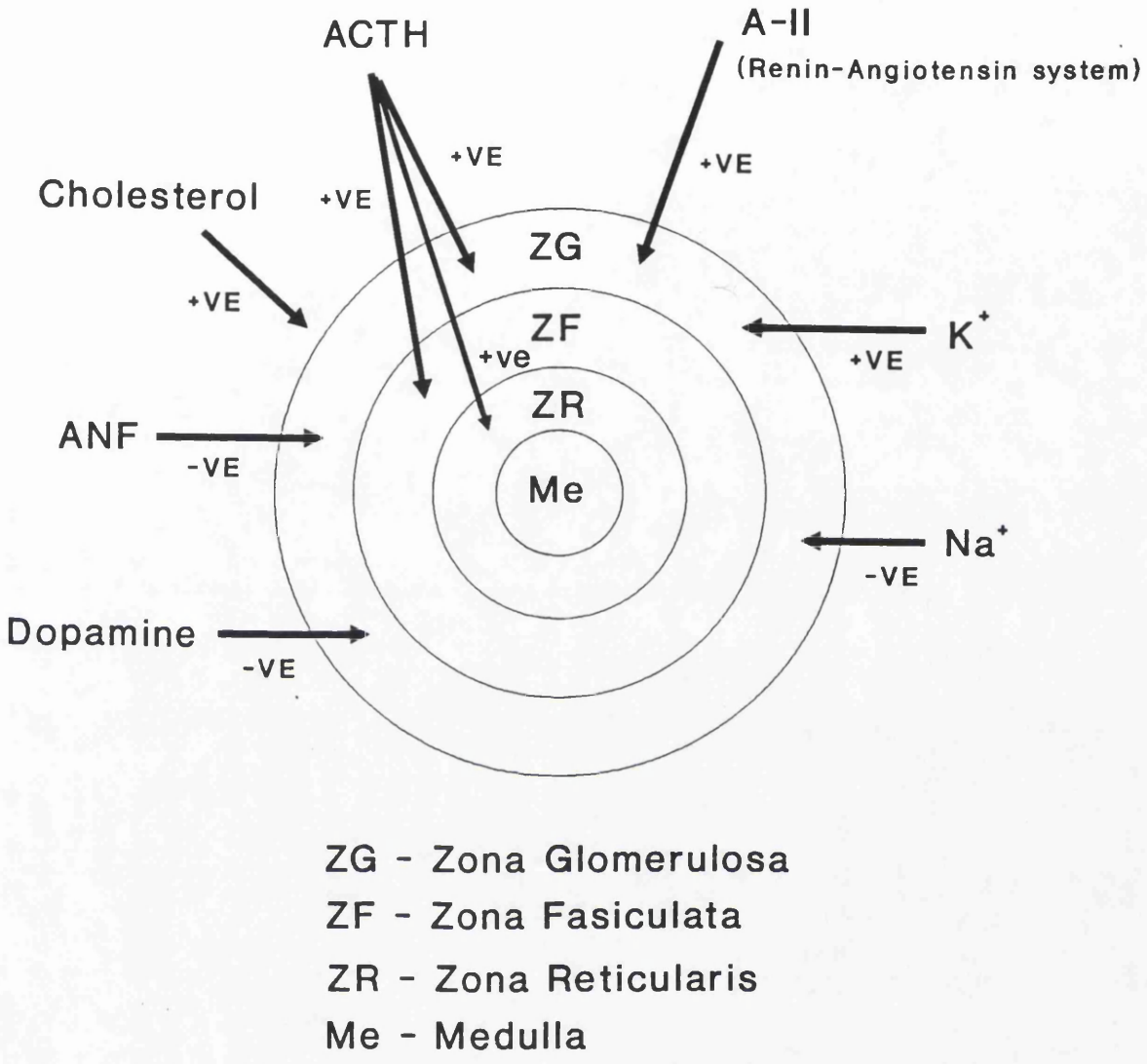


Fig.1.5

The adrenal gland and zonal regulation of steroid synthesis.

particularly in the lungs, a potent pressor agent, angiotensin II (A-II), is formed. Circulating A-II initiates a complex series of physiological events which work together to restore plasma volume, sodium concentration and blood pressure. These events include vasoconstriction, stimulation of catecholamine release, increased renal blood flow, thirst stimulation and inhibition of renin release (27). Circulating A-II is also a key factor in the control of aldosterone secretion. Recently, it has also become clear that local renin-angiotensin systems are present within many tissues (31,32). Certainly, individual components of such a system, adrenal renin and ACE, have been demonstrated to exist in the adrenal gland (33,34) and are known to respond to other physiological effectors of A-II secretion such as changes in electrolyte balance (35,36). Such a self-contained adrenal renin-angiotensin system may exert powerful autocrine and paracrine influences on steroid synthesis (37).

1.7 Stimulation of Aldosterone Synthesis by Angiotensin-II.

A-II increases the rate of aldosterone biosynthesis by increasing the rates of both the early (conversion of cholesterol to pregnenolone) and late pathways (conversion of corticosterone to aldosterone) (38).

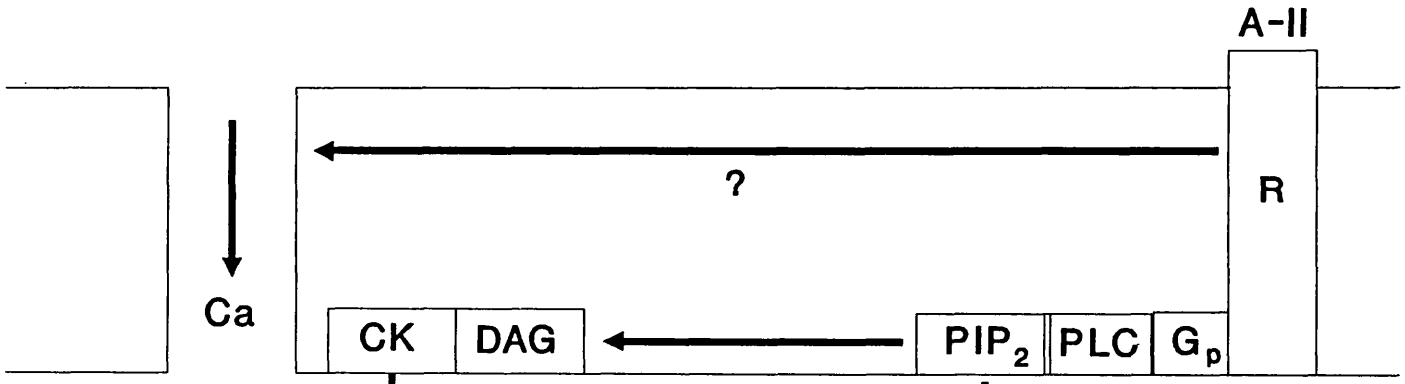
Two classes of A-II receptor have been described in bovine, canine and human glomerulosa cells (39);a high affinity and a low affinity site. Rats possess high affinity sites only. Their relative numbers are modulated by electrolyte balance.

The A-II receptor in glomerulosa cells is coupled to at least two cell-signalling systems, the polyphosphatidylinositol system and the adenylate cyclase system. (39,40).

.1 The Polyphosphatidylinositol System.

The binding of A-II to its receptor results in rapid activation of phospholipase C (PLC), a phosphinositide-specific phosphodiesterase. This enzyme catalyses the breakdown of phosphatidylinositol 4,5 bisphosphate (PIP_2) to form the second messengers inositol(1,4,5) trisphosphate (IP_3) and 1,2 diacylglycerol (1,2 DAG) (Fig.1.6) (41). This phosphoinositide response to A-II can be enhanced by non-hydrolysable GTP analogues and is mediated by an as yet uncharacterised, pertussis-insensitive regulatory GTP-binding protein (G-protein), designated G_p (G-proteins are located within the plasma membrane and play a crucial role in signal transduction mechanisms by coupling occupied receptors to their associated effector systems). Both IP_3 and 1,2 DAG appear to be elevated throughout the duration of A-II stimulation and each seems to be necessary for a normal physiological response (39).

IP_3 formation is rapid, biphasic and is dependent upon A-II concentration (27). An early peak is observed within a few seconds of exposure to A-II, followed by a nadir at about 30 seconds and then a continuous increase which lasts for at least 30 minutes (40). The sustained increase in IP_3 is dependent upon extracellular calcium $[Ca^{2+}]_o$, whereas the initial generation of IP_3 is not (42). Both $[Ca^{2+}]_o$ and IP_3 are essential for a sustained aldosterone secretory response. It has been demonstrated that IP_3 is inactivated by successive dephosphorylations giving consecutively inositol 1,4 bisphosphate, inositol phosphate and finally inositol which can be used to replenish PIP_2 (Fig.1.7) (41). Alternatively, IP_3 can first be phosphorylated by a 3-kinase to yield inositol 1,3,4,5 tetrakisphosphate and then dephosphorylated to give inositol 1,3,4 trisphosphate which may itself be further phosphorylated or dephosphorylated. After stimulation with A-II the rise in cytoplasmic free calcium concentration $[Ca^{2+}]_i$, activates 3-kinase and more

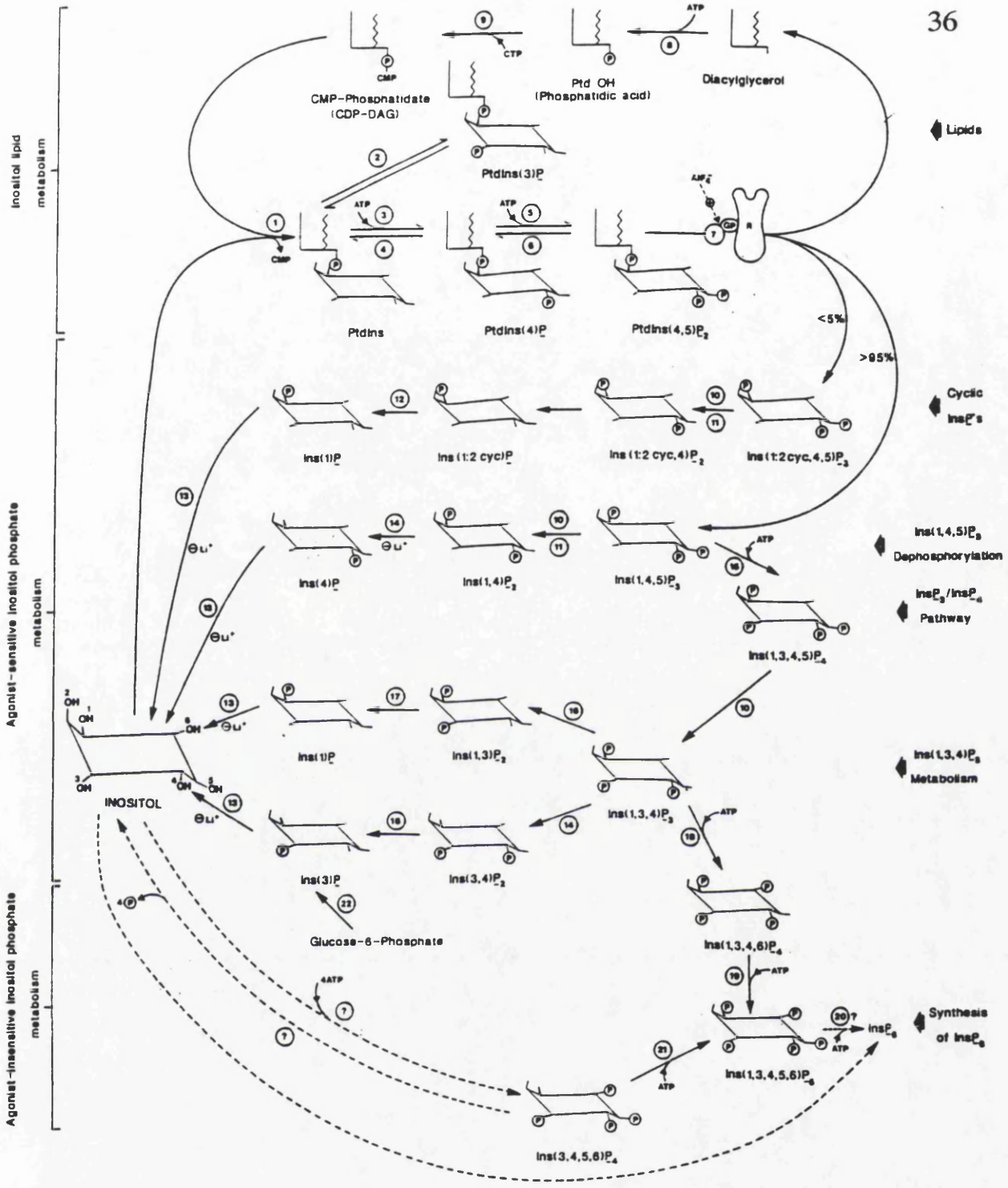


KEY

R - RECEPTOR
G_p - G-PROTEIN
PLC - PHOSPHOLIPASE C
PIP_2 - PHOSPATIDYLINOSITOL 4,5 BISPHOSPHATE
IP_3 - INOSITOL 1,4,5 TRISPHOSPHATE
DAG - 1,2 DIACYLGLYCEROL
CK - PROTEIN KINASE C

Fig.1.6

Angiotensin-II-activated inositol phosphate signalling in zona glomerulosa cells.



Summary of known (solid arrows) and suspected (dashed arrows) routes of metabolism of compounds containing inositol and phosphate. To avoid further complexity, the phosphatidylinositol glycans are not illustrated. myo-inositol is represented in its chair configurationand all the inositol phosphates are numbered in the D-isomer configuration. The enzymes are 1. PtdIns synthetase; 2. PtdIns-3-kinase; 3. PtdIns-4-kinase; 4. PtdIns(4)P phosphomonoesterase; 5. PtdIns(4)P-5-kinase; 6. PtdIns(4,5)$_2$ phosphomonoesterase; 7. phosphoinositidase C; 8. diacylglycerol kinase; 9. CMP-PA synthetase; 10. Ins(1,4,5)P_3/Ins(1,3,4,5)P_4-5-phosphatase; 11. Ins(1,4,5)P_3-5-phosphatase; 12. Ins(1:2 cyc)P phosphodiesterase; 13. InsP phosphatase; 14. inositolpolyphosphate-1-phosphatase; 15. Ins(1,4,5)P_3-3-kinase; 16. inositolpolyphosphate-4-phosphatase; 17. Ins(1,3)P_2-3-phosphatase; 18. Ins(1,3,4)P_3-6-kinase; 19. Ins(1,3,4,6)P_4-5-kinase; 20. Ins(1,3,4,5,6)P_5-2-kinase; 21. Ins(3,4,5,6)P_4-1-kinase; 22. Ins(3)P-synthetase.

Fig.1.7

Metabolism of inositol phosphates.

From (44), Berridge and Irvine, 1989.

inositol 1,3,4 trisphosphate is converted into higher inositol phosphates. The significance of these higher inositol phosphates in glomerulosa cells, stimulated long-term with A-II, is still unknown but they may be involved in steroidogenic control

The rate of 1,2 diacylglycerol (DAG) formation is similar to that of IP_3 production. Sustained DAG production in response to A-II is calcium-sensitive (39,40). However, unlike IP_3 formation, the sustained DAG response to A-II is not totally abolished by low $[Ca^{2+}]_o$ (42). This difference in $[Ca^{2+}]_o$ dependency between IP_3 and DAG responses indicates that a substantial amount of DAG may be formed from sources other than PIP_2. A-II has also been shown to stimulate phospholipase D activity which catalyses the breakdown of a variety of phospholipids to form phosphatidic acid which can then be readily converted to DAG (43). DAG can be removed by two metabolic pathways: either by conversion to phosphatidic acid for re-entry into the phosphoinositide cycle (44) or by hydrolysis which releases arachidonic acid (45). Arachidonic acid provides substrate for cycloxygenase and lipoxygenase enzymes, the products of which, including thromboxanes and prostaglandins, have been implicated in steroidogenic control (see later).

1.7.2 The Adenylate Cyclase System.

This well-characterised signal transduction pathway (46) couples a receptor, via one of two main types of G-protein, G_s or G_i, to the enzyme adenylate cyclase (Fig.1.8). Activation of G_s stimulates enzyme activity and activation of G_i inhibits it. Adenylate cyclase activation results in production of cAMP which mediates its effect on cell function through activation of protein kinase A. In adrenal cells, A-II reduces both basal and stimulated production of cAMP through G_i, although at the present time this inhibitory effect has not been shown to be of physiological

significance (39).

1.7.3 The Calcium Response to Angiotensin-II.

$[Ca^{2+}]_i$ serves as a messenger during both the initiation and maintenance of an aldosterone secretory respose to A-II. The mechanism of messenger generation, the spatial domain in which the messenger operates and the molecular targets of calcium action differ between two temporal phases which are termed the initial phase and the sustained phase (39,40).

(i) Initial phase: There are intracellular binding sites in the ZG which are specific for IP_3 and have a very low affinity for other inositol phosphates (27). In bovine cells, these binding sites are located on vesicles loosely attached to the plasma membrane, which contain stored calcium (39). Binding of IP_3 to its receptor, a glycoprotein which requires binding of four IP_3 molecules for activation, is followed by Ca^{2+} release through an opened membrane channel (47). In bovine glomerulosa cells, this transient rise in $[Ca^{2+}]_i$ achieves a maximum within 0.5 - 3min before falling to a sustained plateau which is still above basal levels (39). The initial calcium spike is sufficient to activate the Ca^{2+}-binding protein calmodulin (48) which affects cell function by phosphorylating target proteins (see later).

(ii) Sustained Phase: After the initial response, $[Ca^{2+}]_i$ remains only minimally elevated. It is widely accepted that the sustained phase of the Ca^{2+} signal is maintained by Ca^{2+} influx across the plasma membrane. However, there is some controversy concerning the participation of voltage-sensitive and insensitive mechanisms in this process.

The resting membrane potential of the glomerulosa cell is -70mv and is similar

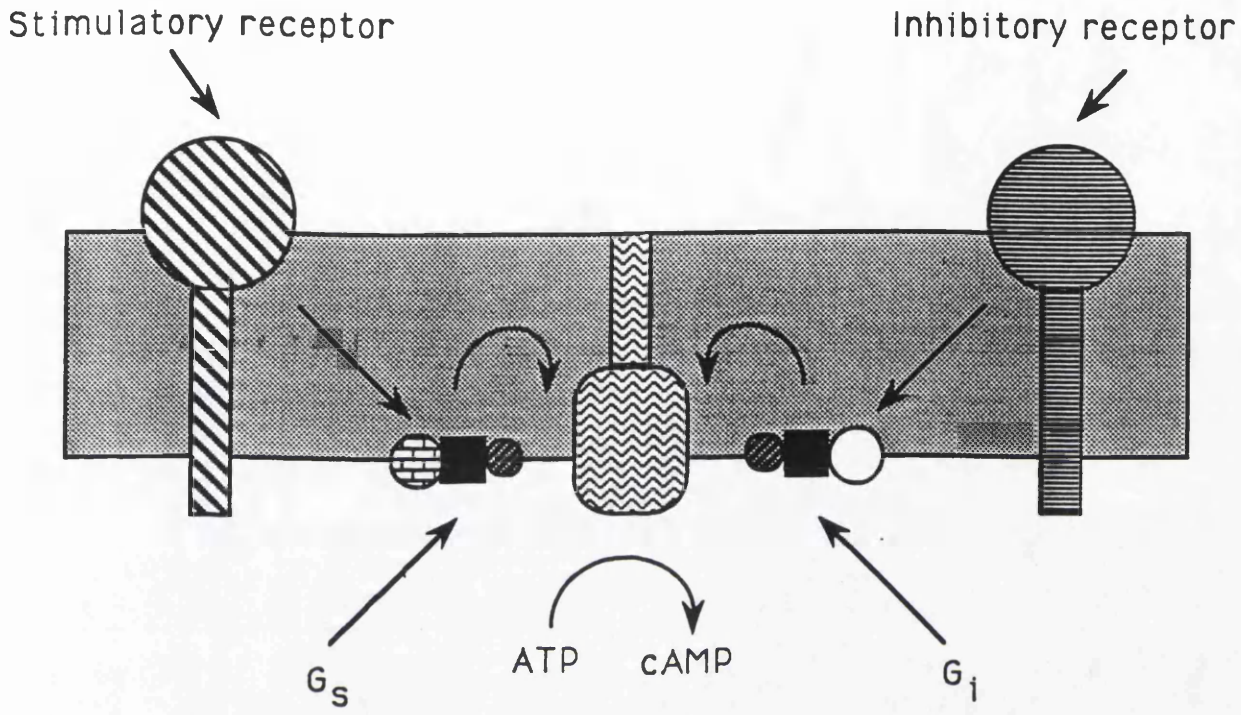


Fig.1.8

Dual control of adenylate cyclase by the regulatory proteins, G_s and G_i.

to that found in other types of excitable cell. This value is totally dependent upon the permeability of the cell membrane to K^+ ions. Any decrease in membrane permeablity to this cation and the cell depolarises. Calcium channels which are activated by cell depolarisation, have been identified in plasma membranes of excitable cells. There is a large literature available on these voltage-sensitive calcium channels (VSCC) but in relation to this study only two types are relevant; the L-type and the T-type (49,50). Both have been identified in the bovine glomerulosa cell (51).

L-type channels have high Ca^{2+} conductance and a prolonged opening time but have a high threshold of activation (-30mv). They are the targets of calcium channel blockers such as the dihydropyridines nitrendipine and nifedipine.

T-type channels have lower activation thresholds (-60mv) than L-type channels but also have lower Ca^{2+} conductance and a shorter opening time. They are insensitive to dihydropyridines but sensitive to catecholamines.

Of these two channel types, only T-type activation may be important in the glomerulosa cell since the L-channel activation threshold (-30mv) is too far from the resting membrane potential to be of physiological relevance (-70mv).

Following A-II stimulation, there is increased $[Ca^{2+}]_o$ uptake by the glomerulosa cell and this uptake is sensitive to pharmacological blockade by VSCC blockers such as the dihydropyridines (52). Electrophysiological evidence suggests that binding of A-II to its receptor leads to depolarisation of the glomerulosa cell by closing membrane potassium channels (53). This conclusion is supported by isotopic studies which measured potassium efflux in isolated bovine glomerulosa cells pre-loaded with ^{43}K (54). Efflux was determined by measuring the radioactivity remaining in cells after the allocated incubation periods. Complex kinetic analysis of experimental

data derived from these studies demonstrated that ^{43}K efflux from glomerulosa cells could be divided into two components, a fast exchange component (t 1/2 approx. 15 mins.) and a slow exchange component (t 1/2 approx. 48 mins.). A-II stimulation produced opposite effects on these two components, inhibiting efflux from the fast exchange component but stimulating efflux from the slow exchange component. These data support a model in which potassium channels close on receptor occupation, leading to membrane depolarisation and increased uptake of $[Ca^{2+}]_o$ through VSCC. The increased $[Ca^{2+}]_i$ concentration then allows opening of Ca^{2+}-sensitive potassium channels (55,56), which have been identified in mouse Y-1 adrenocortical cells (57), and the repolarisation of the plasma membrane.

In contrast, several reports have suggested that $[Ca^{2+}]_o$ uptake during the sustained phase of the A-II response occurs through dihydropyridine-insensitive mechanisms (40). Membrane Na^+/K^+ ATP'ase activity has been implicated in this process. A-II inhibits this ion transport system (58) thereby increasing intracellular Na^+ concentration which, in turn, increases $[Ca^{2+}]_i$ through increased Na^+/Ca^{2+} exchange. However, given that Na^+/K^+ ATP'ase activity is also involved in potassium transport it is likely that A-II inhibition, like ouabain which is an inhibitor of Na^+/K^+ ATP'ase (58), would depolarise the cell, also inducing calcium influx.

Whether voltage sensitive or insensitive mechanisms of Ca^{2+} entry are more important during the sustained phase of A-II action is difficult to ascertain. Possibly a model involving both mechanisms is more accurate (40).

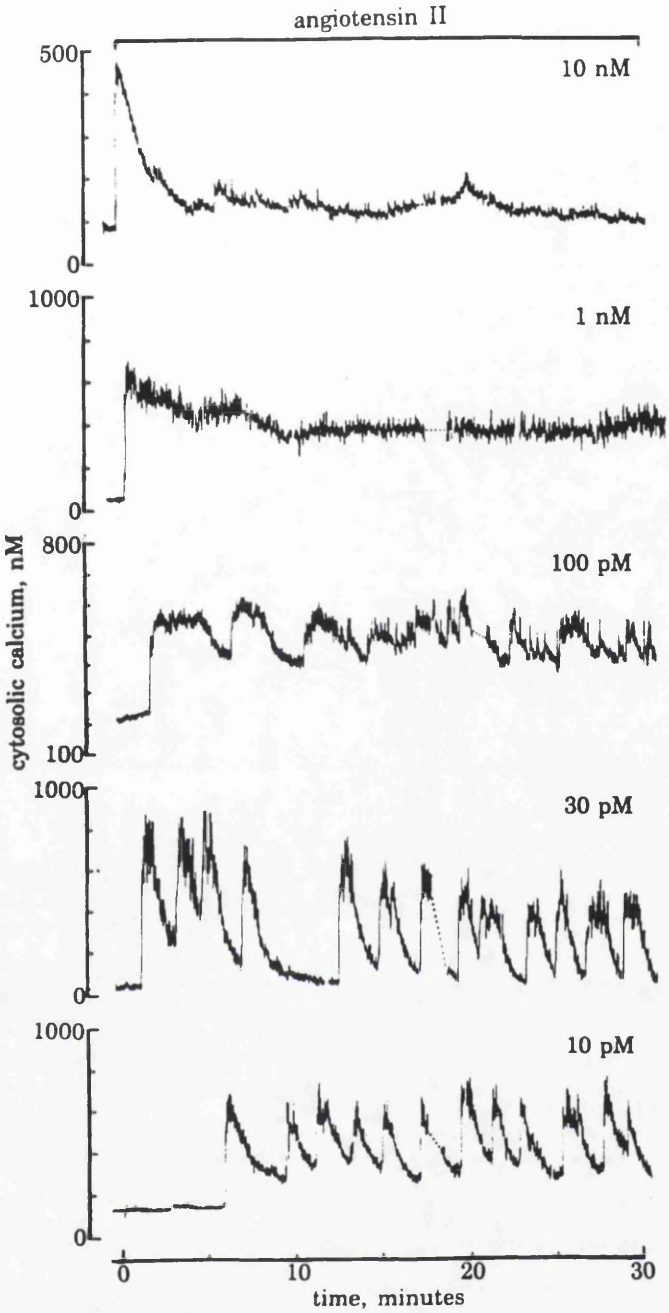
(iii) $[Ca^{2+}]_i$ oscillations: Oscillations of cytosolic Ca^{2+} have been observed in many cell types during hormonal stimulation and it has been suggested that these oscillations represent a process whereby initial agonist-stimulated release of calcium from an IP_3-sensitive pool acts as a primer for further calcium release from IP_3-

insensitive pools, producing a calcium spike that spreads like a wave throughout the cell (59). Individual calcium responses of single glomerulosa cells to A-II have been examined using the calcium-chelating fluorescent dye, Fura-2 (60), monitored either by digital video or by microspectrofluorometric methods (39). In general, these studies have demonstrated the heterogeneity of the intracellular calcium response to A-II. In bovine and rat cells, the concentration of A-II used influences not only the lag-time preceeding the initial IP_3-mediated Ca^{2+} spike but also determines the frequency of subsequent $[Ca^{2+}]_i$ oscillations which merge to form peak plateau waveforms at higher hormone concentrations (Fig.1.9). In the glomerulosa cell, it has been suggested that the frequency of oscillation governs the steroidogenic response (61).

1.7.4 Role of $[Ca_{2+}]_i$ in Angiotensin-II-Stimulated Aldosterone Synthesis.

Increases in $[Ca^{2+}]_i$ are thought to stimulate aldosterone synthesis by activating protein kinases, thereby altering cellular protein phosphorylation. The sequential production of different inositol phosphates and the complexity of the calcium message have led to the proposal that the protein kinases involved in the initial and sustained response of the glomerulosa cell to A-II may be different (39).

Ca^{2+}-activated Calmodulin: Many of the actions of calcium are mediated by calmodulin. The catalytic activity of this widely distributed protein, which has been identified in the adrenal cortex, is calcium-dependent. Following a brief exposure to A-II, several cellular proteins become transiently phosphorylated through the actions of Ca^{2+}/calmodulin-dependent protein kinase(s) (39). Some possible candidates for calmodulin action have been identified in the adrenal Y-1 cell line and include several kinases and phosphatases (39). It has also been proposed that



Time course of the Ca_i responses to different Ang-II concentrations. Traces are representative Ca_i recordings from 5 separate ZG cells during puffer superfusion with the indicated concentrations of Ang-II. The duration of stimulation was 30 min, as indicated by the *horizontal bar above the traces*. Similar results were found for 10–15 cells at each hormone concentration.

Fig.1.9

Dose and time-dependency of the intracellular calcium response of rat zona glomerulosa cells to angiotensin-II.

From (61), Quinn et al, 1991.

calmodulin alters cholesterol transport into the mitochondrion by modifying cytoskeletal architecture (27). Further evidence for a role for calmodulin in activating aldosterone synthesis comes from studies using the calmodulin inhibitors W7 and Calmidazolium (63). Both compounds markedly reduce A-II-stimulated synthesis in calf glomerulosa cells.

Protein Kinase C (PKC): PKC is a cytosolic enzyme whose activation requires two effectors (64), Ca^{2+} and DAG. Upon stimulation of bovine adrenocortical cells with A-II, PKC is translocated from the cytosol to the plasma membrane (65). This not only positions the enzyme in a phospholipid envirnoment, which enhances calcium binding (64), but also brings it into close proximity to high local concentrations of Ca^{2+} and DAG (66). In concert, Ca^{2+} and DAG induce PKC autophosphorylation, causing a conformational change in the enzyme that allows substrate binding and subsequent catalysis (64). It has been reported that during the sustained secretory response, there is a group of proteins which are phosphorylated after a 30 minute but not a 1 minute incubation period (39). It is likely that these phosphoproteins are substrates of PKC, since this pattern of phosphorylation can be reproduced by pharmacological agents, such as phorbol esters and Ca^{2+} ionophores, which substitute for A-II-induced DAG and Ca^{2+} influx respectively. Taken together, these data suggest that PKC mediates the sustained A-II induced elevation of aldosterone secretion whereas the A-II-elicited calcium transient mediates the initial response via activation of Ca^{2+}/calmodulin-dependent proteins.

However, not all evidence points to a central role for PKC in the sustained aldosterone response. Membrane translocation of PKC has not been demonstrated in rat glomerulosa cells and the authors of recent studies in calf cells using inhibitors of PKC and Ca^{2+}/calmodulin concluded that the latter factor is the critical mediator

of both the initial and sustained phases of aldosterone secretion (63). Therefore a role for PKC in the control of aldosterone synthesis by A-II has yet to be confirmed.

1.7.5 Role of Mitochondrial calcium in Angiotensin-II-Stimulated Aldosterone Synthesis.

Changes in mitochondrial free calcium concentration may contribute directly to the regulation of the mitochondrial steroidogenic enzymes. There are reports that Ca^{2+} ions activate P-450_{scc}(67) and P-45011ß (27), although others claim that Ca^{2+} ions inhibit P-450_{scc} (68). Mitochondrial calcium fluxes also affect the concentration of mitochondrial pyridine nucleotides which supply the reducing power for steroid biosynthesis (69). Recent work has demonstrated that A-II, whilst increasing $[Ca^{2+}]_i$ also lowers total mitochondrial calcium content (70). It is interesting to speculate that mitochondrial calcium may play a role not only in activation of steroidogenic enzymes but also in the control of cytosolic calcium.

1.8 ACTH Stimulation of Aldosterone Synthesis.

ACTH is synthesised in the pituitary under the control of corticotropin releasing factor, ANP and circulating glucocorticoids. It is undoubtedly the major regulator of inner zone adrenocortical function. However, its role in the physiological control of aldosterone secretion is less clear and administration of ACTH in vivo has been demonstrated to both increase and decrease aldosterone synthesis (27).

In vitro, ACTH is a most effective stimulus to aldosterone secretion giving a maximal response which is often greater than that of other glomerulosa stimulants (71). The ACTH receptor is linked to adenylate cyclase and receptor occupation

stimulates the enzyme to increase intracellular cAMP via a regulatory G_s protein. Raised levels of cyclic nucleotide then stimulate cAMP-dependent protein kinase which is located in the cytosol of the adrenocortical cell. This activated protein kinase phosphorylates several cytosolic and microsomal proteins. For example, the enzyme cholesterol ester hydrolase, when activated, increases the pool of intracellular free cholesterol (27). cAMP also has an effect at the gene level, promoting transcription of steroidogenic enzymes and their associated electron transport systems (72).

It has been demonstrated that low concentrations of ACTH can also stimulate steroid synthesis in the absence of any measurable rise in cAMP (27). This may be explained by the discovery of two forms of ACTH receptor; a high affinity site and a low affinity site. It has been proposed that high concentrations of ACTH stimulate cAMP formation by activating the low affinity receptor, while low concentrations of ACTH acting via the high affinity receptor stimulate steroid synthesis by a mechanism less dependent on adenylate cyclase (27). The high affinity receptor may be coupled to the calcium messenger system. An intracellular calcium response to ACTH, albeit different to that elicited by A-II, has recently been demonstrated in both rat and bovine glomerulosa cells (73). In most cases, addition of ACTH leads to a slow increase in $[Ca^{2+}]_i$ after a long-lasting delay period (10-15mins). This response is dependent on $[Ca^{2+}]_o$, is dihydropyridine-sensitive and is inhibited by blockers of cAMP-dependent protein kinase suggesting that the rise in $[Ca^{2+}]_i$ induced by ACTH results from calcium influx through voltage-sensitive channels which need to be phosphorylated by cAMP-dependent protein kinase for full activation. Further evidence of a role for calcium in mediating steroidogenic responses to ACTH comes from work with murine Y-1 tumour cells which showed that calmodulin inhibitors

markedly reduced production of both steroids and cAMP in response to ACTH and forskolin. This would imply that the ACTH receptor, acting via G_s, can stimulate steroidogenesis through activation of Ca^{2+}/calmodulin (74). Additionally, in rat and bovine cells, acute (mins) but not long-term (hours) exposure to ACTH increases intracellular DAG and induces PKC translocation to the plasma membrane, implicating a possible for phosphatidyl inositol metabolism in corticotropin action (27).

1.9 Effects of Eicosanoid Metabolites on Aldosterone Synthesis.

Both A-II and ACTH stimulate production of DAG (see above) which provides a common pathway through which both hormones may influence aldosterone secretion. DAG, released in response to hormonal stimulation, can be metabolised by two pathways. It can be converted to phosphatidic acid and then re-enter the phosphoinositide cycle (44) or it can be hydrolysed to release arachidonic acid (45). Arachidonic acid can be further metabolised by three pathways (75) (Fig.1.9);

(a) The cyclo-oxygenase pathway giving prostaglandins (PG's) and thromboxanes.

(b) The lipo-oxygenase pathway giving hydroxyperoxyeicosatetraenoic acids (HPTET's), hydroxyeicosatetraenoic acids (HETE's) and leukotrienes (LT's).

(c) The cytochrome-P450 pathway giving epoxyeicosatrienoic acids (EET's) dihydroxyeicosatrienoic acids and HETE's.

Many studies indicate that arachidonic acid metabolites such as prostaglandins (76) and, more recently, the 12-lipoxygenase product 12-HETE (77,78) augment aldosterone production in the adrenal cortex. However, much of this work was done by using pharmacological inhibitors of the cyclo-oxygenase and lipo-oxygenase pathways rather than examining direct effects of prostaglandins and 12-HETE on

steroid synthesis. Therefore a role for these metabolites in the regulation of aldosterone secretion remains controversial.

1.10 Potassium Ion Activation of Aldosterone Synthesis.

The glomerulosa cell is an extremely sensitive sensor of extracellular potassium such that small elevations in plasma potassium within the physiological range can induce significant increases, not only in aldosterone secretion but also in the expression of steroidogenic enzymes (79,80). Physiologically, the most important action of potassium on the glomerulosa cell is probably to affect its sensitivity to A-II (27); low plasma potassium concentrations decrease the aldosterone response to A-II and high plasma potassium increases it (39). The opposite is true for plasma sodium. This is consistent with aldosterone's role in maintaining electrolyte balance; stimulation of sodium reabsorption and potassium excretion.

In vitro, potassium has a direct action on the glomerulosa cell. Electrophysiological studies have demonstrated that elevated extracellular potassium, $[K^+]_o$, triggers aldosterone secretion exclusively by depolarising the cell membrane and opening voltage-sensitive calcium channels (VSCC's) (40). The involvement of these channels is substantiated by studies in which dihydropyridines were found to inhibit calcium influx rate, $[Ca^{2+}]_i$ elevation and the aldosterone secretory response (81,82). It has been proposed that both the L and T-type VSCC's are involved in mediating the potassium response and that the extent of their involvement is dependent upon membrane potential (83). However, considering that the threshold potential of the L-channel (-30mv) can hardly be attained not only in vivo but also with high potassium concentrations in vitro, their participation in the generation of the calcium signal is unlikely and it is probably the T-channels that mediate this response (40).

In rat, the concentration of $[K^+]_o$ is around 3.6mM. Stepwise increases induce stepwise elevations in $[Ca^{2+}]_i$ and a corresponding increase in aldosterone secretion up to a maximum at around 10mM $[K^+]_o$. Above this concentration, although $[Ca^{2+}]_i$ remains elevated, aldosterone production decreases, resulting in a bell-shaped dose-response curve and a loss in correlation between intracellular calcium and steroid synthesis (84). It is interesting to note that concentration of reduced pyridine nucleotides, which supply reducing power to steroidogenic enzymes, mirrors this aldosterone response to potassium (69). The increase in $[Ca^{2+}]_i$ appears to mediate potassium-stimulated aldosterone synthesis principally through activation of calmodulin (63,85). It has also been suggested that potassium may activate PLC which is Ca^{2+}-sensitive, thereby inducing a moderate production of DAG and an increase in the activity of PKC (86). However, others have shown that potassium-stimulated $[Ca^{2+}]_i$ elevation is independent of inositol phosphate formation and have concluded that potassium neither activates PLC directly nor secondarily (87). Translocation of PKC to the plasma membrane has not been observed in glomerulosa cells in response to potassium (65,88) and PKC inhibitors have little or no effect on potassium-stimulated aldosterone synthesis (63,85).

The role of cAMP in mediating a response to potassium is also questionable; small increases in cAMP or no response at all to elevated potassium have been reported (85,87).

1.11 ANP Inhibition of Aldosterone Synthesis.

ANP is synthesised and stored in atrial muscle cells and released in response to a variety of stimuli including high plasma sodium concentration, atrial tachycardia and atrial streching (27). The active peptide which is formed in the plasma by proteolytic

cleavage from a pro-hormone (pro-ANP) has potent natriuretic, diuretic and vasodilatory properties (89). ANP decreases renin output and is a potent inhibitor of A-II, ACTH and K^+-stimulated aldosterone secretion in vivo and in vitro (90,91,92). The exact mechanism of ANP action on the glomerulosa cell remains to be identified but a number of cellular events associated with ANP action have been observed including increased concentrations of intracellular cGMP (39), increased or decreased intracellular cAMP, decreased uptake of extracellular calcium and, in the longer term, adrenal atrophy (93,39).

1.12 Other Effectors of Aldosterone Synthesis.

There are many other factors which have been reported to influence adrenocortical function (27). Those agents which have a negative effect include dopamine and somatostatin while serotonin, noradrenaline, adrenaline, acetylcholine, vasopressin, α-MSH and vasoactive-intestinal peptide are claimed to be positive effectors. Most of these agents have yet to be shown to be physiologically important in the regulation of aldosterone. A detailed analysis of their actions is beyond the scope of this preliminary review.

1.13 Substrate Supply for Steroidogenesis.

The availability and delivery of cholesterol to the side-chain cleavage enzyme, located in the inner mitochondrial membrane of adrenocortical cells, is the major rate-limiting step in steroid biosynthesis (94,95). This substrate can be derived from a variety of sources (27):

(i) De novo synthesis from acetate.

(ii) Hydrolysis of stored intracellular cholesteryl esters.

(iii) Uptake from circulating plasma lipoproteins.

The relative contribution of each source varies according to species and the overall rate of gland activity.

De novo synthesis: The synthesis of mevalonate from acetyl-CoA is the rate-determining step in this pathway and is catalysed by 3-hydroxy 3-methyl glutaryl CoA reductase (3HMG CoA reductase). The activity of this enzyme is regulated by ACTH and by feedback inhibition of intracellular cholesterol (96).

Hydrolysis of stored cholesteryl esters: Cholesterol is stored in esterified form within cytosolic lipid droplets (27). The interconversion of cholesterol ester and cholesterol is regulated by two enzymes; Acyl CoA cholesteryl acyltransferase (ACAT) and cholesterol ester hydrolase (CEH). CEH is known to be phosphorylated and activated in response to ACTH stimulation (97). The concentration of cholesterol which is available for steroidogenesis (ie-free cholesterol) is dependent upon the quantity and relative activity of these two enzymes.

Uptake of circulating lipoproteins: Cholesterol, triacylglycerols and other lipids are transported in body fluids by a series of lipoproteins classified according to their density. These lipoproteins consist of a core of hydrophobic lipids surrounded by polar lipids and then by a shell of apoproteins. Many of the apoproteins have been characterised and shown to carry the signal sequence that receptors on the appropriate target cell recognise (98).

Low-density lipoprotein (LDL) is rich in cholesterol, most of which is esterified to the polyunsaturated fatty acid, linoleate. In most species LDL appears to be the primary source of cholesterol and is metabolised by the following pathway (99)(Fig.1.10):

(i) LDL binds to specific receptors in the plasma membrane.

(ii) The receptor-LDL complex is internalised by endocytosis.

(iii) The cholesteryl esters are hydrolysed by lysosomal enzymes.

(iv) Free cholesterol is used for steroid synthesis or re-esterified by ACAT and stored in cytosolic lipid droplets.

This pathway regulates cellular cholesterol concentration in two ways. Firstly, released cholesterol supresses de novo synthesis by feedback inhibition of HMG CoA reductase and secondly, the LDL receptor is down-regulated by cholesterol.

Whereas LDL is responsible for the bulk of extracellular cholesterol transport to peripheral tissues, high density lipoprotein (HDL) exerts its main physiological role on reverse cholesterol transport, a process in which cholesterol from peripheral tissues is transported back to the liver for catabolism and excretion (100). This process is essential for homeostasis as most cells cannot metabolise any cholesterol that is accumulated. Steroidogenic cells are a notable exception. They can use both LDL and HDL-cholesterol. The mechanisms involved in HDL binding and transport of cholesterol to and from cells are complex and have not yet been fully elucidated. Two components appear to be involved. Firstly, a high affinity, saturable cell surface component which may interact with HDL apoproteins A-I, A-II (101) and E (102) and secondly, a lower affinity, non-saturable exchange component which seems to be a process of simple bidirectional diffusion of cholesterol between lipoprotein and cell membrane (100). The uptake pattern of the different lipoproteins by the adrenal cortex is complex and exhibits not only species specificity (103), but may also vary between zones (104). In vivo studies indicate an important role for LDL in the sustained supply of cholesterol to the bovine and human adrenal cortex whereas HDL is the prefered donor in the rat (99,105). However, recent work has suggested that acute (1h) cholesterol requirements for steroidogenesis may also be met by HDL

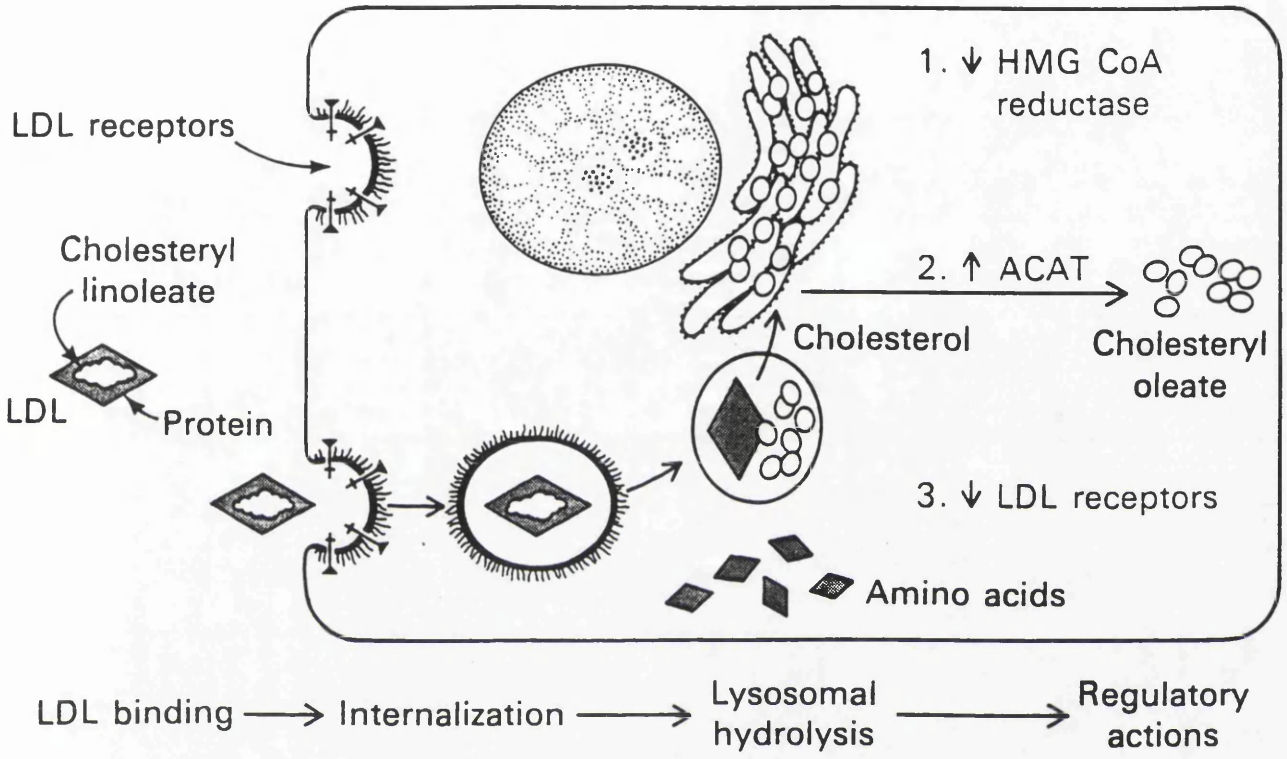


Fig.1.10

Uptake and metabolism of Low Density Lipoprotein (LDL) by adrenocortical cells.

From (98), Stryer, 1981.

in incubations of bovine glomerulosa and human granulosa cells (103,106).

1.13.1 Cholesterol Transport in the Adrenocortical Cell.

Cholesterol transport from cytosol to the inner mitochondrial membrane can be considered in two steps, both of which are accelerated by ACTH (107). The first process is cycloheximide-insensitive and involves transport to the outer mitochondrial membrane. Integrity of the cell cytoskeleton seems to be important since inhibitors of microtubule and microfilament formation, such as cytochalasin B, inhibit adrenal steroidogenesis (27). The second step is the transfer of cholesterol from the outer to the inner mitochondrial membrane. Despite the fact that a knowledge of this process is essential to the understanding of steroidogenic regulation, neither its mechanism of action nor the functional components involved have been positively identified. Intra-mitochondrial transport is cycloheximide-sensitive (108). It is proposed that a protein(s) with a rapid turnover rate is required to mediate this cholesterol translocation and that stimulatory hormones may either increase the level of or activate this protein (27). A number of peptides have been identified as potential candidates. Hepatic sterol carrier protein 2 (SCP_2) enhances intra-mitochondrial transport, reversing the effects of cycloheximide (109) and a protein similar to hepatic SCP_2 , which is inducible by ACTH, has been identified in the adrenal gland (110). Another cycloheximide-sensitive protein, termed protein i (MW 28000), has also been identified in adrenocortical cells (111). Protein i is produced in response to ACTH with a time-course and dose-dependency which parallels the steroidogenic response. A third peptide (MW 2200) has been identified in rat adrenals which is induced by ACTH and which stimulates side-chain cleavage activity (112,113).

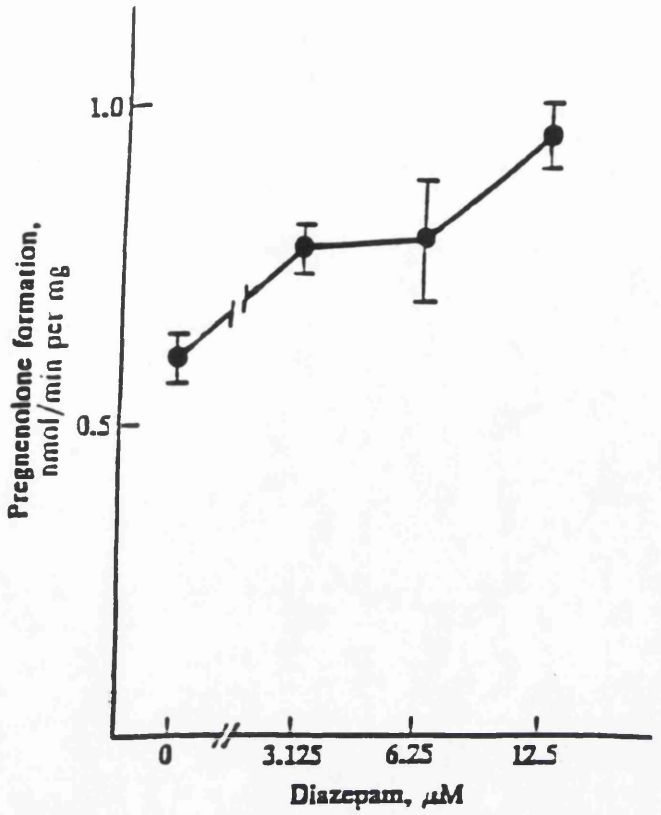
1.13.2 Endozepines and Benzodiazepines.

Recently a new group of proteins, the endozepines or diazepam binding inhibitors, have been proposed as the endogenous regulators of intra-mitochondrial transport (114). These claims originated from studies of the steroidogenic effects of a group of tranquillising drugs, called the benzodiazepines. Benzodiazepines are widely used as anxiolytic and anticonvulsant drugs (115). Specific binding sites in the central nervous system (CNS) appear to mediate their physiological action through the activation of the γ-aminobutyric acid (GABA) receptor (116,117). In addition, binding sites for benzodiazepines have been identified in many peripheral tissues and these sites are pharmacologically distinct from the receptors in the CNS (118). Peripheral binding sites are found in the outer mitochondrial membrane and have been designated mitochondrial benzodiazepine receptors (MBR's) (119). They have been identified in many tissues including heart, lung, kidney and in endocrine tissues such as the testis and the adrenal cortex (118,119). Since there appeared to be no correlation between MBR density and the activities of constitutive mitochondrial enzymes, it has been suggested that these receptors are required to fulfil a more specialised physiological function in the adrenal gland and testis, perhaps relating to control of steroidogenesis (120). Drugs selective for the MBR produce a spectrum of pharmacologic actions including alterations of immune function, cell proliferation and cardiac action potential (121). Of relevance to this review, a variety of MBR ligands (eg. diazepam and RO5-4864) enhance corticosteroid synthesis in cultured rat and bovine ZF/R cells (122). Thus, MBR may be a target for endogenous ligands which mediate the action of steroid-stimulating hormones (114). Proteins which inhibit the benzodiazepine, diazepam from binding to CNS receptors (114) have been

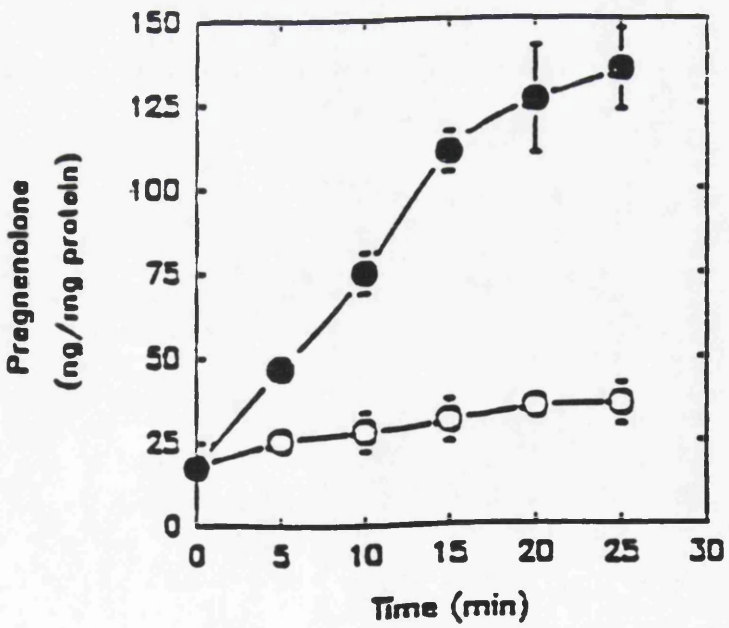
purified to homogeneity from bovine, human and rat brain (123) and have been termed endozepines or diazepam-binding inhibitors (DBI's). Native DBI fragments when added to cholesterol loaded Y-1 mitochondria produce a dose-dependent stimulation of pregnenolone formation which can be antagonised by flunitrazepam (114) implicating the MBR.

Immunocytochemical and biochemical studies have demonstrated the presence of DBI and DBI-like peptides in the periphery with notably high concentrations identified in the adrenal cortex (124). Des-[gly-ile]-DBI has been isolated from bovine ZF/R cells and been shown to stimulate pregnenolone formation in cholesterol-loaded adrenocortical mitochondria in a manner similar to diazepam (114,123). Thus both may operate through the same effector system, the MBR (Fig.1.11). Spectral-shift assays showed that addition of Des-[Gly-Ile]-DBI to mitochondria also increased substrate-bound fraction of P-450_{scc} in the absence of added cholesterol (123). This implies that the peptide may not only influence mitochondrial translocation but may also mobilise inner membrane cholesterol. If DBI are involved in steroidogenic regulation then their synthesis and activity should be under hormonal control. In vivo studies done with hypophysectomised rats demonstrated that administration of ACTH increased adrenal DBI immunoreactivity (125). This increase was blocked by cycloheximide suggesting that DBI synthesis was under the control of ACTH. Conversely, pulse-chase experiments performed with ^{35}S-methionine in Y-1 and LH10 cell lines, failed to show any effects of hormonal stimulation (ACTH for Y-1 cells, LH for LH10 cells) on DBI synthesis or turnover, although steroid synthesis was significantly stimulated (126). It may be that, in vitro, hormone action is directed at DBI-receptor interaction, possibly through receptor phosphorylation, rather than regulating DBI activity and synthesis directly.

Fig.1.11



Effect of diazepam on pregnenolone formation by cholesterol-loaded adrenal mitochondria. (Redrawn from Besman et al, Proc. Nat. Acad. Sci. (1989) 86 4897-4901).



Time course of the effect of maximally stimulating 0.13μM diazepam-binding inhibitor (DBI) on pregenolone formation in adrenal mitochondria prepared and incubated with (●) or without (○) 0.13μM DBI for the indicated time periods. (Redrawn from Papadopoulos et al, Endocrinology (1991) 129 1481-1487).

Such a mechanism would still require DBI for control of steroidogenesis but without the neccesity for any increase in synthesis of the peptide (126).

As yet there is no direct evidence to show that DBI or related proteins are the short-lived, cycloheximide-sensitive factors which regulate aldosterone synthesis. In any case, cholesterol translocation is probably more complex than previously suspected, involving multiple transport mechanisms regulated by more than one protein over different time courses (127).

1.14 Project Aims.

The present study has used a number of benzodiazepine ligands, in particular diazepam, to examine the effects of DBI's on aldosterone synthesis in bovine adrenocortical cells.

Benzodiazepines are metabolised by cytochrome P-450 enzymes in the liver and other drugs which are similarly metabolised are potent inhibitors of steroidogenic enzymes. For example, dantrolene which has been used in signal transduction studies as a specific inhibitor of IP_3-mediated calcium release, interferes strongly with P-450_{scc} (See chapter 2). These drugs also affects depolarisation-induced calcium influx in other excitable cell types. Therefore it was decided to characterise the effects of benzodiazepines in three areas of adrenocortical cell function:

(i) Interaction with steroidogenic enzymes.

(ii) Mobilisation of extracellular calcium.

(iii) Cholesterol-stimulated aldosterone synthesis

All studies were carried out in bovine whole cells and mitochondria. A readily-available source of cholesterol was necessary and HDL was chosen. However, as reports in other systems had suggested that HDL-target cell interaction may be

associated with alterations in cell calcium metabolism, this possibility had to be excluded in adrenocortical cells.

CHAPTER 2.

EFFECTS OF DANTROLENE ON ADRENAL STEROIDOGENIC ENZYMES.

2.1 Introduction

When using supposedly specific drugs to investigate cell function it is important to realise that they may have more than one mode of action. For example, proposed modes of action for benzodiazepines include effects on mitochondrial cholesterol transport and on cell calcium metabolism (122,128). When investigating adrenocortical cell function it is quite likely that drugs interact with steroidogenic cytochrome P-450 enzymes in the same way that they would be metabolised by mixed fuction oxidases in the liver. One such compound is the skeletal muscle relaxant dantrolene. This drug, widely used in the treatment of muscle spasticity, exerts its therapeutic effect by inhibiting the release of calcium from the sarcoplasmic reticulum (129). This property has made dantrolene an obvious tool to study the role of calcium in mediating hormone action (130,131). However, adverse clinical effects, most notably hepatic injury, have been reported following treatment with a high daily dose of the drug (132). This liver damage was associated with direct inhibition of hepatic, cytochrome P-450 enzymes (133,134). Since dantrolene had been demonstrated to inhibit glucocorticoid secretion in vivo (135), I decided to investigate whether the drug affects steroidogenesis in vitro other than by inhibiting intracellular calcium release and, if so, whether key cytochrome P-450 dependent mitochondrial steps are affected.

2.2 Materials and Methods

Unless stated otherwise all reagents were obtained from the Sigma Chemical

Company, Poole, Dorset.

(i) Isolation of Adrenocortical Cells.

Six adrenal glands taken from freshly slaughtered cattle were obtained at a local abattoir. Thin slices of tissue were taken from the outer regions of these defatted glands and minced. They were then subjected to collagenase digestion (1.5mg/ml; Worthington Biochemical Corp., N.J., U.S.A.) with mechanical agitation in Kreb's bicarbonate Ringer's solution (NaCl 120.6 mmol/l, $NaHCO_3$ 24.8mmol/l, KCl 2.6mmol/l, $MgSO_4$ 1.15mmol/l, $CaCl_2$ 2.54mmol/l and KH_2PO_4 1.15mmol/l) containing 10mmol/l glucose and bovine serum albumin (20g/l). The resulting cells were filtered twice , firstly through a tea strainer and then through 100μm gauze (Henry Simon Ltd, Stockport) before being applied to a discontinuous gradient of isotonic Percoll (Pharmacia LKB, Uppsala, Sweden). Following centrifugation (10 min., 2800g) the cells with specific gravity >1.039 and <1.052 were resuspended in medium 199 (Gibco Ltd, Paisley, Scotland), modified so that the ionic composition was the same as that of the Ringer's solution, and containing 10mmol/l glucose and BSA (2g/l). It has been demonstrated previously that, although these preparations are enriched with aldosterone-producing zona glomerulosa cells, they do contain significant ammounts of zona fasiculata/reticularis cells (136). The final concentration of cells used in the following experiments was approximately $3x10^5$ cells/ml.

(ii) Isolation of Adrenocortical Mitochondria

Thin slices were removed from the outermost region of bovine adrenal glands. Adrenocortical tissue was scraped from the capsule and homogenized (3x10s.) using a Polytron PTA20S homogenizer (Kinematic, Denmark) in ice-cold 0.25mol/l sucrose, pH 7.2, containing triethanolamine hydrochloride (10mmol/l),

ethylenediaminotetra-acetic acid (0.1mmol/l) and BSA (10g/l). The homogenate was centrifuged at 600g for 10 min. to remove cellular debris and the supernatant centrifuged at 10000g for 10 min. The pellet was then washed twice by resuspending in sucrose and recentrifuging. Mitochondria were not subject to further purification and so preparations may have included contamination from other sources eg-plasma membrane. Finally, prior to incubation, the mitochondria were suspended in 0.25mol/l sucrose, pH 7.1, containing KCl (20mmol/l), triethanolamine hydrochloride (15mmol/l), $MgCl_2$ (5mmol/l) and BSA (10g/l) at a concentration of 3-15mg protein/ml (137).

(iii) Steroid Incubations

Cell and mitochondrial suspensions (0.5ml) were incubated in sextuplicate in 1.5ml microcentrifuge tubes at 37°C in an atmosphere of 95% O_2, 5% CO_2 in medium 199 (cells) or sucrose solution (mitochondria) containing dantrolene and/or various steroids. The steroids used in these experiments, 20α-hydroxycholesterol, pregnenolone, progesterone, 11-deoxycorticosterone, corticosterone, 18-hydroxycorticosterone and 11-deoxycortisol (dissolved in methanol), were added to the appropriate tubes and the solvent allowed to evaporate prior to the addition of incubation medium. The reactions were initiated by the addition of cells, or for mitochondrial incubations, by the addition of a solution composed of sodium succinate (2.5mmol/l), isocitrate (5mmol/l) and $NADP^+$ (0.16mmol/l). Reactions were stopped after 1h for cells by transferring tubes to ice-cold water. The cell suspensions were centrifuged at 800 g for 10 min. at 4°C and the supernatant stored at -20°C for later analysis. Mitochondrial reactions were stopped after 15 min. by dilution with methanol (1:20) prior to storage.

(iv) Steroid Assays

Steroids were measured by direct radioimmunoassay. All samples from each experiment were analysed in one assay. The coefficients of variation were 13.2%, 14% and 8.7% for aldosterone, cortisol and pregnenolone assays respectively.

(a) **Aldosterone assay**: Duplicate assay standards were made up in 10µl methanol (0.31, 0.62, 1.25, 2.5, 5, 10, 20 and 40pg aldosterone/10µl) together with a volume of medium 199/sucrose solution equal to that of the samples. Sample tubes contained 10µl methanol plus incubation medium. Aldosterone antiserum (a gift from Prof. Th. J. Benraad, Division of Endocrinology, Dept of Internal Medicine, University of Nijmegen, The Netherlands) was diluted 1:300000 in Borate buffer (boric acid 0.13mol/l, sodium hydroxide 0.068mol/l and hydrochloric acid 0.05mol/l), pH 7.5, containing 0.1% γ-globulin and 18Bq/100ml of the aldosterone conjugate, aldosterone-3-(o-carboxymethyl)oximino-(2-[^{125}I]iodohistamine) (Amersham International, Amersham, Bucks). Antiserum mixture (200µl) was added to both standards and samples. The assay was left at room temperature for 1 hour and then overnight at 4°C. Charcoal solution (0.5% Dextran T-70 and 0.5% methanol-washed charcoal in borate buffer, pH7.5) at 4°C was added to each tube (150µl), mixed well and centrifuged at 2000g for 10mins. Supernatants were aspirated from all tubes and the radioactivity in the free fraction measured using a gamma scintillation spectrometer.

(b) **Cortisol assay**: Duplicate assay standards were made up in 100µl of borate buffer (7.8, 15.6, 31.2, 62.5, 125, 250, 500 and 1000pg/100µl) together with a volume of medium 199/sucrose solution equal to that of the samples. Sample tubes contained 100µl borate buffer plus incubation medium. Cortisol antiserum (Scottish Antibody Production Unit, Law Hospital, Carluke, Lanarks., Scotland) was diluted 1:20000 in borate buffer containing 0.1% γ-globulin and 60KBq/100ml of the cortisol conjugate, cortisol-3-(o-carboxymethyl)oximino-(2[^{125}I]iodohistamine)

(Amersham International, Amersham, Bucks.). Antiserum mixture (150µl) was added to all tubes, the contents mixed and the incubations and charcoal separations performed as above.

(c) **Pregnenolone Assay**: Duplicate assay standards were made up in 100µl methanol (6.25, 12.5, 25, 50, 100, 200, 400, 800 and 1600pg/100µl) together with a volume of sucrose/methanol equal to that of the samples. Sample tubes contained 100µl methanol plus sucrose/methanol incubation medium. [^{3}H] pregnenolone (3000cpm in 100µl methanol) and ethylene glycol solution (1%vol/vol in methanol, 100µl) were added to all tubes and the incubations evaporated to dryness at 37°C under a continuous stream of nitrogen. Antiserum to pregnenolone (138), was diluted 1:5000 in borate buffer (containing 0.5% wt/vol γ-globulin and 2% vol/vol methanol) and 500µl added to each tube. After overnight incubation at 4°C, dextran-coated charcoal (0.188% Dextran T-70 and 0.188% methanol-washed charcoal) was used to separate bound and free radioactivity. Supernatant (bound fraction) was then removed from each sample (500µl), added to 10ml Ecoscint A (National Diagnostics, Manville, N.J., U.S.A.) and counted for 10 mins. in a liquid scintillation spectrometer.

Data were compared by analysis of variance using the Newman-Keuls multiple range test (139) or by unpaired t-tests where indicated.

2.3 Results

(i) Effects of dantrolene on basal and A-II-stimulated aldosterone synthesis in isolated adrenocortical cells.

Figure 2.1 shows aldosterone output in basal and AII-stimulated cells in the presence of increasing concentrations of dantrolene; 0.02mmol/l reduced and 0.2mmol/l abolished the aldosterone response to A-II ($P<0.01$).

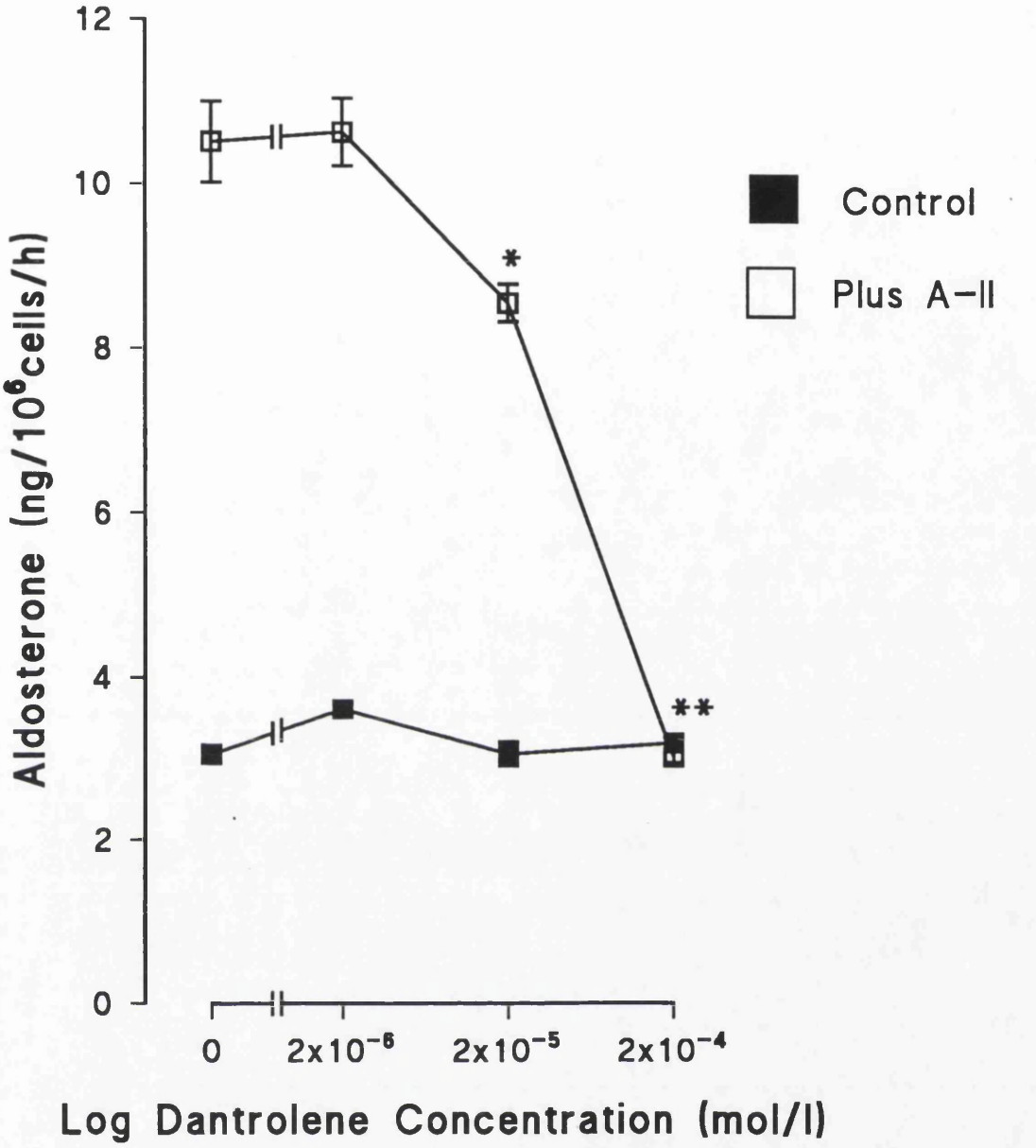


Fig.2.1

Effect of increasing concentrations of dantrolene on aldosterone synthesis by bovine adrenocortical cells incubated in the absence or presence of A-II (10nmol/l).

Values shown are means ± S.E. of six replicate incubations. * and ** indicate significant differences with $p<0.05$ and $p<0.01$ respectively (Newman-Keul's multiple-range test) for aldosterone output from dantrolene-treated cells compared with that from cells treated with vehicle.

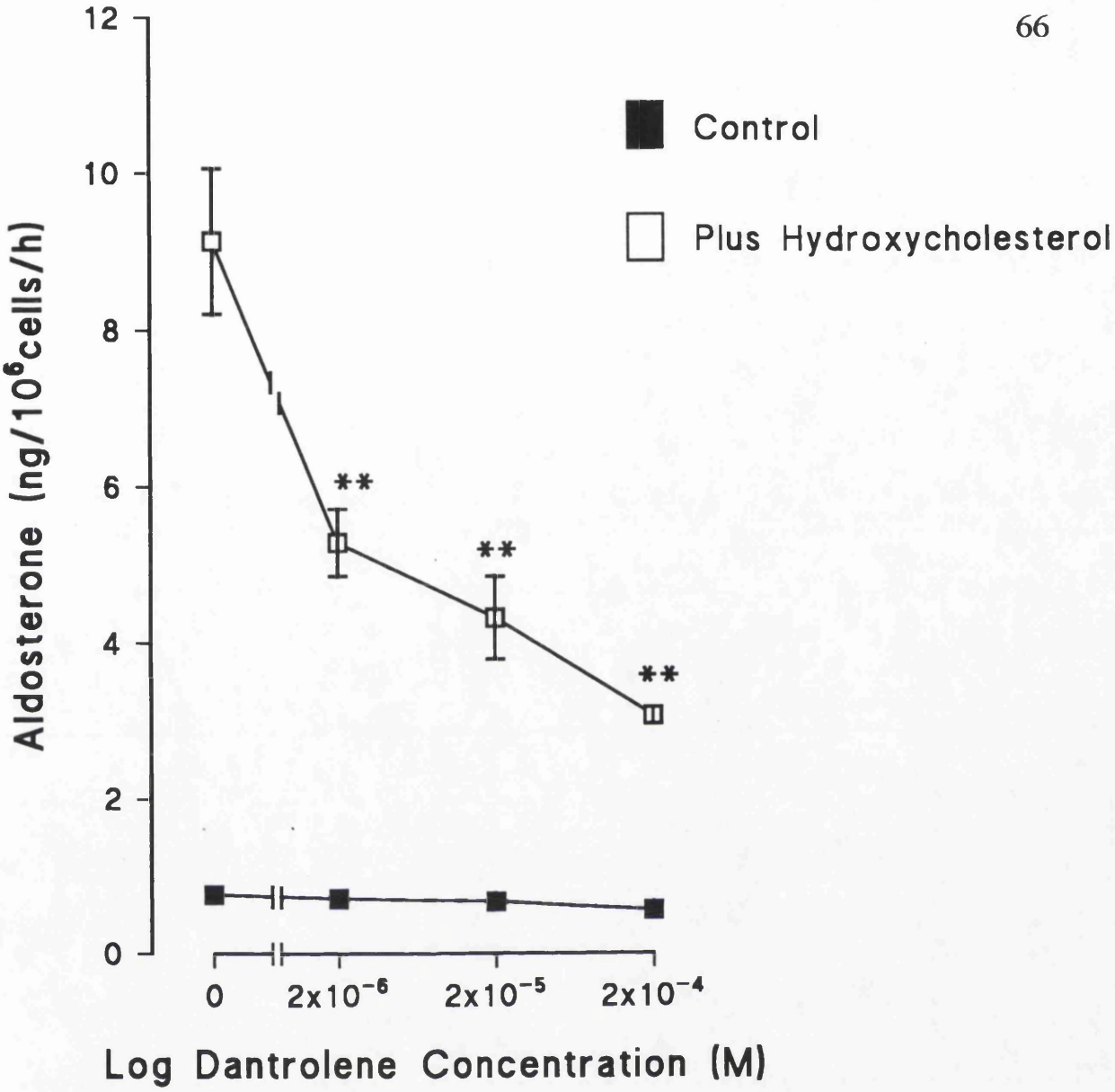


Fig.2.2

Effect of increasing concentrations of dantrolene on aldosterone synthesis by bovine adrenocortical cells incubated in the absence or presence of 20α-hydroxycholesterol (5μmol/l).

Values shown are means ± S.E. (n=6). ** indicates inhibition by dantrolene ($p<0.01$). Aldosterone output from dantrolene-treated cells is compared with that from vehicle-treated controls using Newman-Keul's multiple range test.

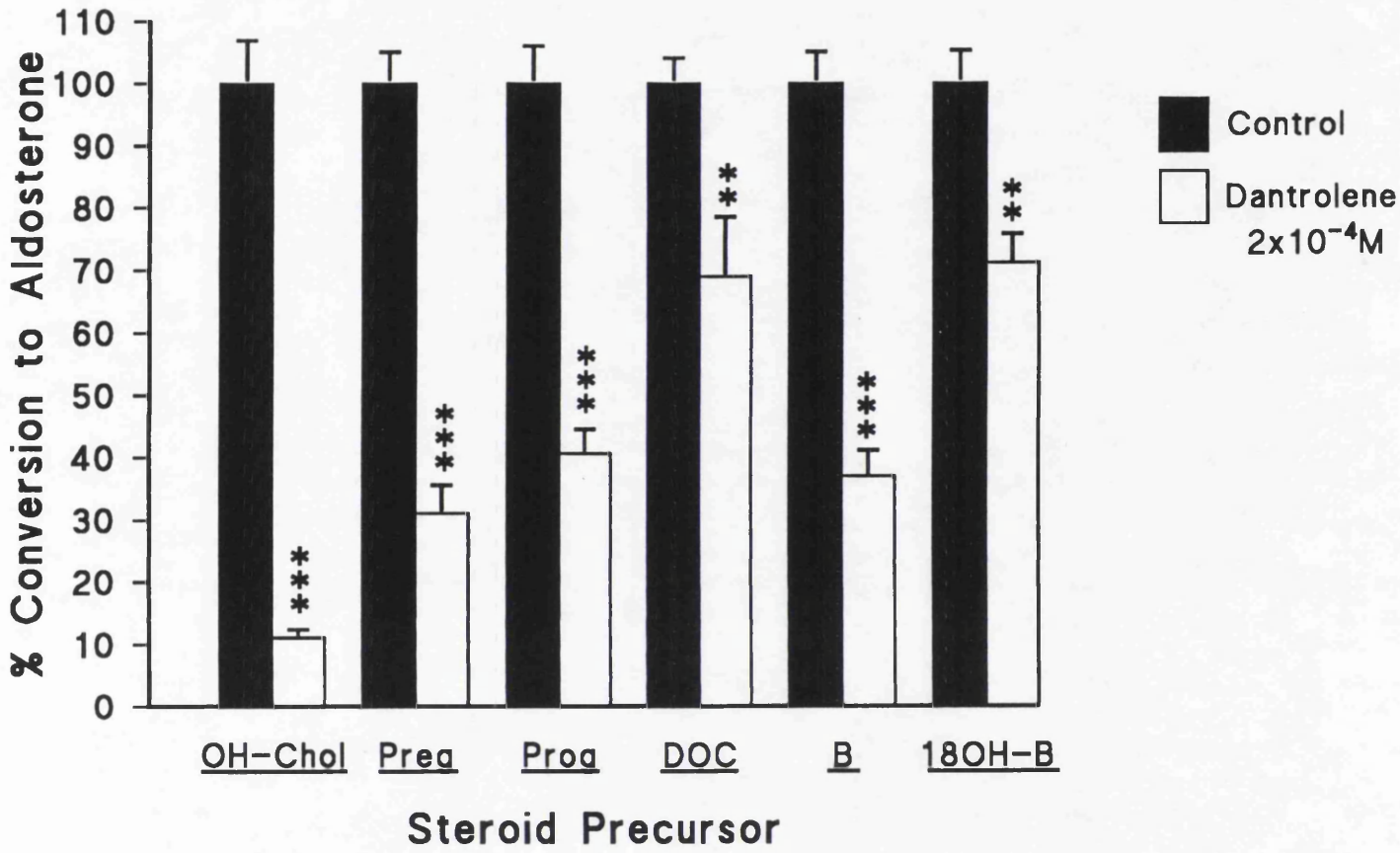


Fig.2.3

Effect of dantrolene on aldosterone synthesis from added precursors in bovine adrenocortical cells.

Values shown are means ± S.E. from six replicate incubations and are expressed as a percentage of vehicle-treated control values (see text for absolute values). Steroid precursors were all added at a concentration of 5μmol/l. Statistically significant inhibition of aldosterone synthesis is indicated by ** ($p<0.01$) and *** ($p<0.001$) (unpaired t-test).

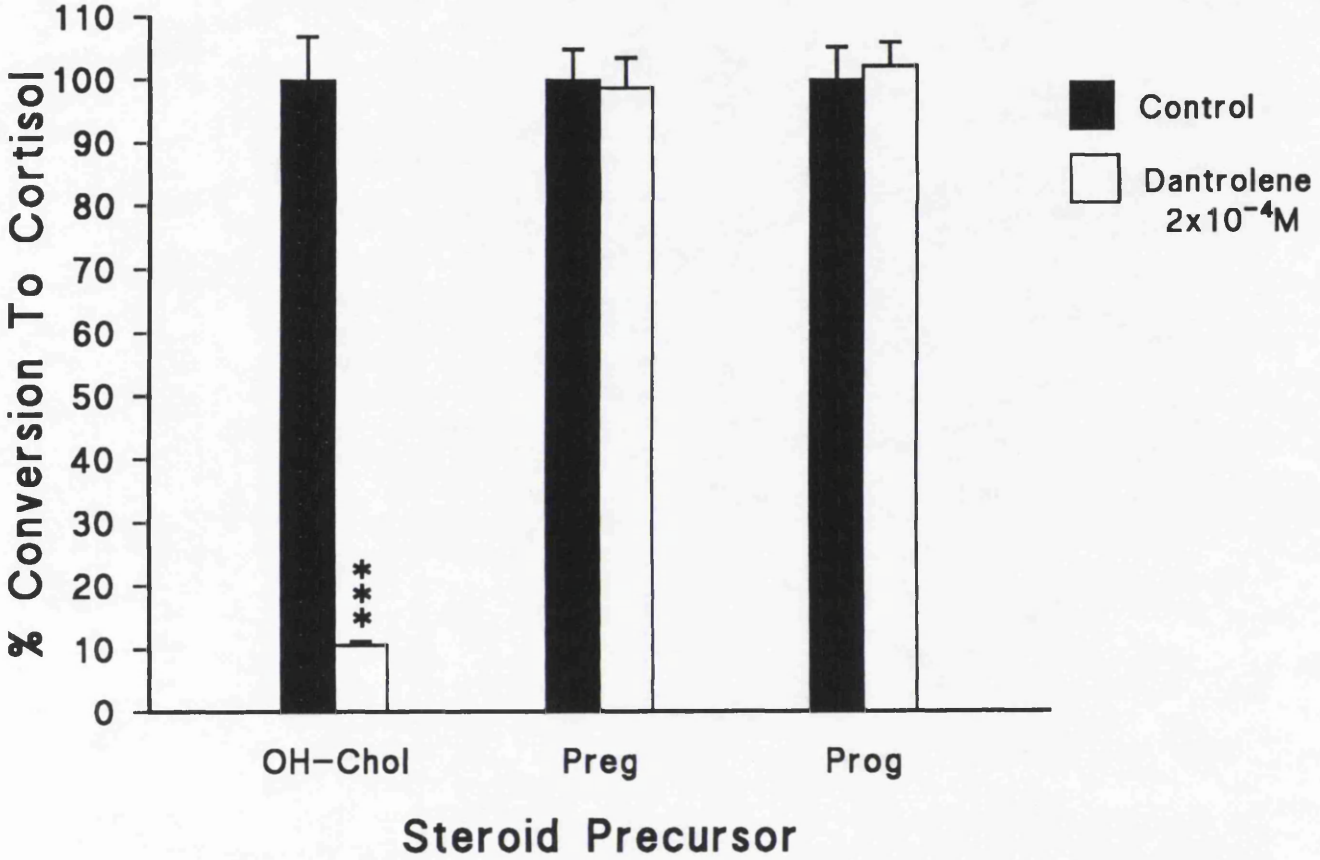


Fig.2.4

Effect of dantrolene on cortisol synthesis from added precursors in bovine adrenocortical cells.

Values shown are means ± S.E. from six replicate incubations and are expressed as a percentage of vehicle-treated control values (see text for absolute values). Steroid precursors were all added at a concentration of 5μmol/l. Statistically significant inhibition of cortisol synthesis is indicated *** ($p<0.001$) (unpaired t-test).

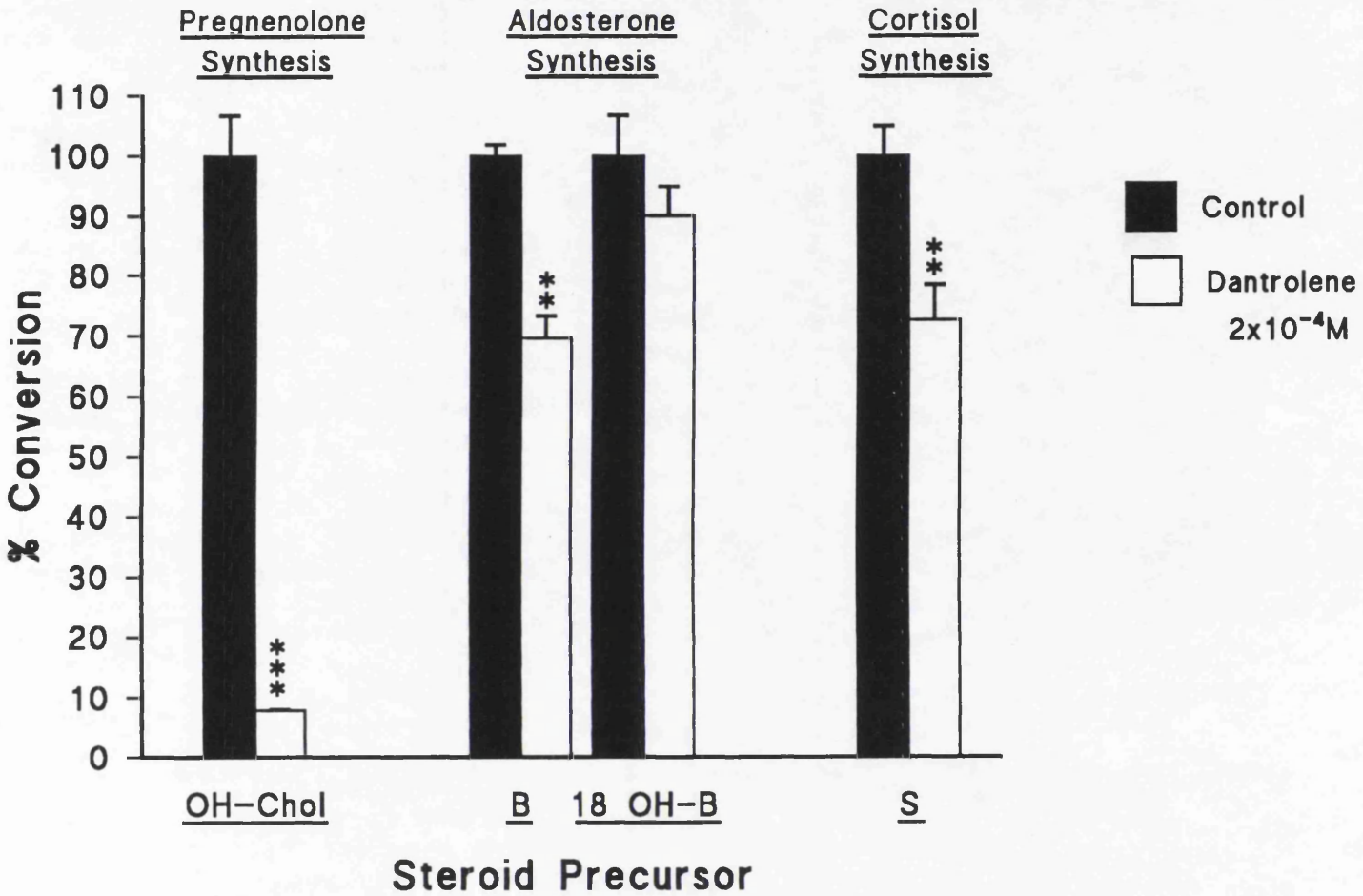


Fig.2.5

Effect of dantrolene on pregnenolone, aldosterone and cortisol synthesis from added precursors in bovine adrenocortical mitochondria.

Values shown are means ± S.E. from six replicate incubations and are expressed as a percentage of vehicle-treated control values (see text for absolute values). Steroid precursors were all added at a concentration of 5μmol/l. Statistically significant inhibition of cortisol synthesis is indicated by ** ($p<0.01$) and *** ($p<0.001$) (unpaired t-test).

(ii) Effects of dantrolene on aldosterone and cortisol synthesis from added precursors in isolated adrenocortical cells.

The effects of the same concentrations of dantrolene on aldosterone synthesis in cells incubated in the presence and absence of hydroxycholesterol is shown in Fig. 2.2. In the absence of hydroxycholesterol, inhibition of aldosterone synthesis was slight and only reached significance ($P<0.05$) at the highest concentration of dantrolene (0.2mmol/l). In the presence of hydroxycholesterol, aldosterone synthesis was inhibited at all concentrations of dantrolene in a dose-dependent manner ($P<0.01$; IC_{50} value approx. 0.02mmol/l). However, aldosterone synthesis was still greater than controls when cells were treated with the highest concentration of dantrolene (0.2mmol/l).

The effects of dantrolene (0.2mmol/l) on aldosterone synthesis in cells which were incubated with various biosynthetic intermediates are shown in Fig. 2.3. Results are expressed as a percentage of controls (i.e. aldosterone synthesised in the absence of dantrolene). Control values (pmol/10^6 cells/h) were 70±5, 250±13, 356±19, 942±39, 667±36 and 65±3 for cells incubated with hydroxycholesterol, pregnenolone, progesterone, 11-deoxycorticosterone, corticosterone and 18-hydroxycorticosterone respectively. In all cases dantrolene significantly inhibited aldosterone synthesis ($P<0.01$). The greatest percentage inhibition by the drug was in cells exposed to hydroxycholesterol (89%) and the least inhibition was with 18-hydroxycorticosterone (28%).

Figure 2.4 shows the effects of dantrolene on cortisol synthesis. The cells used in this experiment were the same as those in Fig. 2.3. Cortisol production was 2.27±0.16, 3.99±0.19 and 6.5±0.32 nmol/10^6 cells/h for control cells incubated with hydroxycholesterol, pregnenolone and progesterone, respectively. Dantrolene only

inhibited cortisol production in cells exposed to hydroxycholesterol (89%, P<0.01).

(iii) Effects of dantrolene on aldosterone and cortisol synthesis from added precursors in isolated adrenocortical mitochondria.

Mitochondrial conversion of added hydroxycholesterol to pregnenolone and of added corticosterone and 11-deoxycortisol to aldosterone and cortisol were inhibited by dantrolene (P<0.01; Fig.2.5). Control steroid production values (pmol steroid/mg protein/15 min) from mitochondria not treated with dantrolene were 1166±150 for pregnenolone, 57±1 and 5±0.3 for aldosterone with added corticosterone and 18-hydroxycorticosterone, respectively and 12880±550 for cortisol from added 11-deoxycortisol. However, it must be emphasised that these results were obtained with only partially purified mitochondria.

2.4 Discussion.

In agreement with previous reports (140) my studies show that dantrolene inhibits AII-stimulated steroid synthesis. Is this inhibition due to prevention of calcium release from intracellular stores or to some other calcium-independent process? In the case of adrenal steroidogenesis, a dantrolene-sensitive component of calcium mobilisation in response to AII has been identified (141,142). It has also been noted that plasma glucocorticoid levels are reduced in vivo by dantrolene (135). Glucocorticoid synthesis is controlled predominantly by ACTH via an intracellular mechanism which does not involve phospholipase C activation and IP_3-induced calcium release ((143); see Chapter 1). My study confirms in vivo findings that dantrolene can inhibit steroidogenesis by a mechanism which is not necessarily dependent upon Ca^{2+}. Whether the mechanisms of in vitro and in vivo inhibition are the same is not clear. Therapeutic plasma concentrations of dantrolene are in the range 75-530nmol/l (144). These concentrations would not inhibit steroidogenesis

using the protocol of the present in vitro studies. It is nevertheless possible that dantrolene is accumulated by adrenocortical tissue during long-term treatment.

Several experimental results show that the effects of the drug in vitro represent more than a block of agonist-induced calcium release. Both aldosterone and cortisol formation from added hydroxycholesterol were inhibited; there were also differences in the patterns of aldosterone inhibition between A-II and hydroxycholesterol-treated cells. The threshold concentration for dantrolene's effects appeared lower in hydroxycholesterol-treated cells. In contrast, the highest concentration of dantrolene (0.2mmol/l) used here completely inhibited the effects of AII but not those of hydroxycholesterol. These results are compatible with the hypothesis that dantrolene interferes with two steroidogenic processes, calcium mobilisation as well as a direct inhibition of one or more enzymes in the steroidogenic pathway.

To determine the site of this latter action, cells were incubated with different steroid precursors. Expressed in relative terms, the greatest effect of dantrolene was on cells treated with hydroxycholesterol. It is perhaps significant that aldosterone synthesis, which is regulated at two sites (cholesterol side-chain cleavage and conversion from corticosterone) (38), was inhibited in cells incubated with a variety of precursors whereas cortisol synthesis, which is regulated by the rate of cholesterol side-chain cleavage, was inhibited only when hydroxycholesterol was used as substrate. Taken together, these results suggest that dantrolene affects two steroidogenic control steps (cholesterol side-chain cleavage and conversion of corticosterone to aldosterone) both of which occur in the mitochondria and are calcium-dependent (145). One possible explanation of these results is that dantrolene blocks both aldosterone and cortisol synthesis by affecting calcium influx across the mitochondrial membrane. Accordingly, dantrolene was tested on isolated

mitochondria. In the absence of added calcium, conversion of hydroxycholesterol to pregnenolone was inhibited, as was the synthesis of aldosterone from corticosterone. Although in intact cells, dantrolene did not affect cortisol synthesis from deoxycortisol, 11ß hydroxylase activity in isolated mitochondria was profoundly inhibited. These data point to an action of dantrolene which is independent of acknowledged, signal-response coupling processes. Inhibitory actions of several drugs on cytochrome P450-dependent steroid hydroxylation reactions have been described in various tissues (146,147). These drugs are also metabolised by cytochrome P450 enzymes in the liver. Since dantrolene is known to be a competitive inhibitor of hepatic P450 enzymes (133), the drug may bind to and inhibit adrenal steroidogenic enzymes in a similar way.

To summarise, dantrolene has an inhibitory effect on steroid synthesis in adrenocortical cells and in isolated mitochondria which is independent of its effects on intracellular calcium. The most likely explanation of this effect is competitive inhibition by the drug of cytochrome P450-dependent hydroxylase enzymes of the steroid biosynthetic pathway. In particular cholesterol side-chain cleavage activity, which catalyses the important rate-limiting conversion of cholesterol to pregnenolone, is sensitive to dantrolene. These data do not deny that dantrolene blocks AII-stimulated, IP_3-mediated calcium mobilisation. However, caution is needed in interpreting experiments in which steroid measurements are used as an end-point when dantrolene, or indeed any class of drug metabolised by hepatic P-450 enzymes is used specifically to study signal transduction in steroidogenic tissues. For example, 4-aminopyridine and cromakalim, two drugs which have known effects on potassium channel function, have, in addition, both been shown to inhibit adrenocortical steroid hydroxylation (148,149).

CHAPTER 3.

EFFECTS OF HIGH DENSITY LIPOPROTEINS ON STEROID SYNTHESIS.

3.1 Introduction.

There are three sources of substrate (cholesterol) for steroidogenesis; (i) de novo synthesis from acetate, (ii) hydrolysis of intracellular stored cholesteryl esters or (iii) uptake from circulating plasma lipoproteins, principally low or high density lipoproteins (LDL or HDL). LDL is responsible for the bulk of cholesterol transport in blood and delivers cholesterol to tissues intracellularly via apolipoprotein B/E receptor-mediated endocytosis. HDL exerts its main physiological role by reverse cholesterol transport, a process critical for homeostasis, whereby cholesterol is transported by HDL from peripheral tissues back to the liver for catabolism and excretion. Apart from the liver, steroidogenic tissues are the only ones which metabolise significant amounts of cholesterol and HDL-cholesterol has been demonstrated to be used as a substrate for steroidogenesis in vivo and in vitro (100). However, the relative dependency of the adrenal cortex on these different lipoproteins is complex and exhibits not only species specificity (105) but also differs between zones of the gland (104). For example, in vivo studies have indicated an important role for LDL in the sustained supply of cholesterol to bovine adrenal cortex (99) whereas HDL is the prefered donor in the rat (105). LDL or HDL utilisation may also vary with time course of experiment. Recent work has suggested that HDL may be the prefered source of steroidogenic substrate in acute in vitro incubations of bovine cells whereas long-term cultures have identified that LDL-cholesterol may be used (103).

I have investigated the role of HDL-cholesterol in adrenocortical steroidogenesis

in more detail by comparing the effects of two HDL subfractions, HDL-2 (cholesteryl-ester rich containing apolipoprotein E) and HDL-3 (smaller, denser predominant plasma type containing a higher proportion of free cholesterol) on both basal and angiotensin II (A-II)-stimulated aldosterone and cortisol synthesis. These effects have been compared acutely (1h) in freshly isolated bovine adrenocortical cells and longer term (24h) in cultured cells.

Previous work in this laboratory (H.D Simpson, unpublished data) demonstrated that unfractionated HDL reduces vasopressin-stimulated platelet aggregation by inhibiting AVP-induced increases $[Ca^{2+}]_i$. In addition, HDL-2 and HDL-3 treated platelets accumulated less ^{45}Ca than appropriate controls when ^{45}Ca uptake was measured both acutely (<2mins) and under steady-state conditions (>30 mins) (D. Graham, unpublished data). As aldosterone synthesis is notably calcium-sensitive (see chapter 1), these data raised the possibility that, in addition to supplying cholesterol, HDL could affect basal and hormone-stimulated steroidogenesis by affecting calcium metabolism. Therefore, I examined the effects of HDL-2 and HDL-3 on ^{45}Ca uptake in freshly isolated zona glomerulosa cells. In addition, I investigated the effects of both subfractions on basal and A-II stimulated $[Ca^{2+}]_i$ concentration in cells which had been in culture for 72h.

3.2 Materials and Methods.

Unless otherwise indicated all reagents were purchased from the Sigma Chemical Company, Poole Road, Dorset.

(i) Isolated Adrenocortical Cells.

Bovine adrenocortical cells were prepared according to the method described in Chapter 2 with the exception that Ham's F-12 medium (Gibco BRL, Paisley,

Strathclyde), containing 2.5mmol/l Ca^{2+} and 0.2% BSA, was substituted for Medium 199 both in cell preparation and in experiments involving measurements of steroid synthesis.

Aliquots of cells (n=6, $2x10^5$cells/ml) were incubated together with A-II and/or HDL-2 and/or HDL-3 in an atmosphere of 95% O_2 and 5% CO_2, at 37°C for 1h. After incubation the cells were centrifuged (800g, 4°C for 10 mins.) and the supernatants removed and stored for later measurement of aldosterone and cortisol by direct radioimmunoassay (see chapter 2).

HDL subfractions were prepared by Dr. Muriel Caslake, Dept. Pathological Biochemistry, Royal Infirmary, Glasgow according to the method based on that of Havel et al (150). Briefly, HDL-2 (1.063-1.125g/l) and HDL-3 (1.125-1.21g/l) were seperated from human serum by repeated ultracentrifugations, after progressively raising solvent density of the serum by the addition of a concentrated salt solution. The total cholesterol content of the HDL subfractions was then measured by an enzymatic colorimetric method (151) according to instructions from and with reagents provided by the Cholesterol C-System assay kit (BCL Ltd, Lewes, East Sussex).

(ii) Cultured Adrenocortical Cells

Following dispersal and Percoll separation the cells were resuspended in modified Ham's F-12 plus 10% fetal calf serum containing the following additions; 50μmol/l butylated hydroxyanisole, 1.2μmol/l α-tocopherol, 100μmol/l ascorbate, 50nmol/l sodium selenite, 300nmol/l glutathione, 10ng/ml insulin, 10μg/ml bovine transferrin, 5μmol/l metyrapone, 2.5mmol/l Ca^{2+}, 4mmol/l $NaHCO_3$ and 25mmol/l HEPES (152). The cells were then seeded into 24 well culture plates (Gibco BRL, Paisley,

Strathclyde) at a concentration of 10^5cells per well. After 5 days in culture, HDL-2 and HDL-3 were added to appropriate wells. On day 6 the plates were washed three times with serum-free Ham's F-12 containing only 2.5mmol/l Ca^{2+} and 0.2% BSA and incubated in the same medium in the presence of appropriate concentrations of A-II and/or HDL-2 and/or HDL-3 in an atmosphere of 95% O_2 and 5% CO_2 at 37°C for 3 hours. The supernatant was then removed and stored for steroid analysis.

Preliminary work had demonstrated that adrenocortical cells achieved confluence after six days and that these cells were then very responsive to A-II (see Fig.3.1).

(iii) ^{45}Ca Uptake Studies

^{45}Ca uptake was measured using a modification of the method of Mauger, Poggioli, Guesdon and Claret (153). Cells (approximately $8x10^5$ per incubation) were preincubated at 37°C for 30 min. in Krebs' bicarbonate-Ringer containing 0.2% glucose and BSA (KRBGA) and 1mmol Ca^{2+}/l. This ensured that measurements of cells were not depleted of calcium prior to ^{45}Ca studies. HDL-2 or 3 was then added and cells were incubated for a further 15min. Following addition of 17kBq ^{45}Ca (Amersham International plc, Amersham, Bucks.) in KRBGA containing 1mmol/l Ca^{2+} (specific activity of the final incubation mixture was 170MBq/mmol), aliquots of suspensions were sampled at appropriate time-points. The cells were collected onto glass fibre filters (GF/C; Whatman International Ltd, Maidstone, Kent) using a Titretek Multi-channel Cell Harvester (Flow Laboratories, Irvine, Strathclyde) and washed for approximately 30s with ice-cold 5mmol Tris buffer/l (approximately 14ml) containing 144mmol/l NaCl and 5mmol/l $CaCl_2$. The filters were then transferred to glass vials for liquid scintillation counting. The results are expressed

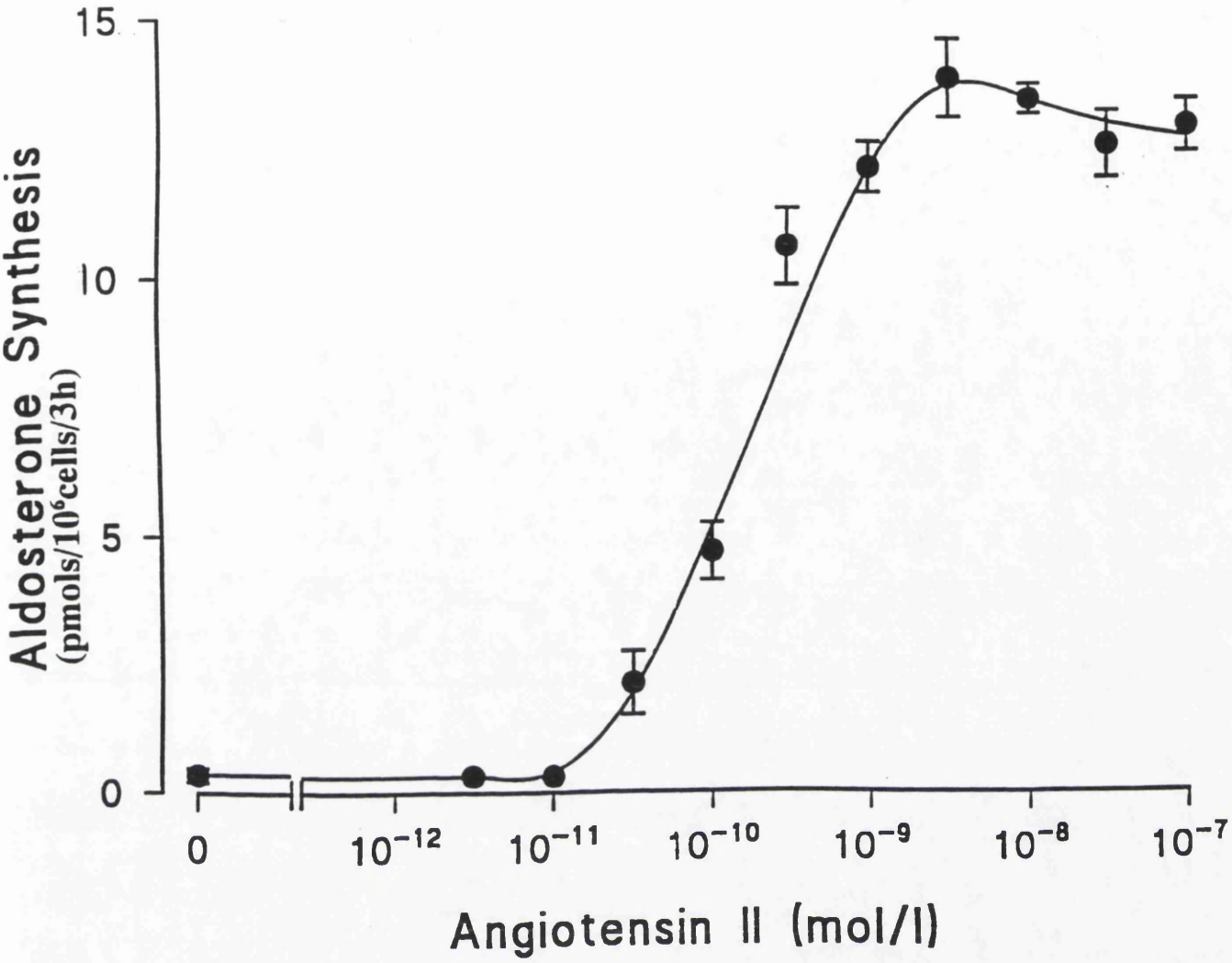


Fig.3.1

Concentration-dependent effects of A-II on aldosterone synthesis in bovine adrenocortical cells which had been in culture for 6 days. Aldosterone response is maximal at 3nmol/l A-II and represents a 45 fold increase over basal synthesis.

Values shown are means ± S.E of 8 replicate incubations.

as percentages of control uptake. Experimental incubations were carried out in octuplicate and results analysed using Student's t-test.

(iv) Measurements of Intracellular Free Calcium.

ZG cells were prepared as previously described in modified Ham's F-12 plus 10% FCS (see above and Chapter 2). They were seeded into sterile bacterial culture plates (Scotlab, Coatbridge, Strathclyde) at a concentration of $5x10^6$ cells/ml and left at 37°C, in the presence of 95%O_2, 5%CO_2, for 72 h (154). Under these conditions the cells, which were only loosely attached to the plate, were removed with gentle scraping and agitation into KRBGA. The Ca^{2+}-sensitive dye, Fura-2 (Sigma Chemical Company, Poole, Dorset) dissolved in dimethyl sulphoxide (DMSO), was added to the cell suspension to give a concentration of 10μmol/l. The final concentration of DMSO in the cell suspension did not exceed 0.2%. The suspension was then aliquoted into 1.5ml Micro-centrifuge tubes containing 1ml aliquots of cell suspension and these were placed in a 37°C water bath. HDL-2 and HDL-2 (both at 0.5mmol/l cholesterol) or control KRBGA were added to the appropriate incubations. The cell suspensions were removed from the water bath after a minimum 30min. exposure to Fura-2 and a precise 15min. exposure to the HDL subfractions. They were spun for 10s. at 2000g in a Microcentaur centrifuge. The supernatant was then removed and the cell pellet resuspended in 2ml of KRBGA. The resulting resuspension was placed in the heated chamber (37°C) of a Perkin-Elmer LS-3B Fluorescence Spectrometer. Fluorescence measurements were carried out at a single excitation wavelength of 340nm, recording the fluorescence signal at the Fura-2 emission wavelength of 505nm. A-II was added in a volume of 20μl to give a final concentration of 10^{-8}mol/l. Details of single wavelength excitation and

emission calibration for Ca^{2+}-chelating dyes are as described by Rink and Pozzan (155) using the following equation:

$$[Ca^{2+}]_i = \frac{F - F_{min}}{F_{max} - F} \cdot K_d$$

F_{max} is the fluorescence signal when all the Fura-2 is calcium-bound. F_{min} is the fluorescence signal when all the Fura-2 is in its free form. The above equation enables the calculation of any $[Ca^{2+}]_i$ denoted by any measured fluorescence, F, between F_{max} and F_{min}. The K_d for Fura-2 has been determined as 224nm at 37°C (61).

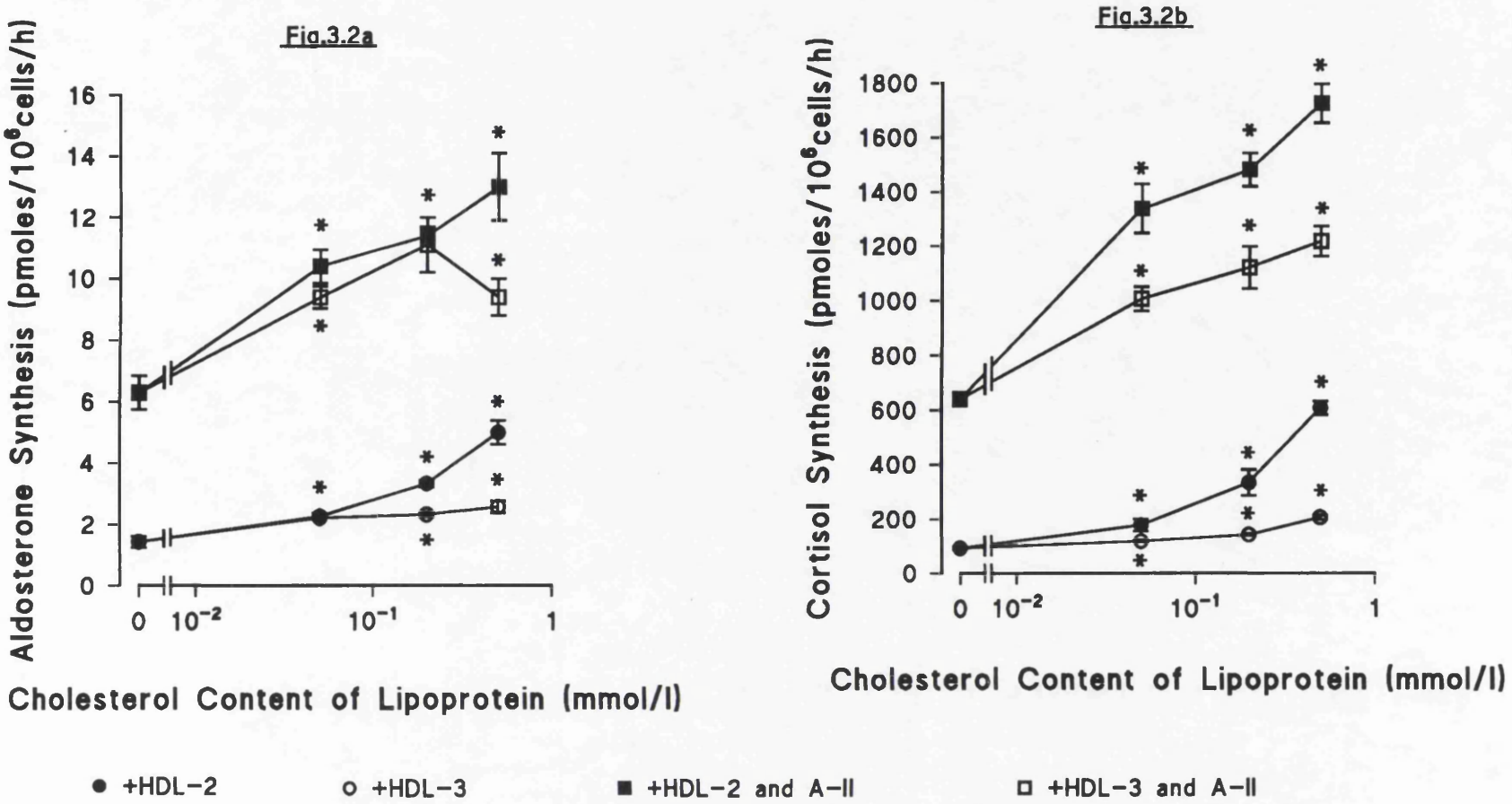
Individual measurements were replicated as indicated in fig.3.6 Results are given as mean ± S.E.M.

3.3 Results

Acute effects (1h) of HDL subfractions on basal and A-II stimulated steroid synthesis in isolated adrenocortical cells

HDL-cholesterol stimulated basal aldosterone (Fig.3.2a) and cortisol synthesis (Fig.3.2b) in a dose-dependent manner, although a much greater stimulatory effect was observed with cells incubated with HDL-2. A-II-stimulated steroid synthesis was also enhanced by HDL-cholesterol. The effects of HDL-2 on A-II-stimulated cortisol synthesis were greater than those of HDL-3. However, any differences in the action of the two subfractions on A-II stimulated aldosterone synthesis are less obvious.

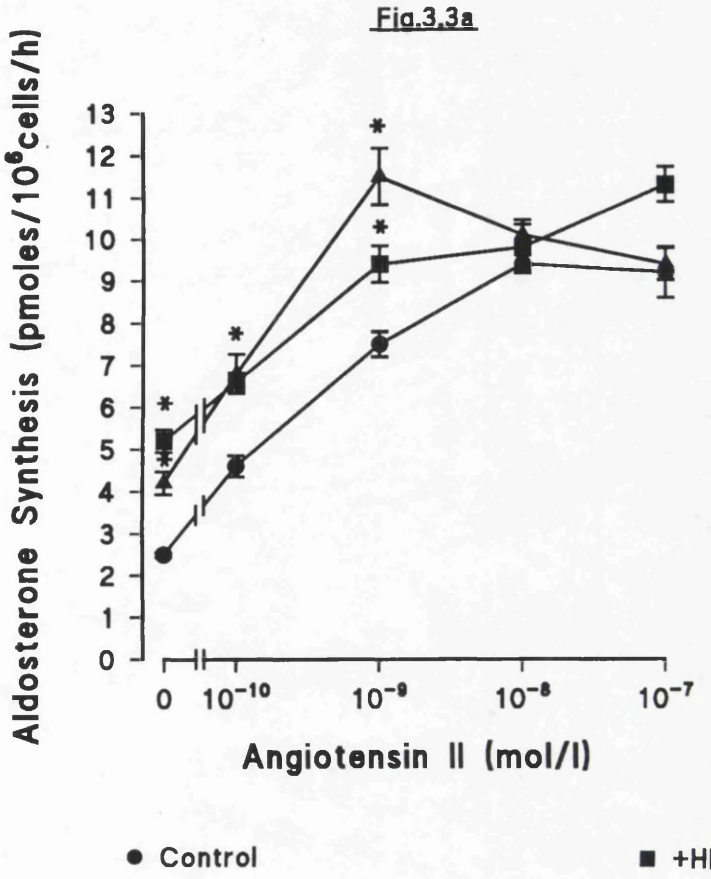
The effects of HDL-cholesterol (0.2mmol cholesterol/l) on the aldosterone and

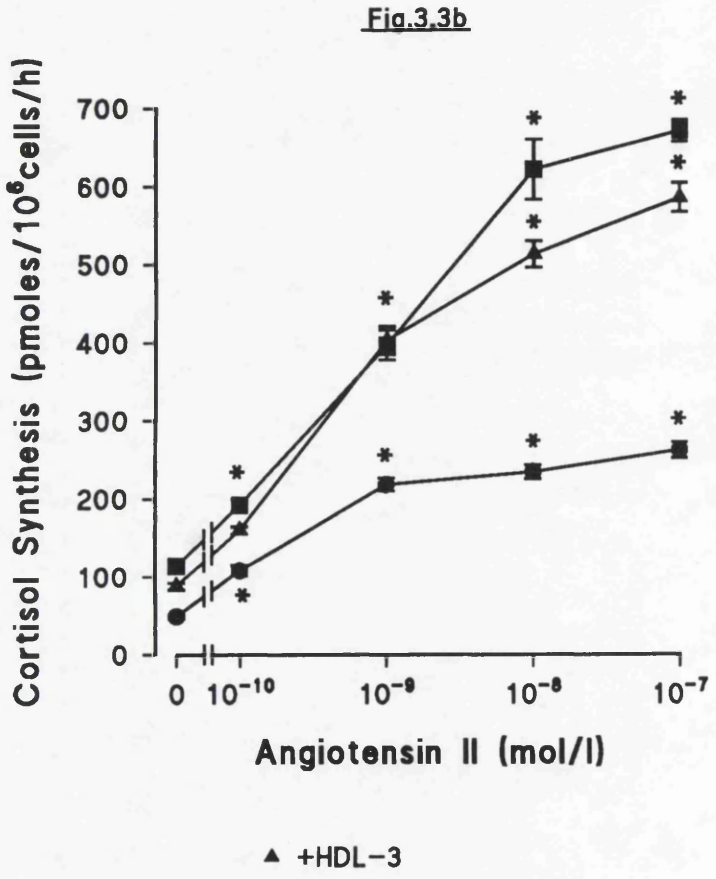


Figs.3.2a and 3.2b

Effects of increasing concentrations of HDL-cholesterol on basal and A-II-stimulated (10^{-8}mol/l) aldosterone (fig.3.2a) and cortisol synthesis (fig.3.2b) in freshly isolated bovine adrenocortical cells.

Values shown are means ± S.E. of six replicate measurements. * indicates significant differences ($p<0.05$) compared with controls (unpaired t-test).





Figs.3.3a and 3.3b

Effects of of HDL-cholesterol (0.2mmol/l) on the aldosterone (fig.3.3a) and cortisol (fig.3.3b) responses of freshly isolated bovine adrenocortical cells to a range of different A-II concentrations.

Values shown are means ± S.E. of six replicate measurements. * indicates significant differences ($p<0.05$) compared with controls (unpaired t-test).

Fig.3.4a

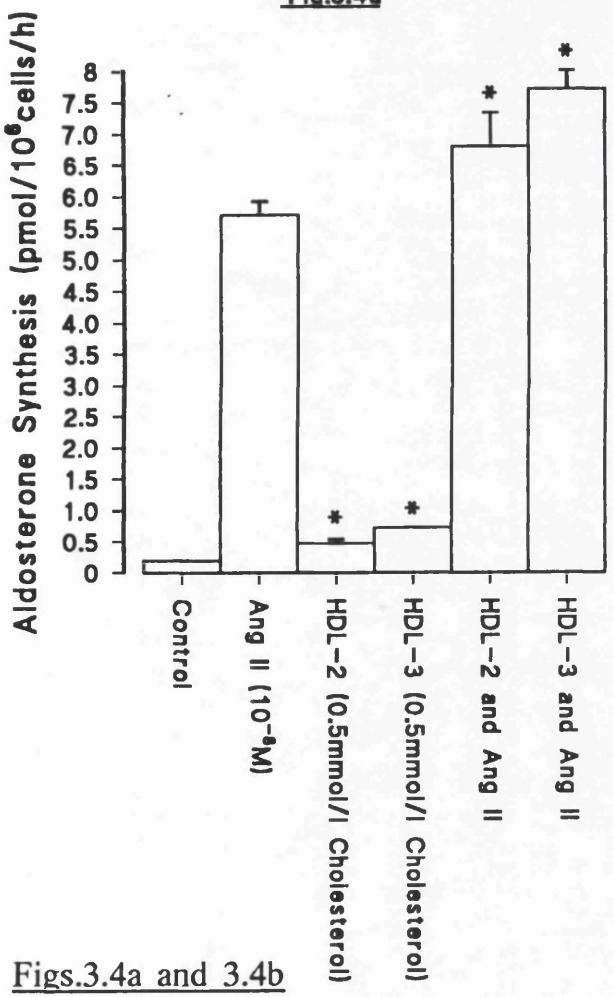
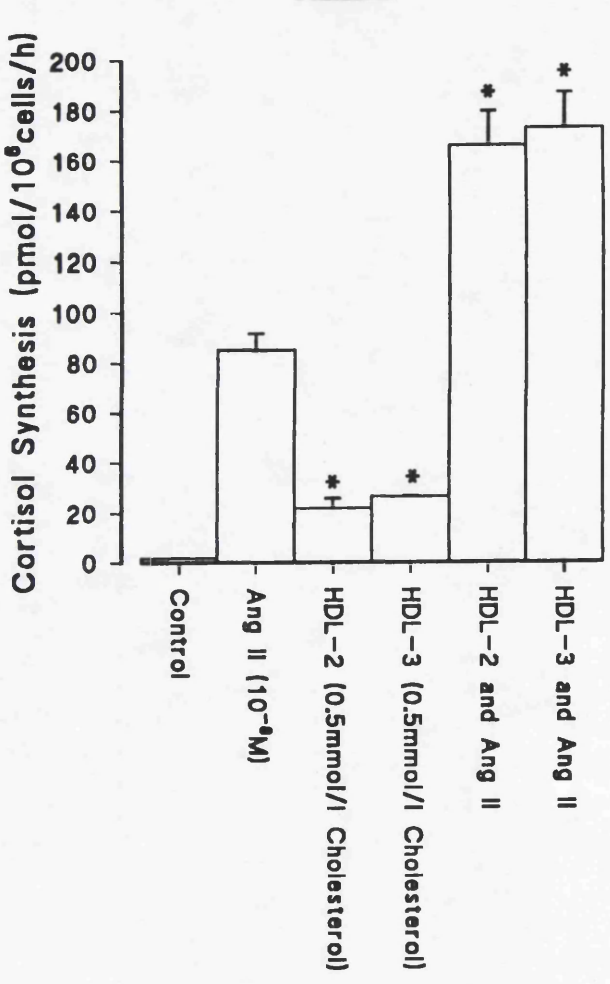


Fig.3.4b



Figs.3.4a and 3.4b

Effects of HDL-cholesterol (0.5mmol/l) on the aldosterone (fig.3.4a) and cortisol (fig.3.4b) responses of cultured bovine adrenocortical cells A-II (10^{-8}mol/l).

Values shown are means ± S.E. of six replicate measurements. * indicates significant differences ($p<0.01$) compared with controls (unpaired t-test).

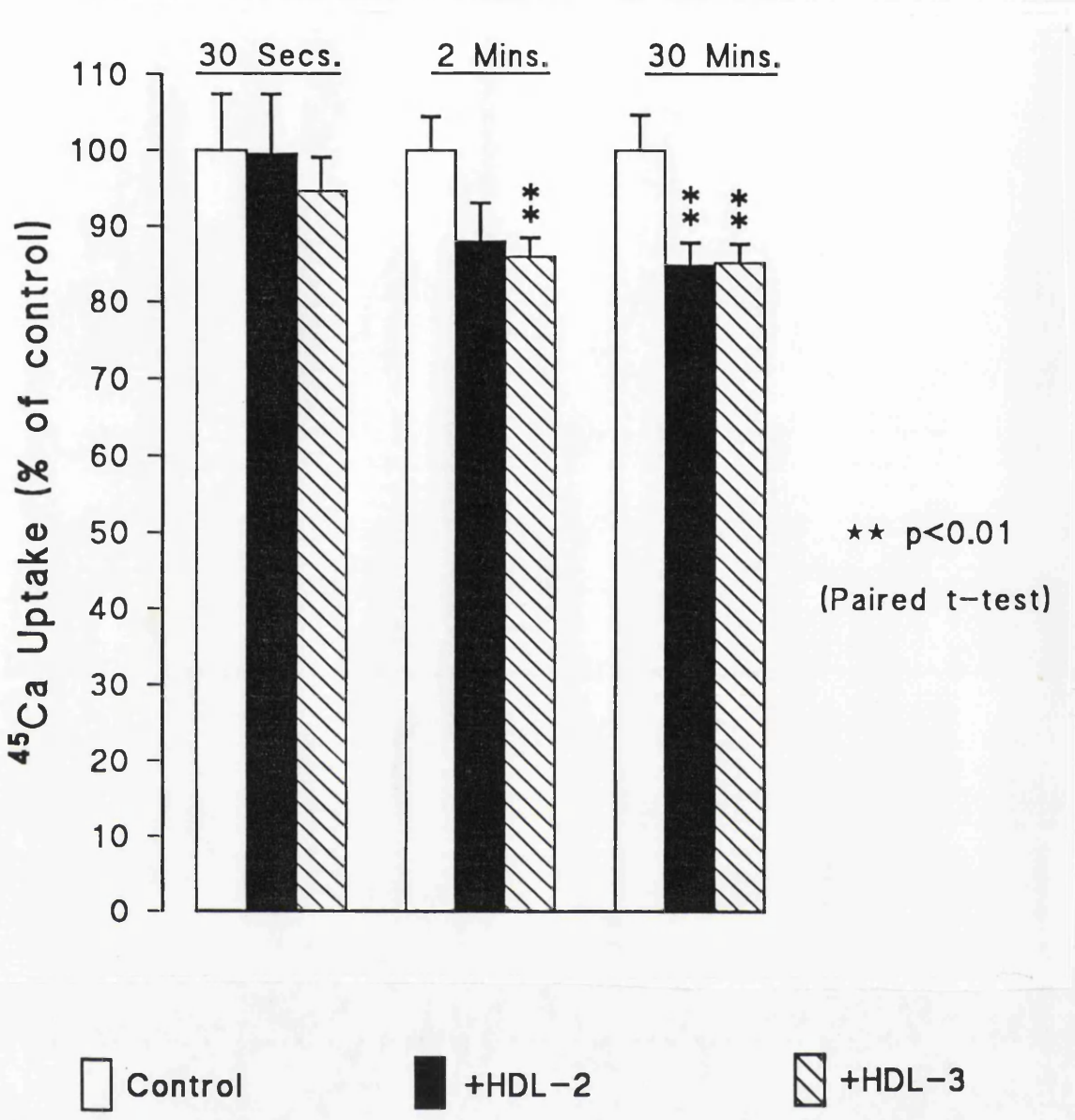


Fig.3.5

Effects of HDL-cholesterol (0.4mmol/l) on basal accumulation of ^{45}Ca in freshly isolated adrenocortical cells which had been pre-treated with lipoprotein/vehicle for 15min. prior to addition of ^{45}Ca.

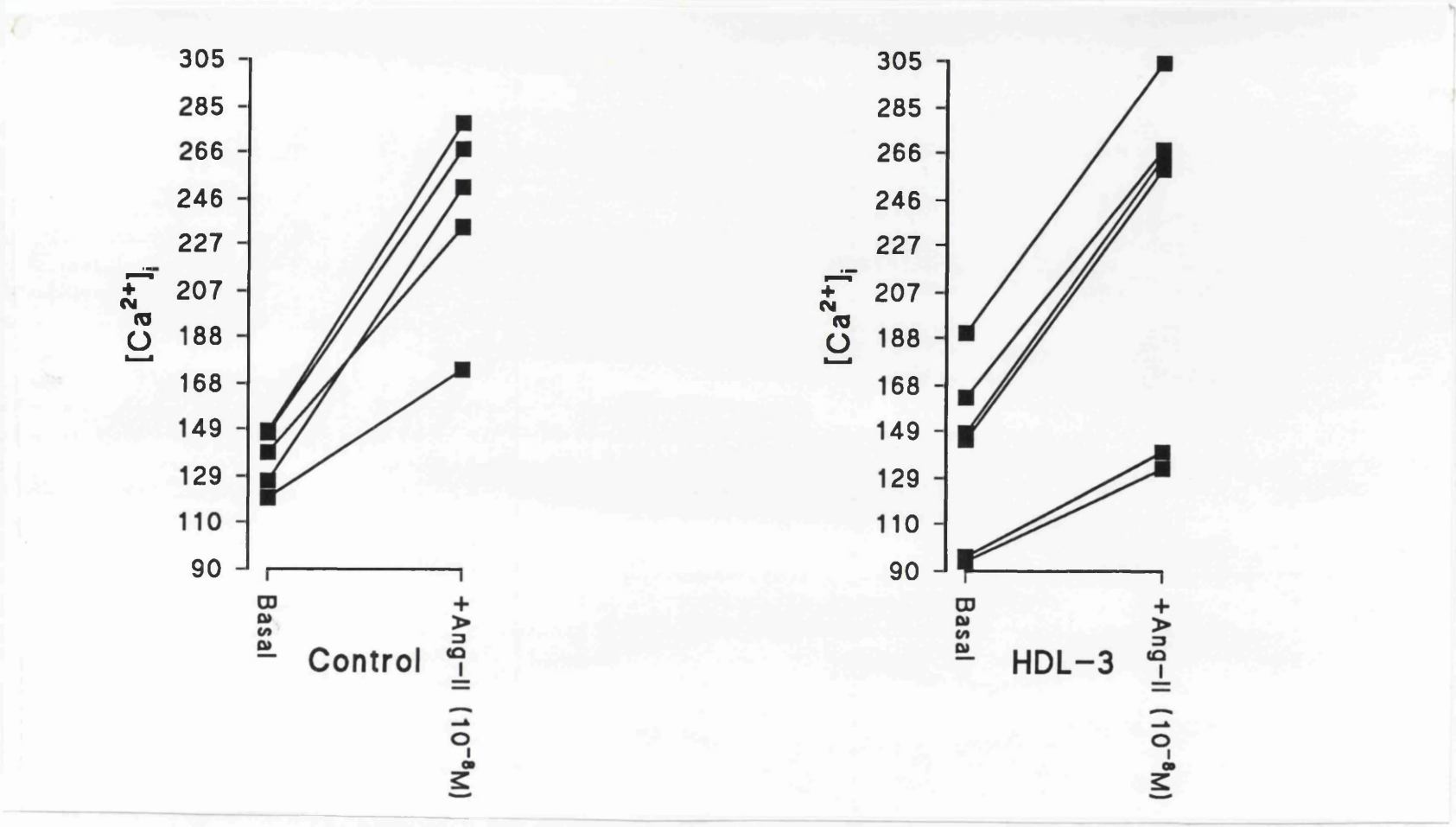


Fig.3.6

Effects of a 15min. pre-exposure to HDL-cholesterol (0.5mmol/l) on basal and A-II-stimulated (10^{-8}mol/l) $[Ca^{2+}]_i$ in adrenocortical cells plated down for 72h prior to experiment. Individual data points are displayed for each type of treatment (Control, n=5; HDL-3, n=6).

cortisol responses to a range of different A-II concentrations are shown in Figs.3.3a and 3.3b. The effects of the two lipoproteins on the aldosterone or cortisol responses to A-II are similar. In cells incubated with HDL-cholesterol, an enhancement of the aldosterone response to A-II ($p<0.01$) was observed at lower concentrations of hormone (10^{-10} and 10^{-9}mol/l) However, at the higher two concentrations of A-II (10^{-8} and 10^{-7}mol/l) this enhancement was not apparent. In contrast, HDL-cholesterol significantly increased A-II-stimulated cortisol synthesis at every concentration used ($p<0.01$).

Sustained effects (24h) of HDL subfractions on basal and A-II-stimulated steroid synthesis in cultured adrenocortical cells

Pre-treatment of cultured cells for 24h with HDL subfractions prior to a 1h acute incubation, markedly affected steroidogenesis. Basal aldosterone (Fig.3.4a) and cortisol synthesis (Fig.3.4b) were increased by factors of 3 and 12 respectively with HDL-2 and by factors of 4.5 and 15 respectively with HDL-3. Without pre-treatment with HDL-cholesterol these cells were very sensitive to hormonal stimulation, addition of A-II increased aldosterone output 30 fold and cortisol output 42 fold when compared with basal synthesis. Pre-treatment with HDL-cholesterol increased total A-II-stimulated production of aldosterone and cortisol. However, the stimulatory response of the cells to A-II was significantly reduced in the presence of HDL-cholestrol because of the enhanced steroid output in the absence of A-II.

Effects of HDL subfractions on basal ^{45}Ca uptake in isolated adrenocortical cells

Neither HDL-2 nor HDL-3 (both at 0.4mmol cholesterol/l) had any effect on acute ^{45}Ca uptake which was measured after 30s. (Fig.3.5). Similarly, HDL-2-treated

samples still showed no difference from control incubations after 2 min. although HDL-3 treated cells had accumulated significantly less radioactivity by this time ($p<0.01$). However, after 30 min., cells treated with either HDL-2 or HDL-3 had taken up approximately 15% less radioactivity than vehicle-treated samples.

Effects of HDL subfractions on basal and A-II stimulated intracellular free calcium in adrenocortical cells

Fig.3.6 demonstrates the effects of HDL-2 and HDL-3 on basal and A-II (10^{-8}mol/l) stimulated free $[Ca^{2+}]_i$ in ZG cells plated down for 72 hours prior to experiment.

In control samples, A-II increased free $[Ca^{2+}]_i$ from 136±5 nmol/l to 241±18 nmol/l (n=5). In samples treated with HDL-2 and HDL-3, basal free $[Ca^{2+}]_i$ was 120±18 (n=2) and 137±13 nmol/l (n=6) respectively. Addition of A-II increased these values to 160±3 for HDL-2 and 216±27 for HDL-3.

3.4 Discussion

The availability of cholesterol to cytochrome $P\text{-}450_{scc}$, located in the inner mitochondrial membrane of adrenocortical cells, is rate-limiting step in steroid biosynthesis. This substrate can be derived from a variety of sources including de novo synthesis from acetate, hydrolysis of stored cholesteryl esters and uptake of circulating plasma lipoproteins. Cholesterol may originate from different lipoprotein sources in different tissues. For instance, serum LDL has been cited as the source of cholesterol in bovine adrenal cells (99) whereas HDL is the major source in rat cells (105). Studies with newborn rat adrenocortical cells have also suggested that HDL may alter the ratio of 21-hydroxylated: 20α-reduced products of progesterone

in these cells; that is the lipoprotein may be involved not only in substrate supply but also in the modulation of steroid pathways away from inactive reduced metabolites towards synthesis of active corticosteroids (156). However, recent in vitro studies have indicated that HDL may be a more efficient cholesterol donor than LDL in short-term incubations of bovine cells (103).

Plasma HDL levels are an important factor in the incidence and prevalence of artherosclerosis (157). This is related to the ability of HDL to remove cholesterol from cells and to transport it to the liver for breakdown and removal from the body. This reverse cholesterol transport is critical for cholesterol homeostasis as most cells cannot metabolise cholesterol they accumulate. Steroidogenic cells are a notable exception and, in vivo, HDL particles deliver cholesterol to them, as well as to liver cells (100). My own data (Figs.3.2a and 3.2b) show significant increases in both basal and A-II stimulated steroid synthesis at a range of HDL concentrations, confirming that HDL supplies substrate cholesterol for steroidogenesis. These same data also suggest that HDL-2 has a greater effect on steroid synthesis (to a lesser extent on A-II-stimulated but certainly on basal) than HDL-3. The reason for this is unclear. The different ratios of free cholesterol: cholesteryl ester ratio in the two subfractions may provide an explanation. HDL-3 is a smaller, denser particle and the action of a plasma-borne lecithin cholesteryl-acyl transferase converts free cholesterol in these particles to cholesteryl ester and forms larger HDL-2 particles (100). In the present experiment the total cholesterol content of HDL-2 and HDL-3 was identical but the cholesteryl ester content of HDL-2 was greater than that of HDL-3. If cholesteryl ester is more efficiently absorbed or transported into the cell, this would account for the greater effect of HDL-2. However, others have suggested that HDL-associated free cholesterol, rather than cholesteryl ester, is preferentialy utilised as

substrate for steroidogenesis (105,106).

My results also demonstrate (Figs.3.3a and 3.3b) a marked difference in responsiveness to HDL of aldosterone and cortisol synthesis in isolated cells treated with A-II. When compared to controls, production of both steroids is enhanced by HDL-2 and HDL-3 at lower concentrations of A-II (10^{-10} and 10^{-9} mol/l). Both lipoproteins also enhance cortisol production at higher concentrations of A-II (10^{-8} and 10^{-7} mol/l). However, at these same higher A-II concentrations, the aldosterone response to HDL-2 and HDL-3 cannot be distinguished statistically from control values. Earlier work in this laboratory may provide an explanation for this phenomenon. It has been established that unfractionated HDL inhibits vasopressin-stimulated platelet aggregation by reducing $[Ca^{2+}]_i$ (Simpson and Graham, unpublished data). Treatment with HDL-2 also reduces ^{45}Ca accumulation by platelets (D. Graham, unpublished data). Others have confirmed that physiological concentrations of HDL-2 are anti-aggregatory and that this may be linked with the occupation of cell-surface receptors by HDL-E, a subclass of HDL-2 which is rich in apolipoprotein E (158). If, as in platelets, HDL decreases $[Ca^{2+}]_{in}$ in adrenocortical cells, either by a receptor-mediated process or by altering plasma membrane cholesterol composition to affect membrane fluidity and perhaps ion-channel activity (159,160), this may explain the differing responsiveness of A-II-stimulated aldosterone synthesis and cortisol synthesis to HDL. Aldosterone synthesis, more so than cortisol synthesis, is sensitive to variations in $[Ca^{2+}]_i$ (see chapter 2).

HDL-2 and HDL-3 had little or no effect on ^{45}Ca uptake in glomerulosa cells measured after 30s and 2 min. It has been demonstrated previously that agonist-sensitive ^{45}Ca uptake is linear during this period (79,158). Significant inhibition of ^{45}Ca accumulation by both subfractions was only measurable after 30min, at a time

when steady-state conditions have been achieved (rate of ^{45}Ca uptake has reached a plateau) and ^{45}Ca content, under these conditions, reflects total intracellular calcium (including Ca^{2+} stored in the endoplasmic reticulum and the mitochondrion) rather than $[Ca^{2+}]_i$. Taken together these data would argue against any cytoplasmic receptor-associated effect of HDL on the uptake of $[Ca^{2+}]_o$. More importantly, HDL subfractions appeared to have no significant effect on the concentration of basal and A-II-stimulated (10^{-8}mol/l) free $[Ca^{2+}]_i$. (Fig.3.6). However, HDL-2 replication was too small (n=2) to draw meaningful conclusions.

Ignoring possible calcium-related effects, the apparent disparity between A-II-stimulated aldosterone and cortisol synthesis in isolated cells incubated with HDL-cholesterol, may simply demonstrate that zona fasiculata/reticularis cells utilise HDL-cholesterol more readily than zona glomerulosa cells. For aldosterone synthesis in the rat, endogenously-synthesised cholesterol appears to be more important than cholesterol derived from exogenous lipoproteins whereas in zona fasiculata/reticularis cells the opposite seems to be the case (104). If a similar preference pertains in bovine cells, it would provide an alternative explanation for my observations.

The actions of HDL-cholesterol on adrenocortical cells are not confined to acute effects (3h) on steroid output. HDL-cholesterol can also provide substrate for adrenocortical steroidogenesis in longer term incubations (Figs.3.4a and 3.4b). Pre-incubating cultured glomerulosa cells for 24h with HDL-cholesterol had a marked effect on steroid output, increasing aldosterone and cortisol synthesis by around 4 and 13 fold respectively. These increases in steroid output were much greater than those obtained in isolated cells (see above) and can be explained by the much lower levels of basal steroid synthesis observed in cultured cells. Although pre-incubation with HDL-cholesterol also increased total A-II-stimulated aldosterone and cortisol

output, the net response of both steroids to A-II (10^{-8}mol/l) was diminished. The differences in responsiveness to A-II probably relates more to changes in unstimulated output than net synthesis in stimulated cells. For example, maximal stimulated output in cultured cells is similar to that in cells which have been freshly isolated. However, unstimulated cultured cells produce less aldosterone than freshly isolated cells and hence the maximum degree of stimulation to A-II is much greater.

In summary, this study confirms that HDL-2 and 3 in vitro are readily usable sources of cholesterol for both basal and, to a lesser degree, A-II-stimulated steroidogenesis. Further studies with ion sensitive fluorescent dyes may answer the question as to whether lipoproteins additionally affect adrenocortical calcium metabolism. The selective use of inhibitors of de novo cholesterol synthesis, in conjunction with lipoproteins, may help to identify any inter-zonal variations in the source of cholesterol for steroidogenesis within the bovine adrenal cortex.

BENZODIAZEPINES AND STEROID SYNTHESIS.

Benzodiazepines, in addition to their sedative, hypnotic and tranquilizing actions, can exhibit both positive and negative effects on steroidogenesis. The next two chapters of this thesis will look at both phenomena in detail. Chapter 4 will focus on the inhibitory effects of two of these drugs, midazolam and diazepam, on steroid synthesis. The next section will then examine the proposed stimulatory effects of several benzodiazepines on adrenocortical cholesterol transport in whole cells by measuring aldosterone synthesis under both basal conditions and in the presence of HDL subfractions to act as cholesterol donors.

CHAPTER 4.

INHIBITORY EFFECTS OF BENZODIAZEPINES ON STEROID SYNTHESIS.

4.1 Introduction

Benzodiazepines can inhibit adrenocortical steroidogenesis (162,163). Two different mechanisms have been suggested to explain this inhibition: (i) direct competition with substrates for steroid-hydroxylating enzymes and (ii) a receptor-mediated process involving cellular calcium metabolism.

Evidence that benzodiazepines inhibit steroidogenic enzymes comes from studies examining cortisol synthesis in isolated bovine adrenocortical cells (138). It was noted that inhibition of ACTH-stimulated synthesis was associated with increased pregnenolone and 17α-hydroxyprogesterone (17-OHP) production. Also, added 17-OHP, pregnenolone and progesterone were converted to cortisol less efficiently in cells treated with benzodiazepine drugs. From these data, it was concluded that, like many other drugs with an imidazole moiety (164,165), benzodiazepines inhibit

adrenal cytochrome P450 enzymes. However, unlike imidazole drugs such as etomidate, ketoconazole and omeprazole (166,167,168), benzodiazepines inhibited microsomal enzymes more strongly than those in mitochondria. Diazepam appeared to be a particularly effective inhibitor of 17α-hydroxylase activity. Since the potencies of benzodiazepines and imidazoles for adrenocortical cytochrome P450 enzymes are related to the affinities that the hepatic cytochrome P450 oxidase enzymes show for these drugs, it seems unlikely that interference with microsomal steroid hydroxylations is receptor-mediated.

Inhibition of aldosterone synthesis in rat adrenocortical cells (162,163) cannot be explained by reduced microsomal 17α-hydroxylase activity since aldosterone synthesis does not involve this step nor does rat adrenal contain 17α-hydroxylase. Also, the concentration of diazepam needed to inhibit microsomal hydroxylation reactions is much greater than that required to inhibit aldosterone synthesis. A more likely explanation of the effects of diazepam on aldosterone synthesis is that they are receptor-mediated. Benzodiazepine receptors have been identified in the adrenal cortex (118,169) and it is significant that the dissociation constant (Kd) value for diazepam binding is appropriate for the effects of diazepam on aldosterone synthesis (162). If, as in the heart, binding of benzodiazepines to adrenocortical receptors closes membrane calcium channels (128), diazepam would tend to oppose the actions of angiotensin II, which stimulates calcium uptake (82,141,170).

In this chapter the inhibitory action of midazolam on steroidogenesis in bovine adrenocortical cells in vitro has been examined more closely. Midazolam has a fused imidazole ring in addition to the more basic benzodiazepine structure exhibited by drugs such as diazepam. One might expect this drug to inhibit aldosterone synthesis by interfering with P-450 enzyme function since previous studies have indicated that

midazolam inhibits 21 hydroxylation as well as 17-α hydroxylation.

First, I compared the concentration-dependent effects of midazolam on adrenocorticophic hormone (ACTH)-stimulated cortisol synthesis with those on potassium-stimulated aldosterone synthesis. Since the mechanism of ACTH action is widely considered to be less dependent on calcium than that of potassium, a difference in sensitivity to benzodiazepines was anticipated. Secondly, I measured the concentration-dependent effects of midazolam on 21-hydroxylase activity, firstly by assaying 17-OHP production in cells incubated with and without ACTH and then more directly, by measuring conversion of 21-deoxycortisol to cortisol in zona fasiculata/reticularis cells and the conversion of 11ß-hydroxyprogesterone to aldosterone in zona glomerulosa cells. Finally, I investigated possible inhibitory effects of both midazolam and diazepam on basal and potassium-stimulated uptake of extracellular ^{45}Ca, and compared the concentration-dependent effects of midazolam on ^{45}Ca uptake in cells isolated from the zona glomerulosa and zona fasiculata regions of the gland.

4.2 Materials and Methods.

(i) Isolation of Adrenocortical Cells

Cell suspensions enriched with aldosterone-synthesising zona glomerulosa cells were prepared as described in Chapter 2 except that Ham's F-12, containing 2.5mmol/l Ca^{2+}, and 0.2% BSA, was substituted for medium 199 during cell preparation and in all experimental incubations.

Cell suspensions containing high proportions of cortisol-synthesising zona fasiculata/reticularis cells were prepared in Ham's F-12, again as described in chapter 2 but in this case isolating cells from the Percoll gradient with specific gravity

>1.052-<1.097.

(ii) Steroid Incubations

All incubations were carried out in sextuplicate for 1h at 37°C in an atmosphere of 95% O_2, 5% CO_2 in a total volume of 0.5ml, containing approximately $2x10^5$ cells. Incubations were carried out in Ham's F12 medium containing 0.2% BSA and 2.5mmol/l Ca^{2+}. The concentration-dependent effects of midazolam (Roche Products, Welwyn Garden City, Herts, U.K.) on basal and ACTH (Synacthen; Ciba, Horsham, Sussex)-stimulated cortisol and 17-OHP synthesis were examined. In addition, the direct effects of the drug on 21-hydroxylase activity were investigated in zona fasiculata/reticularis cells using 21-deoxycortisol and in zona glomerulosa cells using 11ß-hydroxyprogesterone (both from Sigma Chemical Company, Poole, Dorset, U.K.) as substrates. To examine the effects of midazolam and diazepam (Phoenix Pharmaceuticals, Gloucester, Gloucs. U.K.) on basal and potassium-stimulated aldosterone synthesis, zona glomerulosa cells were incubated in Krebs' bicarbonate Ringer (KRBGA), containing calcium at 2.5mmol/l plus glucose and BSA at 0.2%, modified to give final potassium concentrations of 3.8 and 10mmol/l. All reactions were stopped by the addition of 0.25ml methanol and then centrifuged at 800g for 10 min before removal of supernatants which were stored at -20°C for steroid analysis.

Measurement of cortisol and aldosterone synthesis were carried out by radioimmunoassay as described in Chapter 2. 17-OHP production was kindly measured by Dr C.E. Gray, Dept. of Clinical Biochemistry, Royal Infirmary, Glasgow, again by direct radioimmunoassay. Results were expressed as nmol steroid/10^6 cells per h and were analysed using Student's t-test.

(iii) ^{45}Ca Uptake Studies

^{45}Ca uptake was measured according to the method described in Chapter 3. Cells were stimulated with K^+ (final concentration 10.8mmol/l) at the time when ^{45}Ca was added. To maintain uniform osmolarity, the potassium content of KRBGA was adjusted by substituting KCl for NaCl. The results are expressed as percentages of control uptake. For each experimental run, cells with all different treatments were incubated simultaneously. Experimental runs were then replicated five times and the results were analysed using Student's paired t-test.

4.3 Results.

Effects of midazolam on cortisol synthesis in isolated adrenocortical cells

Figure 4.1a shows the concentration-dependent inhibition of basal and ACTH-stimulated cortisol synthesis by midazolam. The thresholds for inhibition of basal and ACTH-stimulated cortisol synthesis were 3.4 and 13.6μmol/l respectively ($p<0.001$). Figure 4.1b shows concurrent changes in 17-OHP synthesis. There was an increase in basal and stimulated 17 OHP synthesis, rising to maxima with 13.6 and 6.8μmol midazolam/l respectively ($p<0.001$). At these concentrations, midazolam presumably inhibits 21-hydroxylase activity in a dose-dependent manner. The subsequent decline in 17-OHP synthesis which occurs over the same concentration range as that for the inhibition of ACTH-stimulated cortisol synthesis (Fig.4.1a) could be explained by an additional effect of midazolam on 17α hydroxylation at higher concentrations.

Figure 4.2a further illustrates the effects of midazolam on microsomal hydroxylation when 21-deoxycortisol is provided as substrate for 21-hydroxylation. Although 21-deoxycortisol is not a normal substrate, it can be 21-hydroxylated to form cortisol. Three concentrations of 21-deoxycortisol were used (1, 10 and

Fig.4.1a

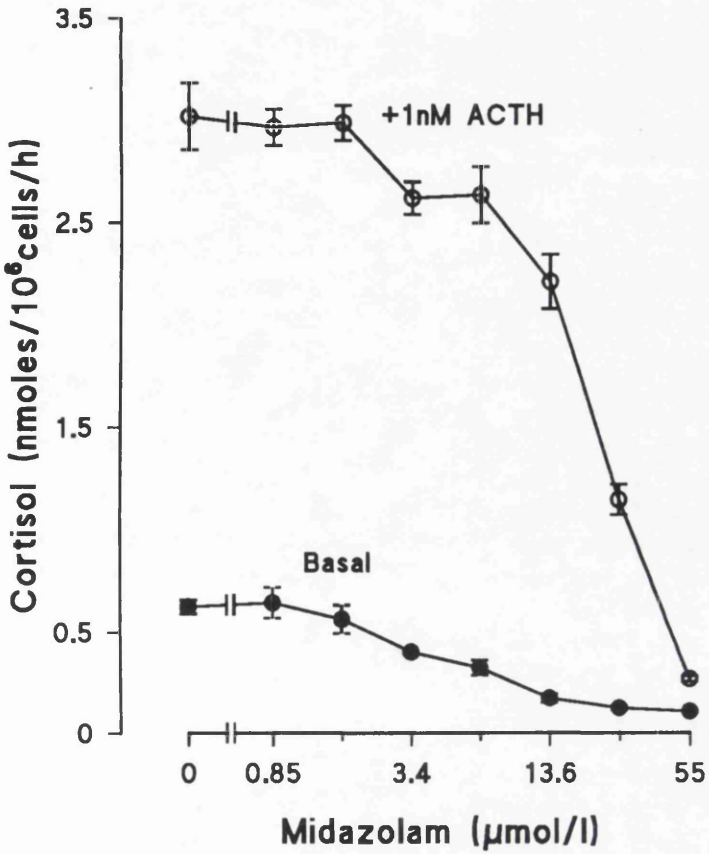
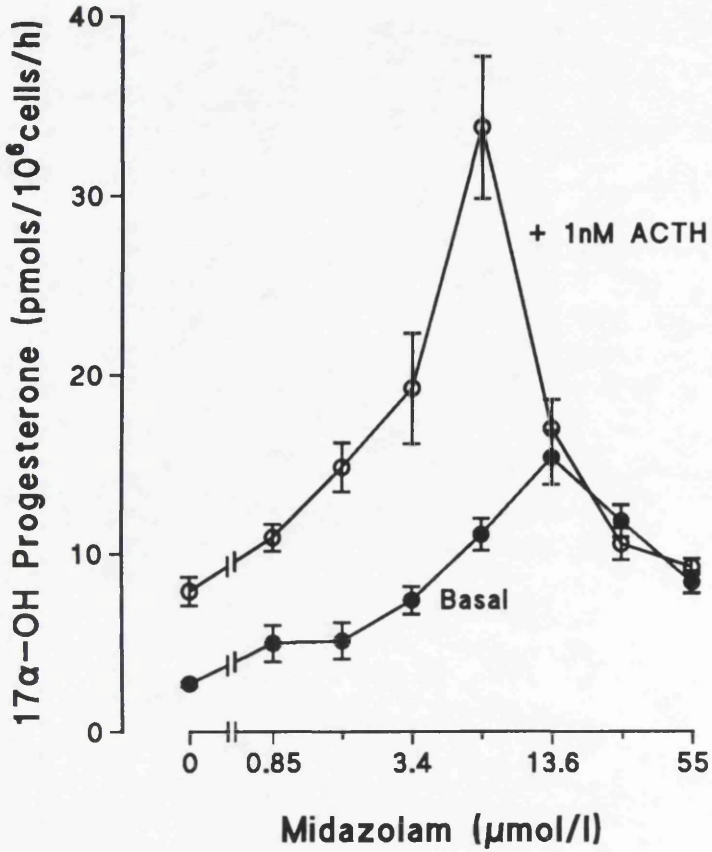


Fig.4.1b



Figs.4.1a and 4.1b

Concentration-dependent effects of midazolam on basal and ACTH-stimulated (1nmol/l) cortisol (fig.4.1a) and 17α-OH progesterone synthesis (fig.4.1b) in isolated bovine zona fasiculata/reticularis cells. Values shown are means ± S.E. (n=6).

Fig.4.2a

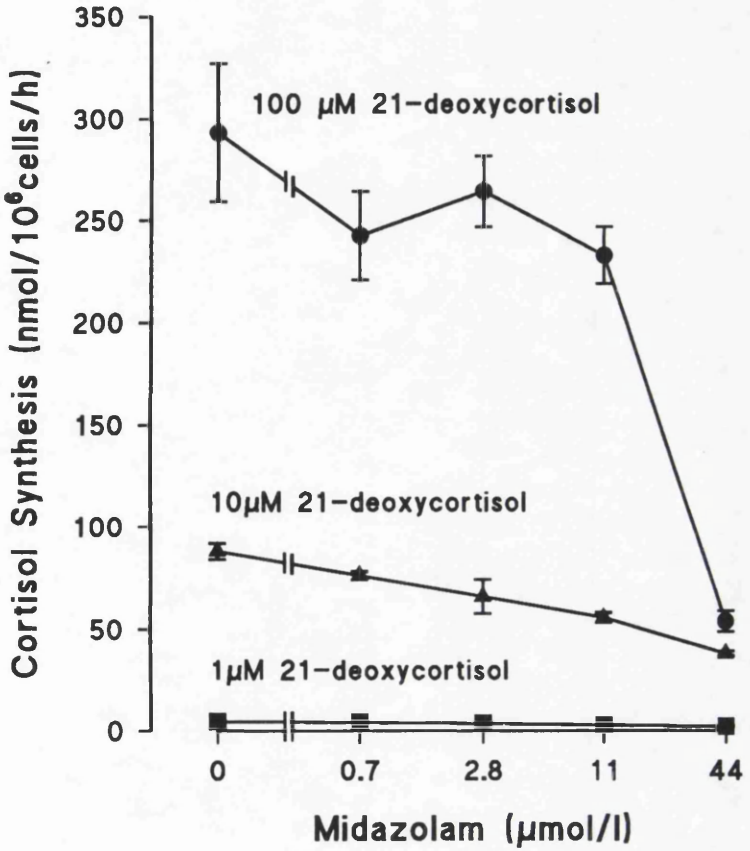
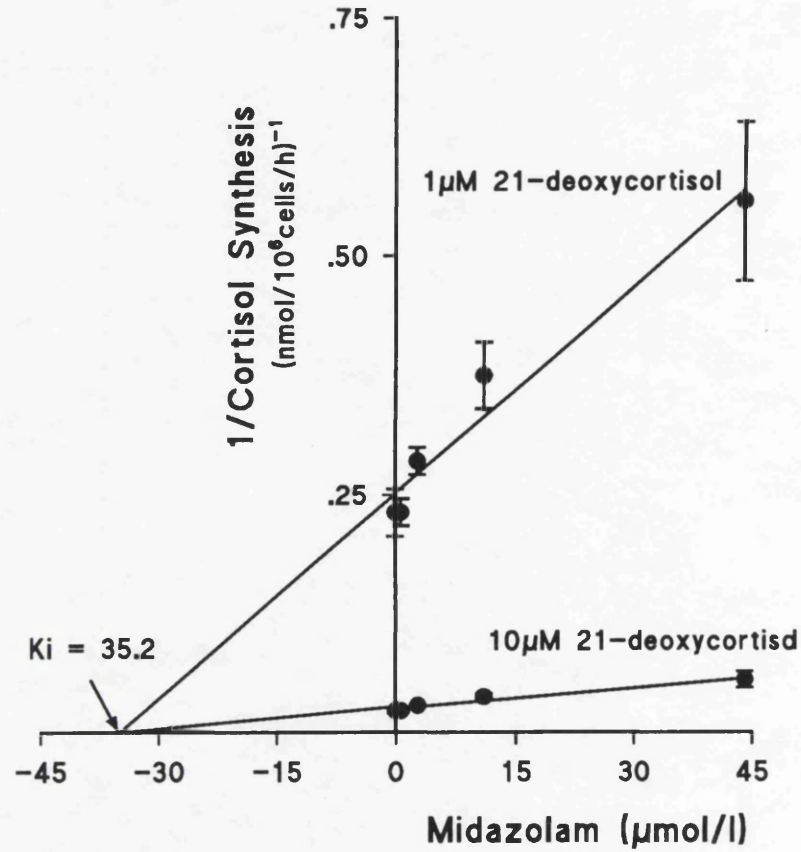


Fig.4.2b



Figs.4.2a and 4.2b

Concentration-dependent effects of midazolam on cortisol synthesis in isolated bovine zona fasiculata/reticularis cells, incubated in the presence of 21-deoxycortisol at 1, 10 and 100µmol/l, are shown in Fig.4.2a. Values shown are means ± S.E. (n=6).

The Dixon Plot (Fig.4.2b) was constructed from data obtained at the lower two concentrations of 21-deoxycortisol.

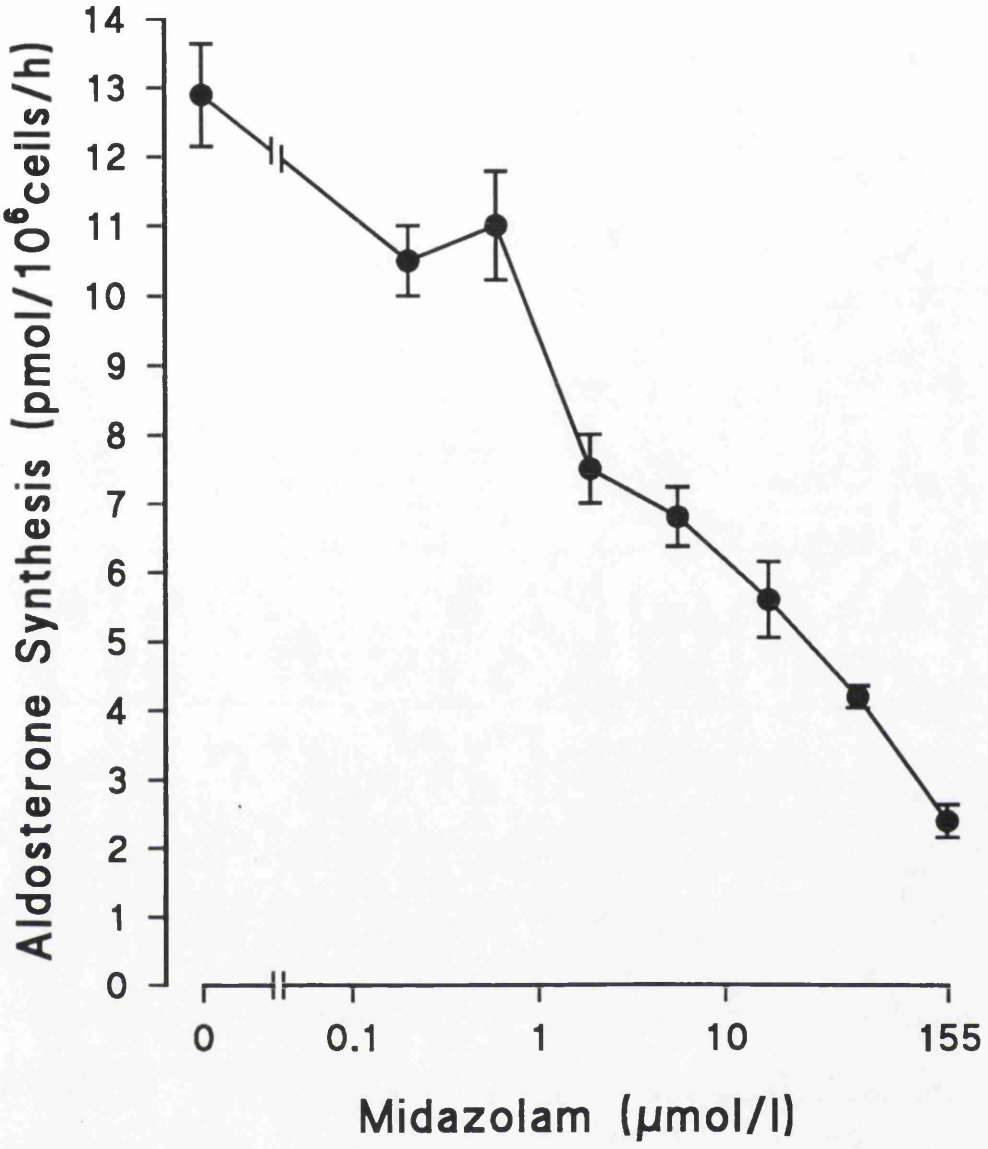


Fig.4.3

Concentration-dependent effects of midazolam on aldosterone synthesis from added 11ß-hydroxyprogesterone (2.5µmol/l) in isolated bovine zona glomerulosa cells.

Values shown are means ± S.E. (n=6).

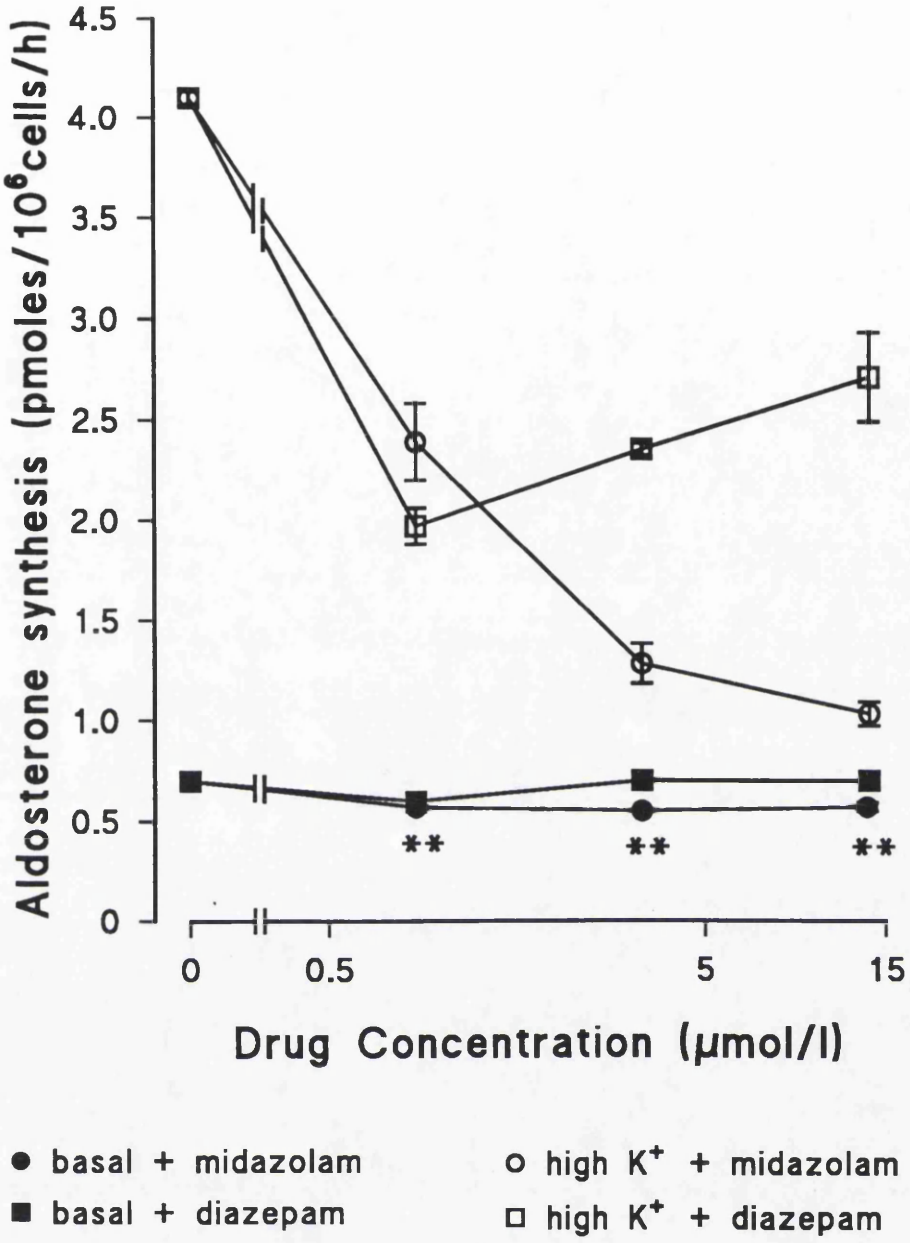
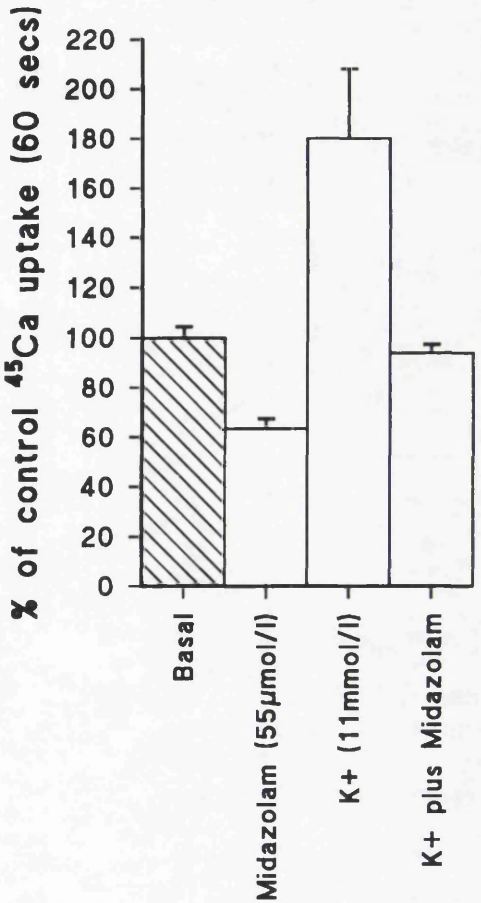


Fig.4.4

Concentration-dependent effects of midazolam and diazepam on basal (3.8mmol K^+/l) and K^+-stimulated (11mmol K^+/l) aldosterone synthesis in isolated bovine zona glomerulosa cells.

Values shown are means ± S.E. (n=6). Statistically significant inhibition by benzodiazepines on basal synthesis is indicated by ** ($p<0.01$) using Student's unpaired t-test.



STATISTICS

Basal : Midazolam p<0.01

Basal : High K^+ p<0.05

High K^+: High K^+ plus Midazolam p<0.01

Midazolam : High K^+ plus Midazolam p<0.01

Fig.4.5

Effect of midazolam (55µmol/l) on ^{45}Ca accumulation over a 1min. period by isolated bovine zona glomerulosa cells under basal conditions (3.8mmol K^+/l; control uptake = 88pmoles $Ca^{2+}/10^6$ cells/min.) and when stimulated with 11mmol K^+/l.

Values shown are means ± S.E. (n=6) and are expressed as percentages of basal ^{45}Ca uptake. Statistical significance was assessed by Student's paired t-test.

Fig.4.6a

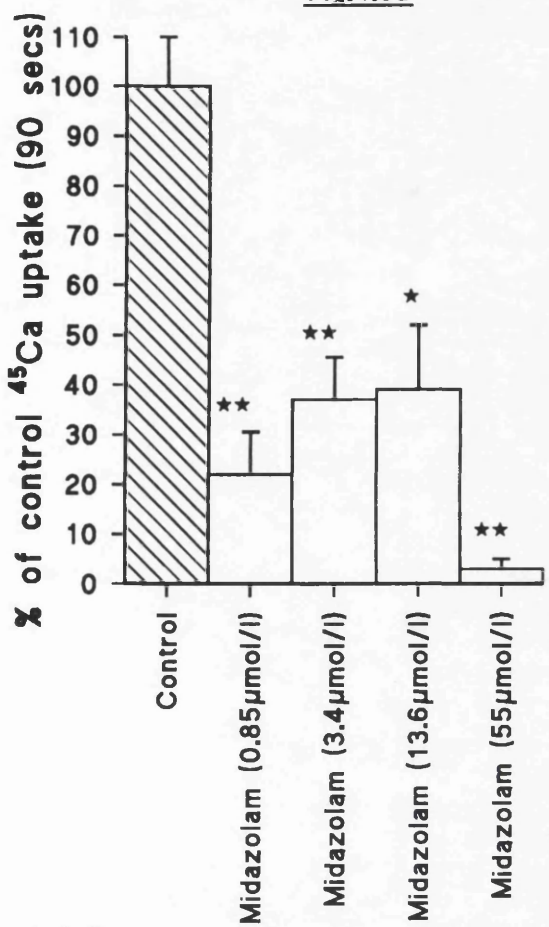


Fig.4.6b

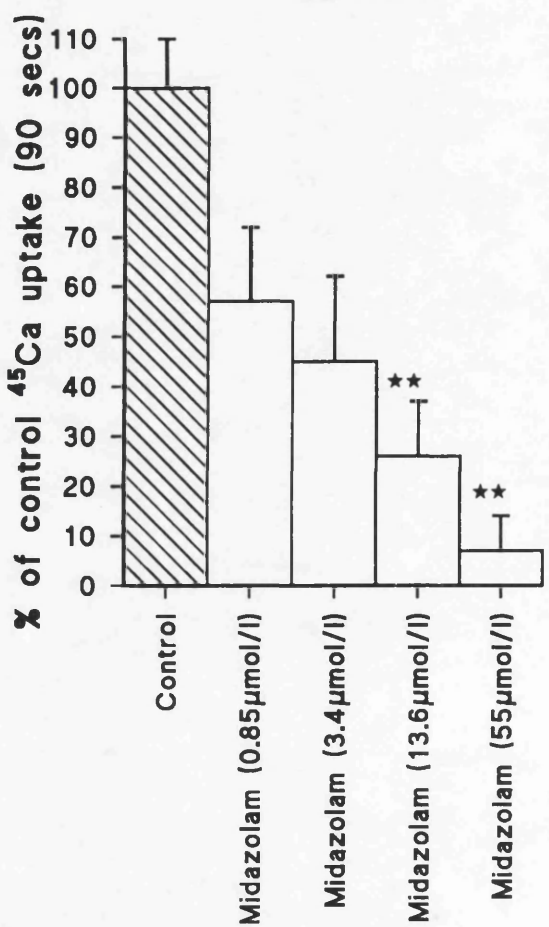


Fig.4.6a and 4.6b

Concentration-dependent effects of midazolam on ^{45}Ca uptake in zona glomerulosa (fig.4.6a) and zona fasiculata/reticularis cells (fig.4.6b). Values shown are means ± S.E. (n=6) and are expressed as percentages of control uptake rates. In the absence of midazolam uptake rates were 42 and 40pmoles $Ca^{2+}/10^6$ cells/min. for zona glomerulosa cells and zona fasiculata/reticularis cells respectively. * ($p<0.05$) and ** ($p<0.01$) indicates significant inhibition by midazolam (Student's paired t-test).

100μmol/l); in each case, cortisol synthesis was inhibited in a dose-dependent manner by midazolam. At the lower drug concentrations (1 and 10μmol/l), the inhibitory thresholds for midazolam were 2.8 and 0.7μmol/l respectively ($p<0.05$). The inhibitory constant (K_I) was 35μmol/l (Fig.4.2b) which corresponds with the 50% inhibitory dose (IC_{50}) of 22μmol/l for the effects of midazolam on ACTH-stimulated cortisol synthesis (Fig.4.1a).

Effects of midazolam and diazepam on aldosterone synthesis in isolated adrenocortical cells

Figure 4.3 shows the effects of midazolam in zona glomerulosa cells on aldosterone synthesis from added 11ß-hydroxyprogesterone (2.5μmol/l). The drug inhibited synthesis in a dose-dependent manner with an inhibitory threshold of 1.9μmol/l and a K_I of 54μmol/l.

The effects of both midazolam and diazepam on basal and potassium-stimulated (3.8 and 10mmol/l K^+ respectively) aldosterone synthesis are shown in Fig.4.4. Midazolam significantly inhibited basal synthesis at each concentration used ($p<0.05$), but no such effect was observed for diazepam. Both drugs markedly inhibited potassium-stimulated aldosterone synthesis at 0.85μmol/l. For diazepam, this was partial and was maximal at this concentration. However, the effects of midazolam were more obviously concentration-dependent, and were greater than those of diazepam at higher concentrations. Note that the inhibitory effects of benzodiazepines on potassium-stimulated aldosterone synthesis occurred at lower concentrations (IC_{50} 1.2μmol/l for midazolam and 0.8μmol/l for diazepam) than those required to inhibit ACTH-stimulated cortisol synthesis (Fig.4.1a), conversion of 21-deoxycortisol to cortisol (Fig.4.2a) or conversion of 11ß- hydroxyprogesterone to aldosterone

(Fig.4.3), which have IC_{50} values for midazolam of 22, 29 and 10μmol/l respectively.

Effects of midazolam and diazepam on ^{45}Ca uptake in isolated adrenocortical cells

In preliminary experiments, we confirmed the findings of several other groups (79,158) that the rate of ^{45}Ca uptake by adrenocortical cells remained linear during the first 2 min of incubation. In the present experiments, uptake was measured over 60s during this initial 2 min period. Figure 4.5 illustrates the effects of potassium stimulation on ^{45}Ca uptake by zona glomerulosa cells in the presence and absence of midazolam. As expected, potassium (10.8mmol/l) stimulated ^{45}Ca uptake, probably by opening voltage-sensitive calcium channels. Treatment with midazolam significantly inhibited both basal ($p<0.01$) and potassium-stimulated uptake ($p<0.05$). However, comparing basal plus midazolam data with potassium-stimulated plus midazolam data indicates that the effects of potassium on calcium uptake are not completely abolished by the drug at this concentration.

The effects of increasing concentrations of midazolam on initial rates of basal ^{45}Ca uptake are illustrated in Fig.4.6a and 4.6b. Figure 4.6a shows data from a preparation containing mainly zona glomerulosa cells; figure 4.6b displays effects on zona fasiculata/reticularis cells. In both cases, midazolam inhibited uptake in unstimulated cells, although the effect on zona fasiculata/reticularis cells only achieved statistical significance at 13.6 and 55μmol drug/l. The zona glomerulosa cells appeared to be more sensitive to midazolam; all concentrations of the drug tested significantly reduced the rate of ^{45}Ca uptake relative to controls.

4.4 Discussion.

Adrenal and hepatic cytochrome P450 enzymes are inhibited by many drugs. In

particular, imidazole derivatives such as etomidate and ketoconazole interfere strongly with these enzymes (165,167). Since the benzodiazepine, diazepam, is a well known inhibitor of hepatic cytochrome P450 enzymes, we tested its adrenocortical effects and found that steroidogenesis was inhibited. Interestingly, midazolam, a benzodiazepine which also contains a fused imidazole ring, proved to be a more potent inhibitor of microsomal steroid hydroxylase activity and hence of ACTH-stimulated cortisol synthesis (138). The present study has confirmed and extended these findings.

Midazolam significantly inhibited 21-hydroxylase activity in basal and ACTH-stimulated zona fasiculata/reticularis cells causing 17-OHP to accumulate. The specific effects of midazolam on 21-hydroxylase activity was assessed by measuring cortisol production from added 21-deoxycortisol in zona fasiculata/reticularis cells and by measuring aldosterone synthesis from added 11ß-hydroxyprogesterone in zona glomerulosa cells. Inhibition of both aldosterone and cortisol synthesis was competitive, with Ki values of 35 and 54 μmol midazolam/l respectively. However, the effects of midazolam on 17-OHP synthesis were biphasic, with a decrease observed at higher drug concentrations. This higher concentration effect corresponded to an observed decrease in stimulated cortisol synthesis (Figs.4.1a and 4.1b) and probably reflects an inhibition of 17α-hydroxylation in addition to 21 hydroxylation. In contrast, diazepam appears to be a more potent inhibitor of 17α- than 21-hydroxylase (138).

Two other groups have described inhibitory effects of diazepam on corticosteroidogenesis (162,163). In both of these studies, aldosterone production by rat adrenocortical cells was used as an end-point and both found inhibitory effects using drug concentrations a magnitude lower than in previous studies carried out in

this laboratory (138). Given that aldosterone synthesis does not involve 17α-hydroxylation and that, at nanomolar concentrations, diazepam would not be expected to affect 21-hydroxylation directly, the nature of this additional inhibition requires further explanation. One possibility may be that rat cells are more sensitive to benzodiazepines than bovine cells. Alternatively, these drugs, when acting on zona glomerulosa cells, could interfere with some aspect of adrenocortical control which is unique to the synthesis of aldosterone. In support of this hypothesis, previous work has reported an inhibition of the conversion of corticosterone to aldosterone by diazepam, although again this was observed at high drug concentrations (138). To investigate this further, I examined the effects of midazolam and diazepam on K^+-stimulated bovine adrenal cells. The results indicate that benzodiazepine inhibition of stimulated aldosterone synthesis in bovine cells is at least as sensitive as previously reported for rat cells and is 10-20 times more sensitive than the inhibition of ACTH-stimulated cortisol synthesis, the conversion of 21-deoxycortisol to cortisol or the conversion of 11ß-hydroxyprogesterone to aldosterone.

Control of zona glomerulosa cells differs from that of zona fasciculata cells. Whereas cortisol synthesis is controlled by ACTH at a single early step only, i.e. the conversion of cholesterol to pregnenolone, aldosterone synthesis is regulated additionally at later points in its biosynthetic pathway when corticosterone is converted to aldosterone (171). Control of aldosterone synthesis is exerted by several agonists which do not stimulate cortisol synthesis. For example, only small increases in extracellular [K^+] are required to stimulate steroidogenesis (172). The stimulatory effects of K^+ at both early and late steps in the biosynthetic pathway of aldosterone are known to be mediated by increased calcium uptake through voltage-sensitive calcium channels following depolarization of the membrane (173,174). These calcium

channels are very sensitive to small negative potentials and show rapid inactivation, resembling T-type channels found in neurones and atrial pacemaker tissues. They also display high sensitivity to drugs such as nitrendipine and nifedipine. Both K^+-stimulated aldosterone synthesis and calcium uptake have been shown to be inhibited by voltage-sensitive calcium channel blockers (82,175). Since benzodiazepines have been shown to inhibit depolarisation-induced calcium uptake in several tissues (176,177,178) we tested whether midazolam interfered with signal-response coupling in adrenocortical cells in a way similar to calcium channel antagonists.

Midazolam reduced ^{45}Ca uptake in K^+-stimulated cells to the level of that in unstimulated cells. This effect is similar to that observed by others with dihydropyridine antagonists (82,175). Notice, however, that midazolam also reduced uptake in unstimulated cells and that, unlike cells treated with nifedipine or nitrendipine, the difference between basal and K^+-stimulated uptake in the presence of midazolam was still statistically significant. So, although midazolam inhibits K^+-stimulated calcium uptake, it does not abolish the effect completely at this concentration.

It is relevant that the relationship between the concentration of midazolam and inhibition of ^{45}Ca uptake by zona glomerulosa cells is similar to that for midazolam and K^+-stimulated aldosterone synthesis. The fact that aldosterone synthesis, more than that of cortisol, is dependent upon changes in cytosolic free Ca^{2+}, may explain why higher concentrations of benzodiazepine are needed to inhibit ACTH-stimulated cortisol synthesis. In line with this argument, the inhibition of calcium uptake by zona fasciculata cells by midazolam appears to be weaker than that in zona glomerulosa cells.

The nature of the low-dose anti-steroidogenic effects of benzodiazepines suggests

a mechanism other than non-specific interference with adrenal cytochrome P450 enzymes. One possibility is that the mechanism is receptor-mediated. Receptors have been characterized in the central nervous system through which benzodiazepines can positively or negatively modulate the effects of the inhibitory neurotransmitter aminobutyric acid (GABA), on neuronal firing (115,179). A different receptor has been identified in peripheral tissues. These latter receptors are present in large numbers in the adrenal cortex, and have been localized to the outer mitochondrial membrane (119). Moreover, natural ligands for these receptors, proteins known as endozepines, have recently been identified (123). The functions of these receptors would seem to be incompatible with any effects mediated by benzodiazepines on calcium metabolism, as their cellular location would rule out a primary influence on plasma membrane calcium transport. They have also been most often linked with increased rather than decreased steroidogenesis (see Chapter 5); endozepines and diazepam have both been shown to increase pregnenolone synthesis in mitochondria from a variety of tissues (123,180) possibly by facilitating cholesterol transport to the inner mitochondrial membrane (181). However, since inhibitory effects of benzodiazepines have also been reported (182), this mechanism cannot be completely discounted at present. In addition, although adrenal studies have demonstrated that the peripheral receptor is primarily associated with the mitochondrion, the possibility that it is also associated with membranes from other organelles, including the plasma membrane cannot be excluded (119).

In summary, I have demonstrated a direct inhibitory effect by midazolam on steroid hydroxylase reactions in vitro. Microsomal 21-hydroxylation is particularly sensitive to this drug; higher concentrations appear to inhibit 17α hydroxylations. This competitive inhibition explains why ACTH-stimulated cortisol synthesis is

inhibited, but does not account for the higher sensitivity of K^+-stimulated aldosterone synthesis to benzodiazepines. The involvement of the peripheral receptor in mediating this effect is unlikely, due to it subcellular localization. The additional inhibitory effects of midazolam on aldosterone synthesis are more likely to be due to a blocking of calcium uptake through one or more types of plasma membrane channel (51). The interrelationships between the actions of endozepines and cation transport in the adrenal cortex merit further investigation.

CHAPTER 5.

STIMULATORY EFFECTS OF BENZODIAZEPINES ON STEROID SYNTHESIS.

5.1 Introduction.

Binding sites for benzodiazepines have been identified both within the central nervous system (CNS) and outside in perpiheral tissues (116,119). These central binding sites are associated with the GABA-gated chloride ion channel and drugs binding at these sites modulate the effects of GABA (the principal inhibitory neurotransmitter in the CNS) on ion channel activity and hence on membrane potential and neuronal firing (115,117). Centrally-acting benzodiazepines are used therapeutically as tranquillisers and sedatives.

Peripheral benzodiazepine binding sites have been identified and localised using 4-chlorodiazepam (R05-4864) which has no known therapeutic effects (118). Peripheral sites are found in many tissues but are present at greatest density in steroidogenic tissues, particularly the adrenal cortex (118). Further investigation in adrenocortical cells has localised the peripheral binding site to the outer mitochondrial membrane (119) and it is most often refered to as the mitochondrial benzodiazepine receptor (MBR). The MBR is pharmacolgically distinct from the benzodiazepine receptors in the CNS. Clinically potent benzodiazepines, such as clonazepam bind with high affinity to central receptors but have negligible potency for the MBR while the reverse is true for peripheral ligands such as RO5-4864 and PK11195 (116,183). Furthermore, in contrast to central receptors, binding of benzodiazepines to the MBR is not affected by GABA or chloride ions (118).

MBR ligands induce a wide range of effects on cell function including alterations in proliferation and proto-oncogene expression (121). However, it has also been

reported that steroid biosynthesis is stimulated in whole cells and mitochondria by a number of these MBR selective drugs (114,122,123). This has led to speculation that benzodiazepines, selective for the MBR, mimic the effects of the endogenous cycloheximide-sensitive "labile" protein proposed to regulate translocation of cholesterol from the outer to the inner mitochondrial membrane (181). This is one of the most important but, as of yet, least understood, steps in steroid synthesis. It is now generally agreed that the major rate-limiting step in steroidogenesis is not P-450_{scc} but rather the incorporation of cholesterol into the mitochondrion and its subsequent transport from the outer mitochondrial membrane to P-450_{scc}, located in the inner (95,107). To date MBR ligands have been demonstrated to stimulate cortisol and corticosterone synthesis in bovine and rat fasiculata/reticularis cells respectively (122). In addition, they also stimulate steroid synthesis in mouse Y-1 adrenocortical and MA-10 Leydig cell lines (122,182). However, no-one has, as yet, examined the effects of these ligands on the synthesis of aldosterone by glomerulosa cells.

Bearing in mind probable difficulties in distinguishing stimulation from the inhibition described in Chapter 4, the possible stimulatory effects of benzodiazepines on aldosterone production in cultured zona glomerulosa cells were examined in the following series of experiments:

(i) Time and concentration-dependent effects of diazepam on basal synthesis.

(ii) Effects of benzodiazepines on synthesis stimulated by the addition of A-II, ACTH or K^+.

(iii) Effects of benzodiazepines and the high affinity MBR ligand PK11195, on steroidogenesis from cholesterol obtained from HDL subfractions.

(iv) In addition to the transport of cholesterol to the inner mitochondrial membrane,

control of aldosterone synthesis also depends on the rate of deoxycorticosterone (DOC) conversion to aldosterone. Since this step also involves transport of precursor into the mitochondrion, I decided to investigate whether benzodiazepines stimulate aldosterone synthesis from added DOC.

5.2 Materials and Methods.

(i) Cultured adrenocortical Cells

Cell suspensions rich in aldosterone-synthesising zona glomerulosa cells were prepared in modified Ham's F-12 medium +10% fetal calf serum (FCS) as described in Chapters 2 and 3. They were then seeded into 96 well plates (Linbro tissue culture plates, Flow Laboratories, Irvine, Strathclyde) at a density of $2x10^4$ cells/well and maintained in culture for 6 days. On day 6, the modified culture medium was removed and the cells were washed x3 with unmodified Ham's F-12 containing 2.5mmol Ca^{2+}/l and 2mg/ml BSA. Steroid production was measured over a 3h incubation period at 95% O_2 and 5% CO_2 in a final volume of 200µl of the same medium.

Experiments were set-up as follows:

(a) Time and concentration-dependent effects of diazepam on basal aldosterone synthesis: Cells were treated with various concentrations of diazepam in one of three ways (i) 24h prior to but not during the subsequent 3h experimental incubation when samples were collected for steroid measurement (ii) A 3h incubation with no pre-incubation (c) For 24h prior to and during a 3h incubation period.

(b) Effects of benzodiazepines on hormone and K^+-stimulated aldosterone synthesis: Cells stimulated by the addition of 0.1nmol/l ACTH (Synacthen, Ciba Laboratories, Horsham, Sussex), 1nmol/l A-II (Sigma Chemical Company, Poole, Dorset) or

32mmol/l K^+ were treated with diazepam and midazolam (10^{-7}mol/l). To maintain uniform osmolarity, the potassium content of the Ham's F-12 was adjusted, substituting KCl for NaCl, by mixing appropriate volumes of Ham's F-12 and a high K^+ KRBGA. Controls were incubated with Ham's F-12 mixed with normal K^+ KRBGA.

(c) Effects of benzodiazepines and PK11195 on HDL-stimulated aldosterone synthesis: Diazepam, RO5-4864 and PK11195, at various concentrations, were added either alone, or in combination, to cells together with HDL-2 or HDL-3.

(d) Effects of benzodiazepines on DOC-stimulated late pathway activity: Diazepam, RO5-4864 and PK11195 were added at a range of concentrations, either on their own or in combination, to cells stimulated by the addition of DOC dissolved in methanol. The final methanol concentration of the incubations did not exceed 0.2%.

(ii) Extraction and Partial Chromatographic Separation of cell incubation medium.

Although the cross-reactivity of the aldosterone antiserum with DOC is slight (0.04%), the medium of cells which had been treated with DOC had concentrations (1 or 5μmol/l) which significantly interfered with measurements of aldosterone. To remove this exogenous DOC, all samples were subjected to solid-phase chromatography prior to aldosterone assay. C-18 Sep-paks (Millipore, Watford, Herts.) were primed by addition of 2ml methanol and then washed with 5ml H_2O. The samples were added to the columns. The columns were again washed with 5ml of H_2O. Finally, 3ml of 55% methanol were added and this elutant collected. Previous studies, using ^3H DOC and ^3H aldosterone, had determined recovery values of 85% and 6% for aldosterone and DOC respectively under these conditions.

Aldosterone was measured by direct radioimmunoassay as described in Chapter 2

with no corrections for recovery loss. All incubations were replicated as indicated and the data analysed using Student's t-test.

5.3 Results.

Time and concentration-dependent effects of benzodiazepines on basal aldosterone synthesis in cultured adrenocortical cells

An acute 3h exposure to diazepam (Fig.5.1) significantly increased basal aldosterone synthesis ($p<0.01$) by between 2 and 4 fold at all concentrations tested (10^{-9}-10^{-5} mol/l). This increase was not dose-dependent and was not enhanced by an additional 24h pre-exposure to the drug. A 24h pre-exposure alone demonstrated an aldosterone response of glomerulosa cells to diazepam which persisted in the absence of drug.

Effects of benzodiazepines on A-II, ACTH and K^+-stimulated aldosterone synthesis in cultured adrenocortical cells

The dose-dependent effects of diazepam on aldosterone synthesis stimulated by a range of A-II concentrations is shown in Fig.5.2. Enhancement of stimulated synthesis by the drug was most marked ($p<0.05$; $p<0.001$) at the lower two concentrations of A-II used (10^{-10} and 10^{-9}mol/l). This enhancement was only observed at concentrations of diazepam <0.1mmol/l and concentrations of drug greater than this inhibited stimulated aldosterone synthesis at every concentration of A-II tested ($p<0.05$; $p<0.001$). There was also little or no enhancement by diazepam, at any concentration, when supra-maximal concentrations of A-II (10^{-8}mol/l) were used to stimulate steroid synthesis.

Fig.5.3 demonstrates the effects of diazepam and midazolam (10^{-7}mol/l) on the

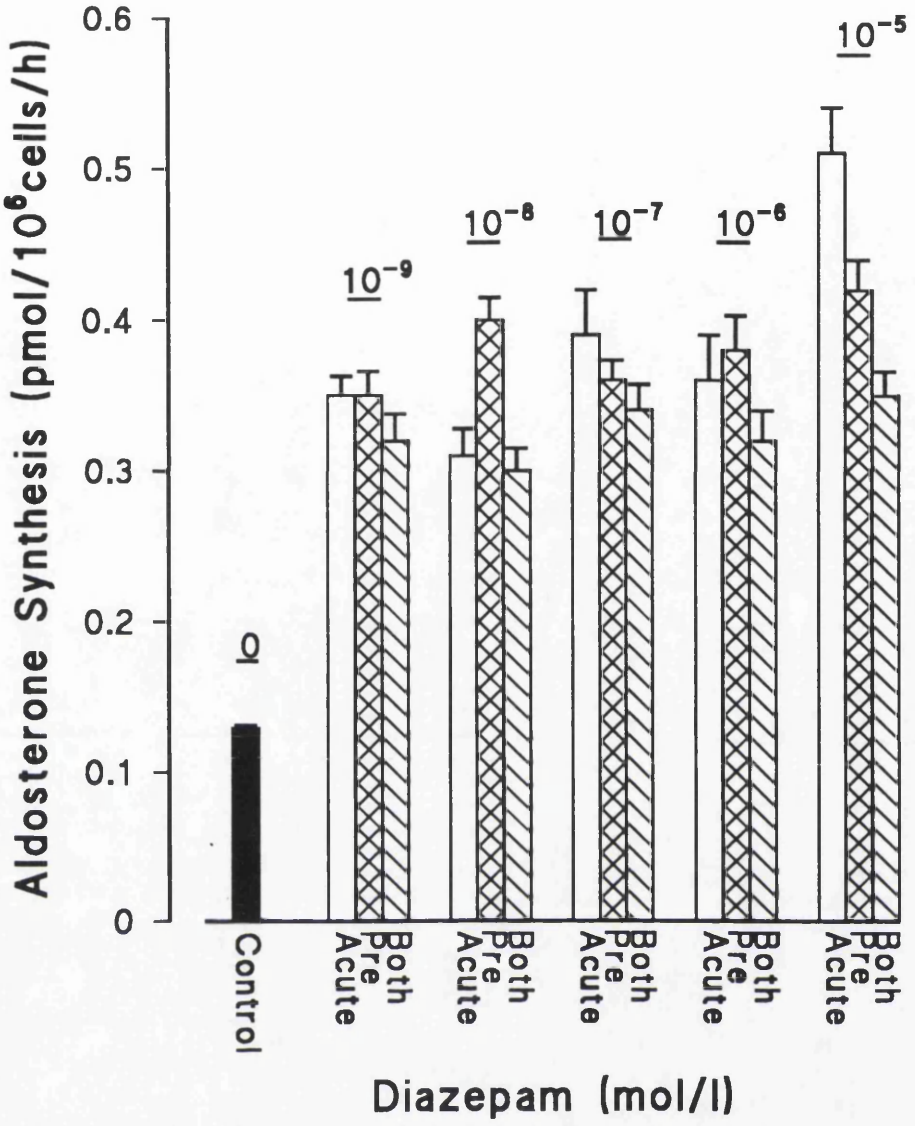


Fig.5.1

Concentration-dependent effects of (i) a 24h pre-exposure to diazepam or (ii) a 3h acute treatment with the drug or (iii) both of the above, on basal aldosterone synthesis in cultured zona glomerulosa cells.

Values shown are means ± S.E. (n=8). Aldosterone synthesis by all diazepam treated samples was statistically greater than control ($p<0.01$; Student's unpaired t-test).

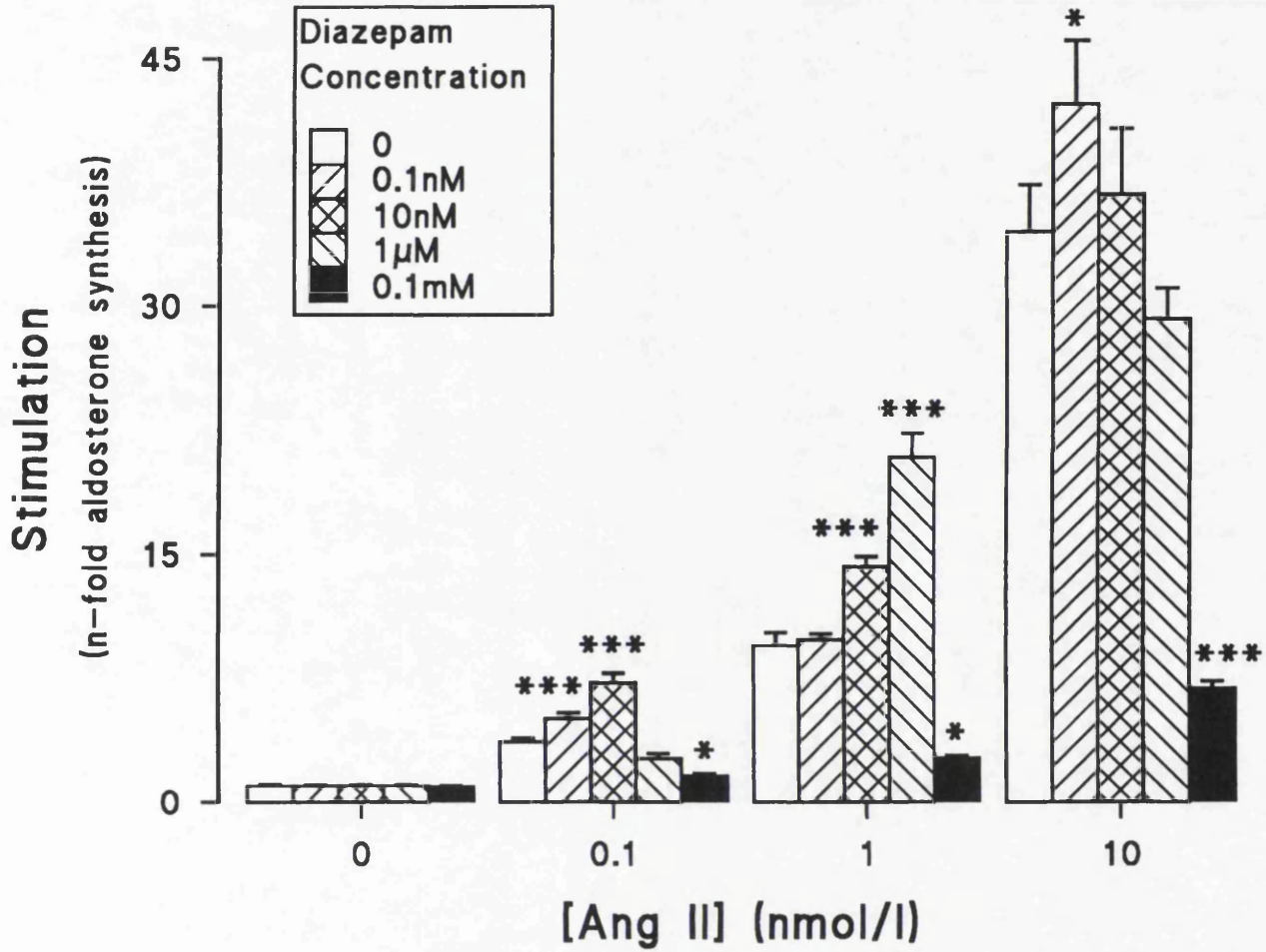


Fig.5.2

Dose-dependent effects of diazepam on aldosterone synthesis in cultured bovine zona glomerulosa cells stimulated by a range of A-II concentrations.

Values shown are means ± S.E. (n=8) and are expresed as a factor of basal synthesis in the absence of drug. Significant differences compared with controls are indicated by * ($p<0.05$) and *** ($p<0.001$) using Student's unpaired t-test.

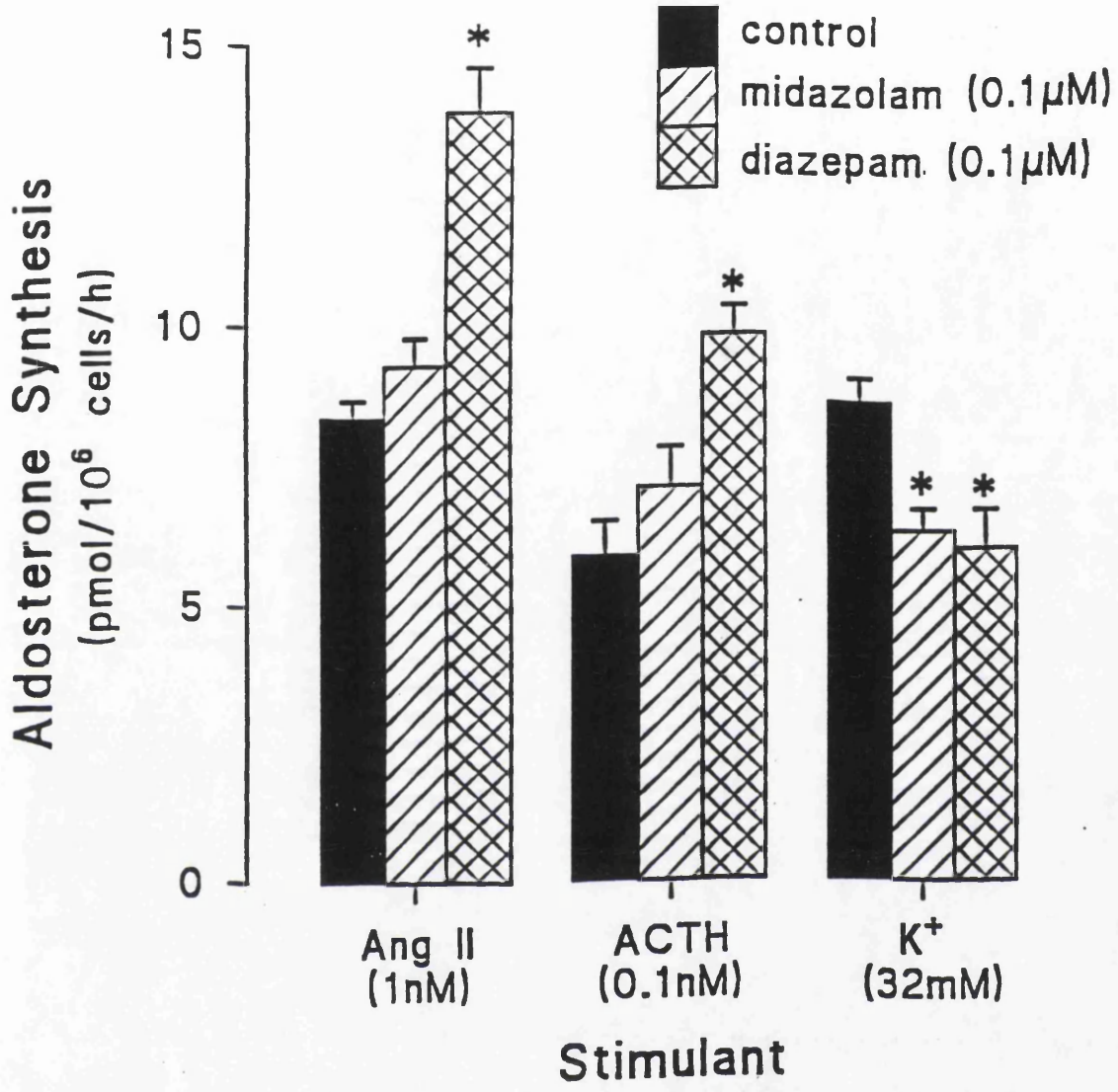


Fig.5.3

Effects of midazolam and diazepam (both at 1μmol/l) on A-II (1nmol/l), ACTH (0.1nmol/l) and K^+ (32mmol K^+/l)-stimulated aldosterone synthesis in cultured bovine zona glomerulosa cells.

Values shown are means ± S.E. (n=8). * indicates significant differences compared with controls ($p<0.05$) using Student's unpaired t-test.

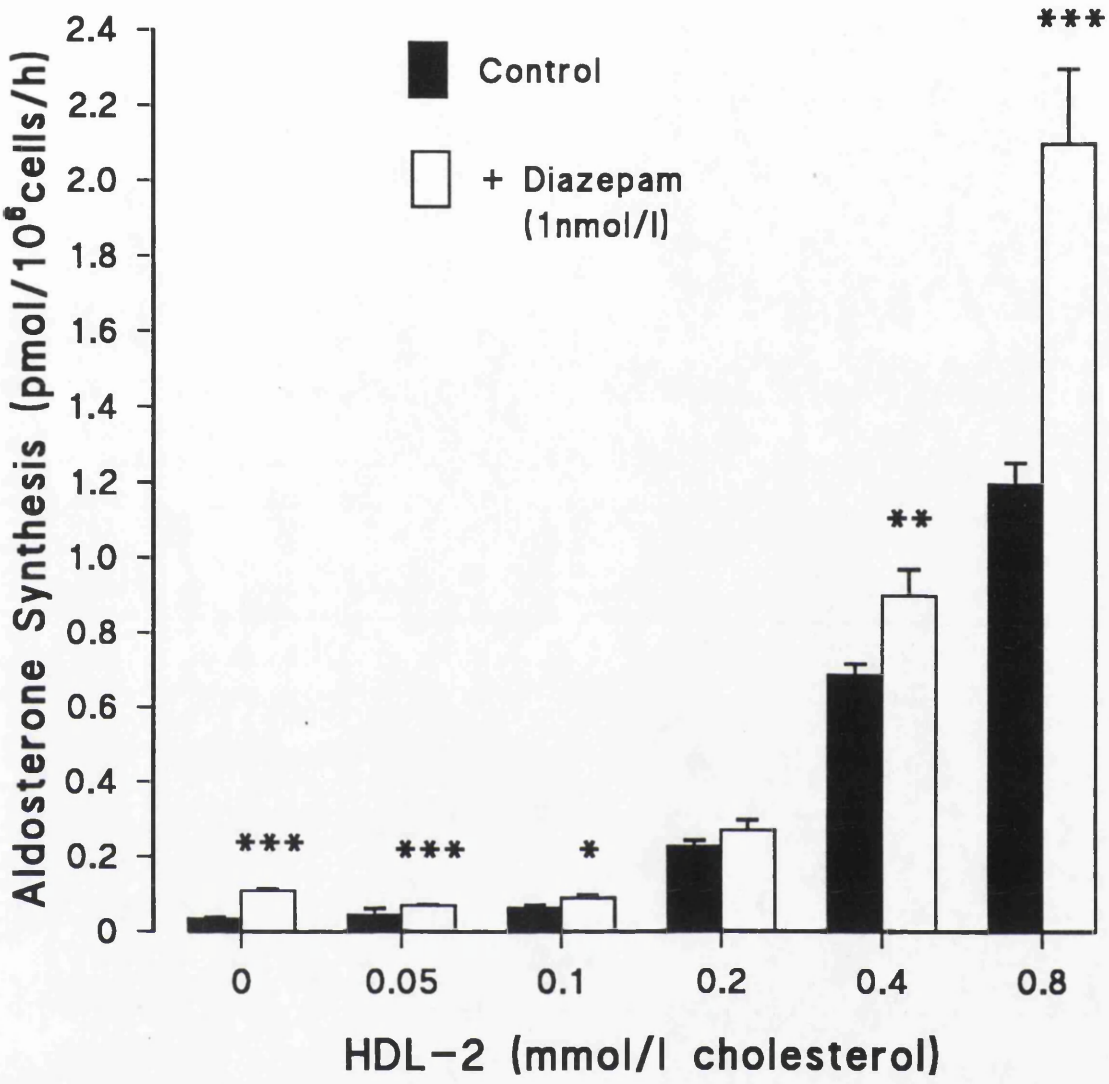


Fig.5.4

Effects of diazepam (1nmol/l) on aldosterone synthesis in cultured bovine zona glomerulosa cells incubated with HDL-2 at a range of concentrations.

Values shown are means ± S.E. (n=8). Statistical differences compared with controls are indicated by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$) using Student's unpaired t-test.

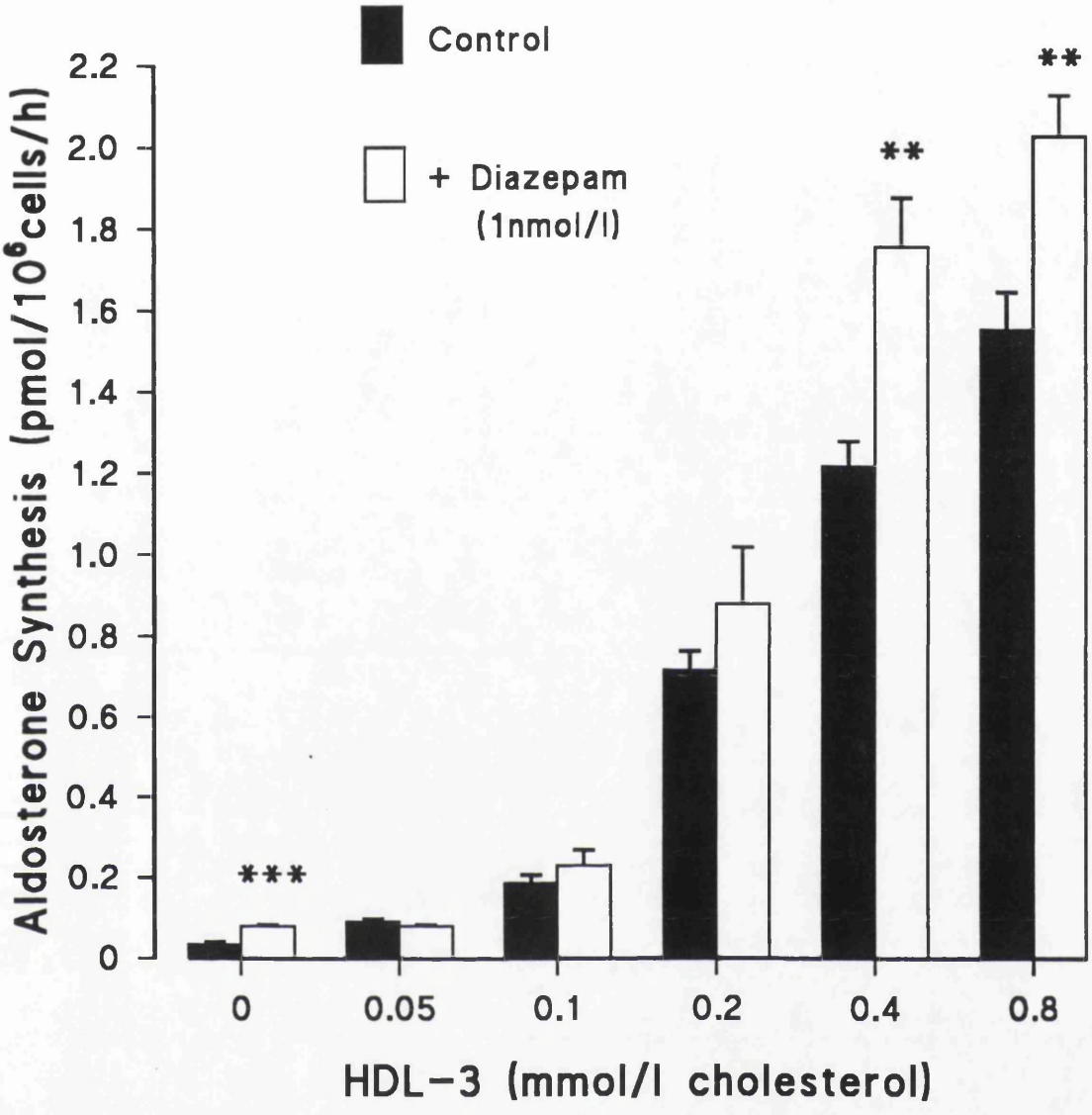


Fig.5. 5

Effects of diazepam (1nmol/l) on aldosterone synthesis in cultured bovine zona glomerulosa cells incubated with HDL-3 at a range of concentrations.

Values shown are means ± S.E. (n=8). Statistical differences compared with controls are indicated by ** ($p<0.01$) and *** ($p<0.001$) using Student's unpaired t-test.

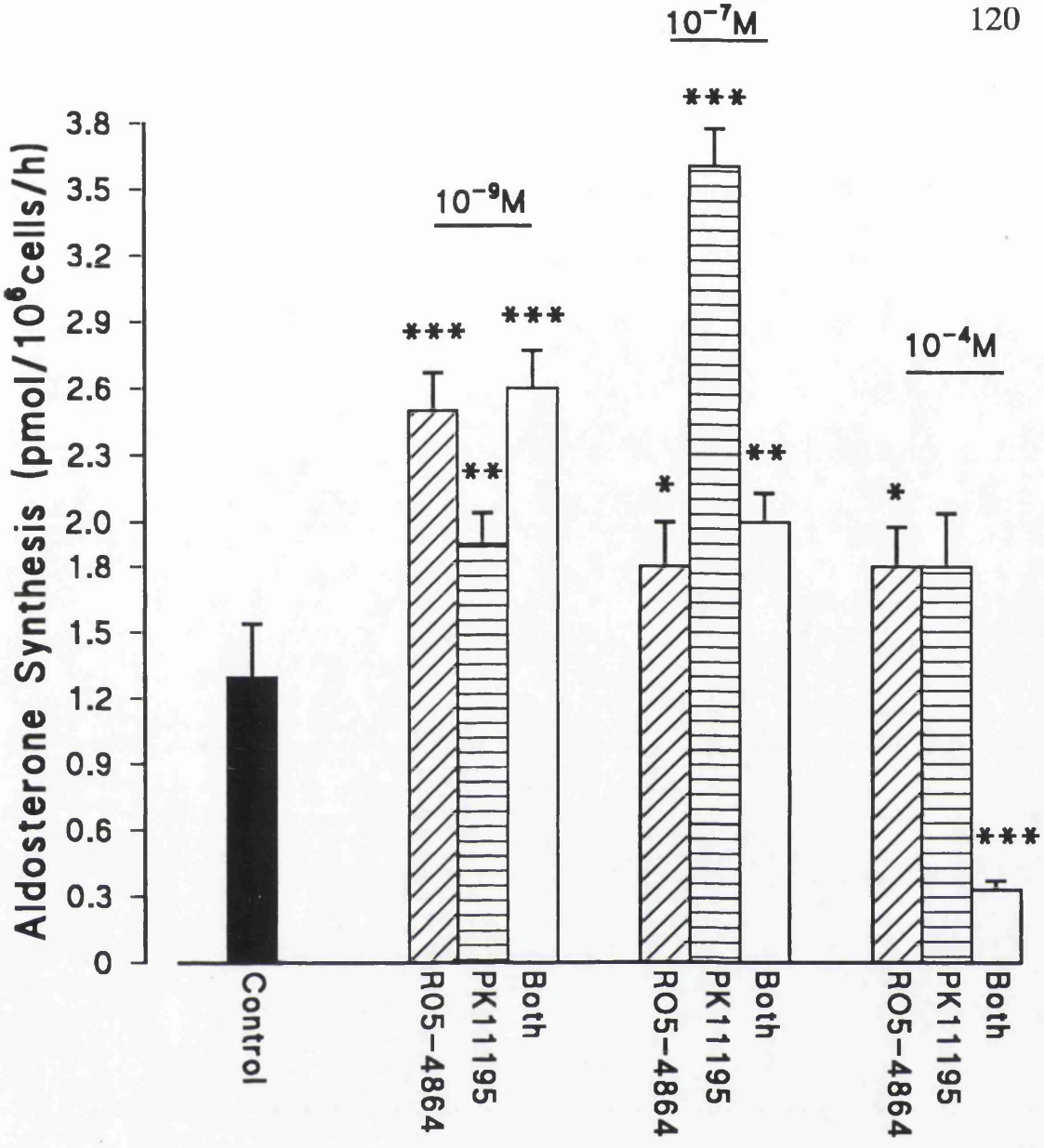


Fig.5.6

Concentration-dependent effects of RO5-4864 and PK11195, added individually or together, on aldosterone synthesis in cultured bovine zona glomerulosa cells incubated with HDL-2 (0.6mmol/l cholesterol).

Values shown are means ± S.E. (n=8). Statistical differences compared with controls are indicated by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$) using Student's unpaired t-test.

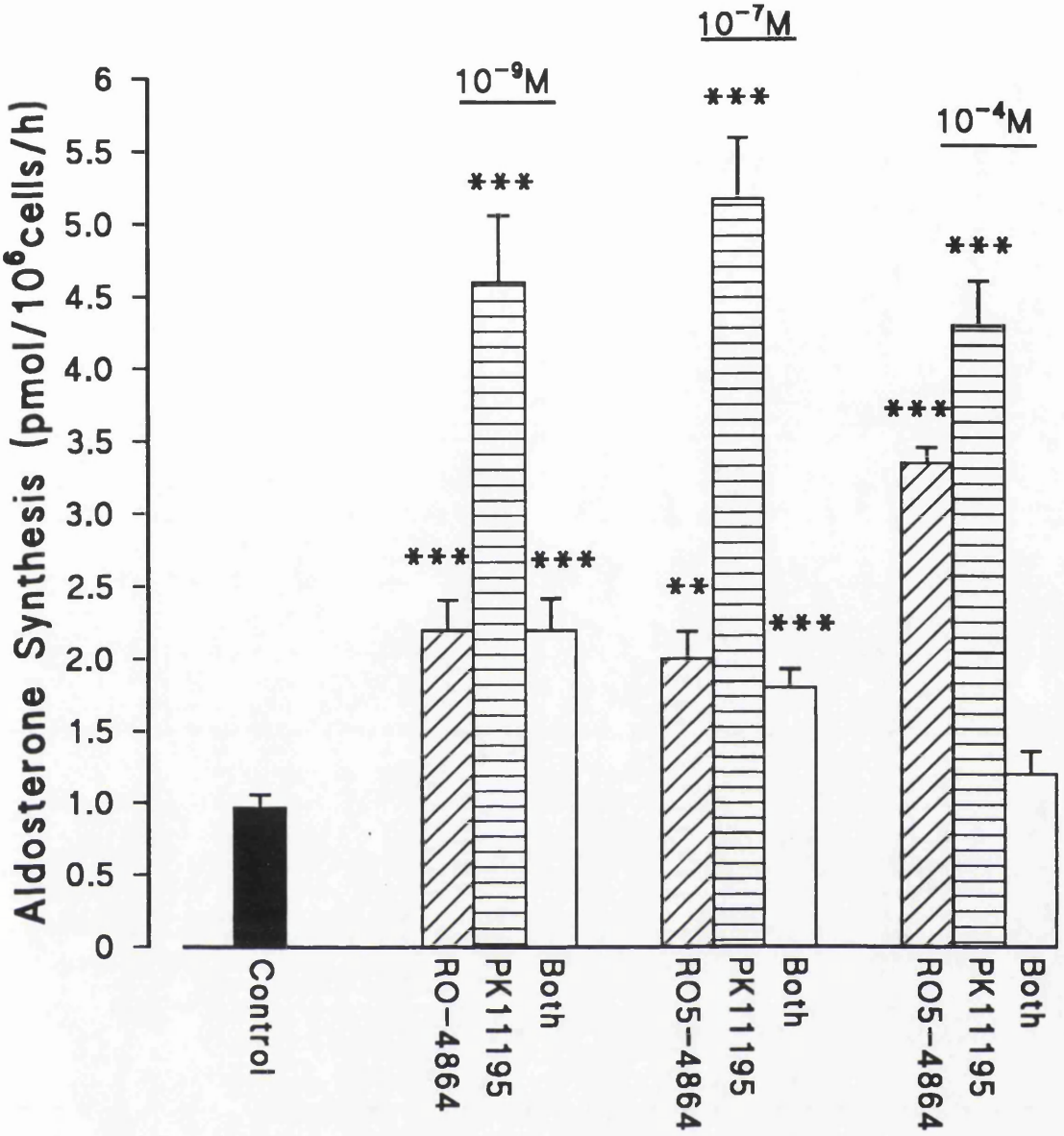


Fig.5.7

Concentration-dependent effects of RO5-4864 and PK11195, added individually or together, on aldosterone synthesis in cultured bovine zona glomerulosa cells incubated with HDL-3 (0.6mmol/l cholesterol).

Values shown are means ± S.E. (n=8). Statistical differences compared with controls are indicated by ** ($p<0.01$) and *** ($p<0.001$) using Student's unpaired t-test.

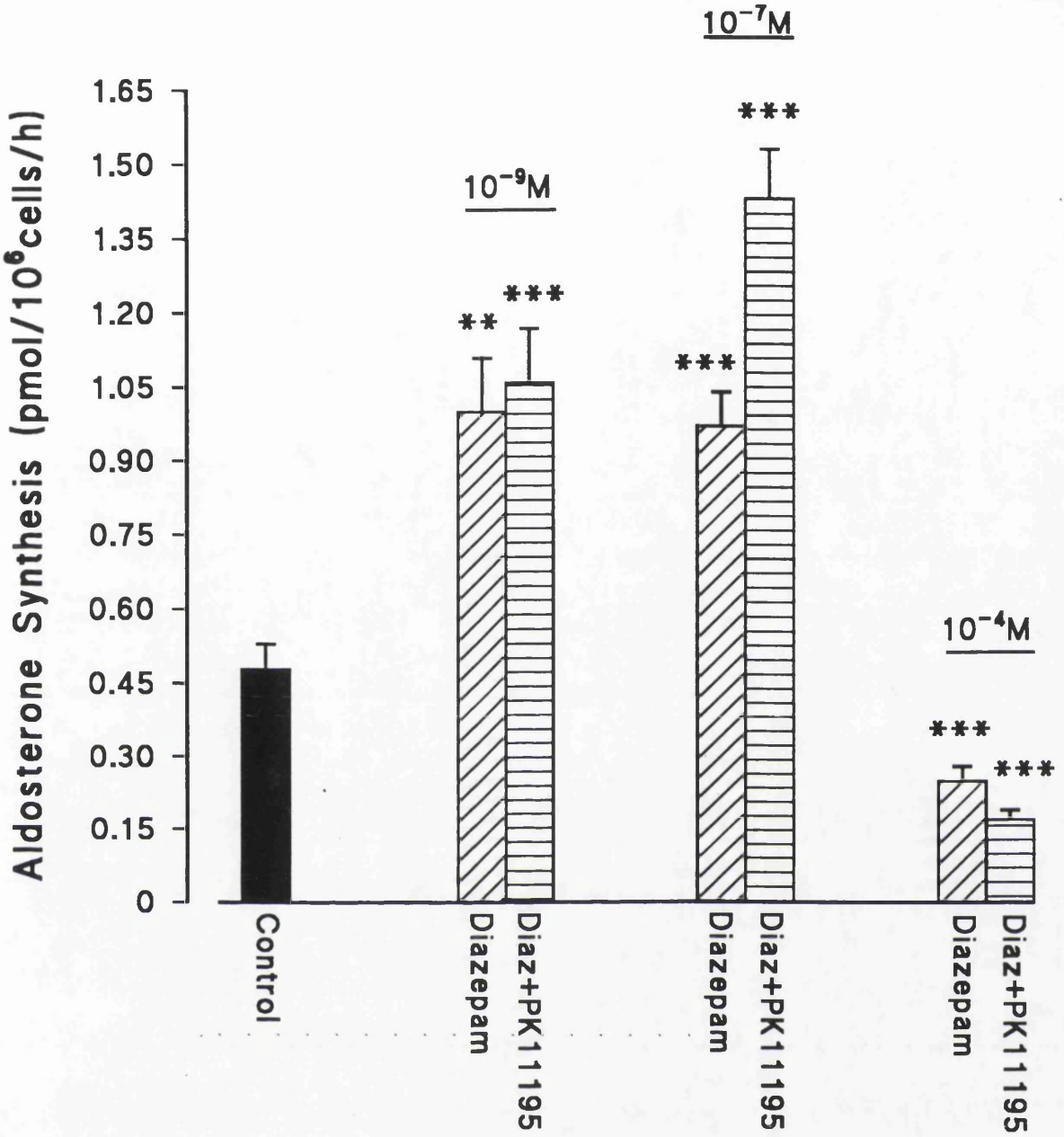


Fig.5.8

Concentration-dependent effects of diazepam added alone and diazepam added together with PK11195 on aldosterone synthesis in cultured bovine zona glomerulosa cells incubated with HDL-2 (0.6mmol/l cholesterol).

Values shown are means ± S.E. (n=8). Statistical differences compared with controls are indicated by ** ($p<0.01$) and *** ($p<0.001$) using Student's unpaired t-test.

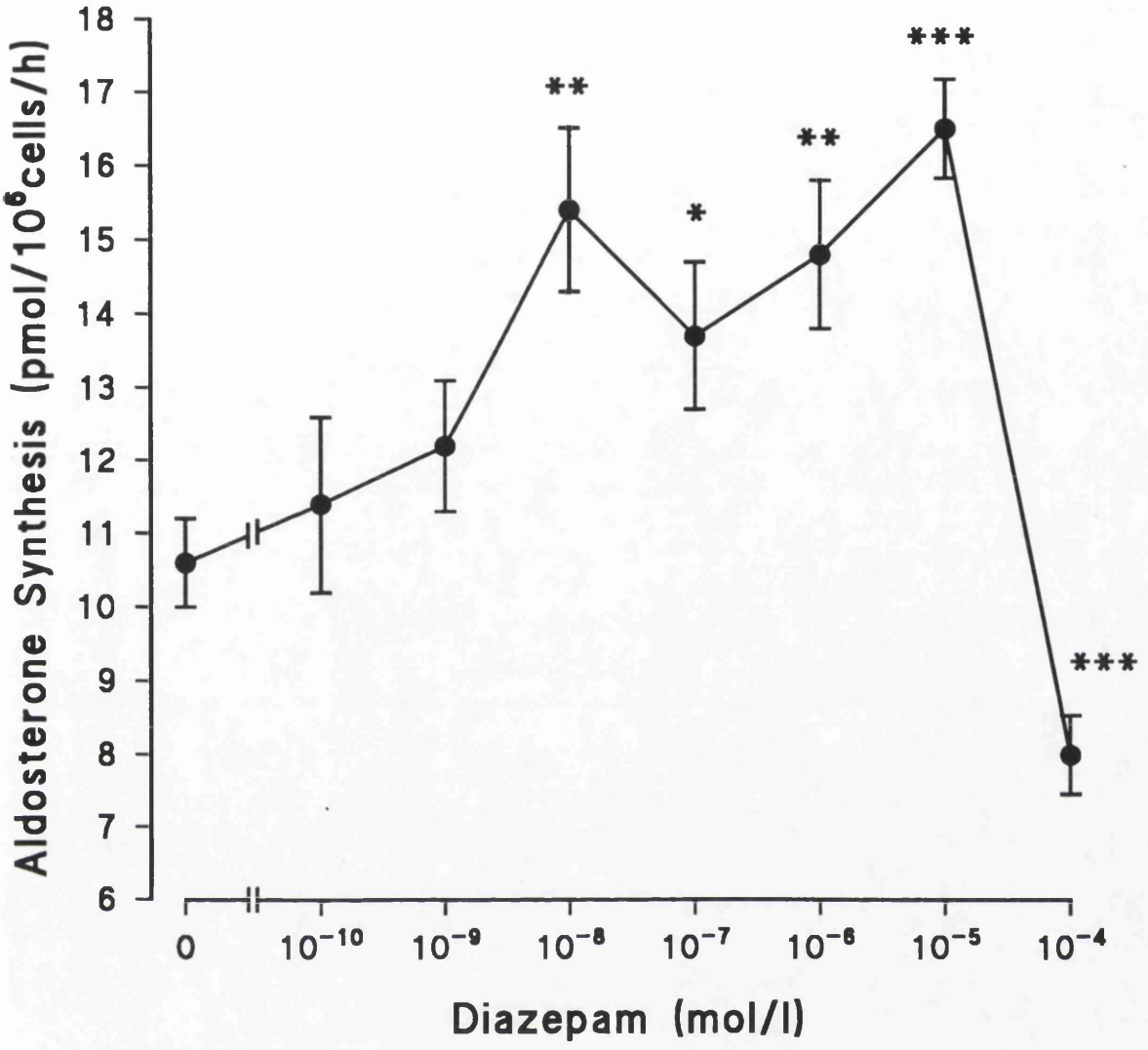


Fig.5.9

Concentration-dependent effects of diazepam on aldosterone synthesis in cultured bovine zona glomerulosa cells incubated in the presence of added DOC (5μmol/l). Values shown are means ± S.E. (n=8). Statistical differences compared with controls are indicated by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$) using Student's unpaired t-test.

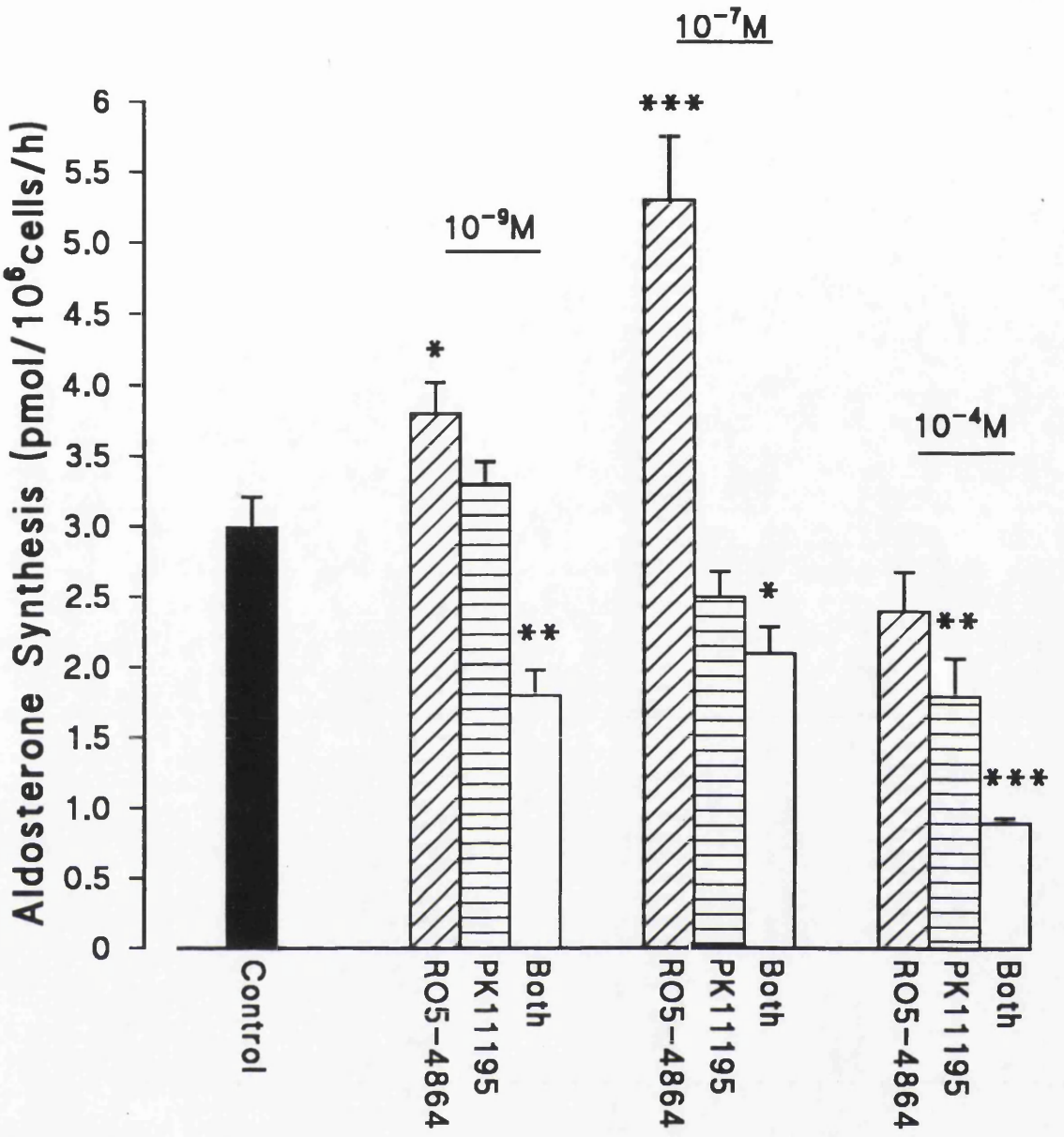


Fig.5.10

Concentration-dependent effects of RO5-4864 and PK11195, added together or individually, on aldosterone synthesis in cultured bovine zona glomerulosa cells incubated in the presence of added DOC (1μmol/l).

Values shown are means ± S.E. (n=10). Statistical differences compared with controls are indicated by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$) using Student's unpaired t-test.

three principal physiological stimuli of aldosterone secretion; A-II, ACTH and K^+. Diazepam, but not midazolam, significantly enhanced both A-II and ACTH-stimulated aldosterone synthesis by factors of 1.6 and 1.7 respectively ($p<0.01$). Both drugs significantly decreased K^+-stimulated synthesis by a factor of 1.4 ($p<0.01$).

Effects of benzodiazepines on conversion of HDL-cholesterol to aldosterone in cultured adrenocortical cells

The effects of diazepam (10^{-9}mol/l) on the aldosterone dose-response to HDL-2 are shown in Fig.5.4. The drug had significant stimulatory effects at all concentrations of lipoprotein used ($p<0.05$, $p<0.01$, $p<0.001$) with one exception (0.2mmol/l cholesterol). The most marked stimulatory effect of diazepam was at the highest concentration of HDL-2 used (0.8mmol/l cholesterol), representing a 1.75 fold increase when compared to the appropriate control ($p<0.001$).

At low concentrations of HDL-3 cholesterol (0.05-0.2mmol/l) diazepam (10^{-9}mol/l) had little or no effect on aldosterone synthesis (Fig.5.5) and a significant enhancement ($p<0.01$) was only achieved at the two highest concentrations of HDL-3 used (0.4 and 0.8mmol/l). This should be noted and contrasted with the very marked stimulatory effect of diazepam on basal synthesis ($p<0.001$).

The dose-dependent effects of RO5-4864 and PK11195 on aldosterone synthesis from added HDL-2 cholesterol (0.6mmol/l) are shown in Fig. 5.6. In the presence of HDL-2, RO5-4864 significantly enhanced aldosterone synthesis at all concentrations used ($p<0.05$; $p<0.001$). This effect was maximal at 1nmol/l RO5-4864. PK11195 also enhanced synthesis by 1.5 and 2.7 fold at concentrations of 10^{-9} and 10^{-7} mol/l respectively ($p<0.001$; $p<0.01$). When both RO5-4864 and PK11195 were added together at 10^{-9} mol/l, the effect on aldosterone synthesis was

similar to that observed when each drug was added individually. With both drugs present at 10^{-7}mol/l, aldosterone synthesis resembled the individual actions of RO5-4864 and was very much less than that elicited by PK11195 alone. At 10^{-4}mol/l the combination of drugs was markedly inhibitory ($p<0.001$).

The aldosterone response to PK11195 and RO5-4864 from added HDL-3 cholesterol was broadly similar to that in cells treated with HDL-2, the major difference being the greater steroidogenic output in the presence of HDL-3 (Fig.5.7). By itself, PK11195 was a more potent stimulus to aldosterone synthesis than RO5-4864. Together at 10^{-9} and 10^{-7}mol/l, steroid output resembled that of RO5-4864 alone. Again with both drugs present at 10^{-4}mol/l, aldosterone synthesis was inhibited.

PK11195 did not block the effects of diazepam (Fig.5.8). Indeed, at 10^{-7}mol/l the stimulatory effects of PK11195 and diazepam appear to be additive and at 10^{-4}mol/l the inhibitory effects of diazepam are more marked in the presence of PK11195.

Effects of benzodiazepines on synthesis of aldosterone from added DOC in cultured adrenocortical cells

The dose-dependent effects of diazepam on the conversion of DOC (5μmol/l) to aldosterone are shown in fig.5.9. At concentrations ranging from 10^{-8} to 10^{-5}mol/l, diazepam-treated cells produced 30-55% more aldosterone than vehicle-treated controls ($p<0.01$; $p<0.05$; $p<0.001$). At a concentration of 10^{-4}mol/l, DOC conversion was inhibited.

At 10^{-9} and 10^{-7}mol/l, RO5-4864, but not PK11195, also enhanced DOC (1μmol/l) conversion to aldosterone ($p<0.05$; $p<0.001$)(Fig5.10). At 10^{-4}mol/l, neither drug had any effect on aldosterone output. Curiously however, when PK11195 and RO5-4864

were added together, aldosterone synthesis was inhibited at each concentration tested ($p<0.01$; $p<0.05$; $p<0.001$), particularly at 10^{-4}mol/l.

5.4 Discussion.

Benzodiazepines exert their clinical anxiolytic and anti-convulsant effects by modulating the actions of γ-aminobutyric acid (GABA). GABA is a major neurotransmitter in the central nervous system (CNS) which inhibits neuronal firing by regulating chloride ion channel activity (115,117). A different type of benzodiazepine binding site has been identified in tissues outside the CNS (119). The concentration of these peripheral sites is especially high in the adrenal gland and, in particular, the adrenal cortex (118) where high affinity sites have been localised to the outer mitochondrial membrane (119). The presence of mitochondrial benzodiazepine receptors (MBR's) in this tissue does not appear to be associated with the activity of any constitutive mitochondrial enzymes (120). Hence, it was suggested that these receptors may fulfil a more specialised role relating to steroidogenesis. Studies in rats demonstrated a radical decrease in the concentration of adrenal MBR's after hypophysectomy (169). The concentration of kidney MBR's in the same rats showed little or no change. These data indicated that in the adrenal, MBR expression was under the control of pituitary gland hormones.

Benzodiazepine ligands can stimulate corticosteroid synthesis in bovine and rat ZF/R cells (122). This stimulation is not cycloheximide-sensitive and is not mediated by direct activation of P-450_{scc}. Further studies, carried out in Y-1 and MA-10 tumour cell lines using a number of benzodiazpine ligands with differing affinities for the MBR, demonstrated that the potency of these drugs as stimulators of steroidogenesis correlates very closely with their affinities for the MBR and not at

all with their binding to central-type receptors.

My own results demonstrate that diazepam, which binds to both the MBR and the central receptor (184), can stimulate basal aldosterone synthesis in bovine ZG cells (Fig.5.1) over a wide concentration range (10^{-9}-10^{-5}mol/l). This stimulation of steroid synthesis at nanomolar concentrations corresponds with the binding affinity of the MBR for the drug. This is not just an acute effect, as pretreatment with the drug is also sufficient to induce an increased steroid response. Indeed steroid output in cells pretreated with diazepam was not significantly different from that in cells exposed to diazepam before and during the period when aldosterone production was measured.

Diazepam potentiated the stimulatory effects of A-II and ACTH (Figs.5.2 and 5.3). For A-II, this enhancement was more effective at lower, sub-maximal concentrations of peptide (Fig.5.2). This might indicate that the stimulatory effects of A-II and diazepam on aldosterone synthesis are limited by a common reaction, possibly P-450_{scc} activity. Below this limit benzodiazepine and A-II effects are additive. Similar additive effects were observed with ACTH-stimulated aldosterone synthesis (Fig.5.3). Midazolam, of which there are no reports as regards affinity for the MBR, has no effect on A-II or ACTH-stimulated synthesis at a concentration of 0.1μmol/l (Fig.5.3).

The stimulatory effects of diazepam on cultured cells are in marked contrast to the inhibition described in chapter four. There are several possible reasons for this discrepancy:

(i) The steroid response of freshly isolated glomerulosa cells to diazepam may be different to that of cultured cells. This is unlikely as diazepam inhibited K^+-stimulated aldosterone synthesis in both freshly isolated and cultured cells (Figs.4.4

and 5.3).

(ii) The stimulatory effects of diazepam on aldosterone synthesis may be time-dependent. The similarity between the acute (3h) and the 24h response to the drug demonstrates that this is not the case.

(iii) The effects of diazepam may be concentration-dependent. Drug concentration appears to be a critical factor in determining the effects of diazepam on steroidogenesis. Diazepam increased basal and stimulated aldosterone synthesis (Figs.5.1, 5.2 and 5.3) at concentrations a magnitude lower (nM) than that which inhibited synthesis (μM) (Fig.5.2 and (138)). This was not observed with K^+-stimulated cells where diazepam also inhibited aldosterone synthesis at sub-micromolar concentrations (Fig.4.4 and (162)). These data may be explained by assuming that diazepam, depending upon its concentration, can affect glomerulosa cells in three ways: (a) By inhibiting steroidogenic enzymes (see chapter 4). (b) By inhibiting K^+-stimulated $[Ca^{2+}]_o$ uptake (see chapter 4) (c) By increasing intra-mitochondrial cholesterol transport at the MBR.

Further evidence that the MBR participates in hormone-stimulated synthesis is provided by studies using the benzodiazepine, flunitrazepam (182). This weak MBR agonist antagonises the steroidogenic effects of ACTH and lutenizing hormone (LH) in Y-1 and MA-10 cell-lines respectively, suggesting that hormone-stimulated synthesis occurs by a mechanism coupled to the MBR.

Although ultimately P-450_{scc} activity is rate-limiting in unstimulated cells, it is the rate at which cholesterol is transported to this enzyme in the inner mitochondrial membrane which governs availability and hence the rate of steroid synthesis (95,107). It is suggested that MBR ligands exert their action by controlling cholesterol transport within the mitochondrion (181). Two mechanisms have been

proposed for intra-mitochondrial cholesterol transport (181), one involving a carrier protein in the inter-membrane space and the other a direct transfer of cholesterol between membrane compartments at intermembrane contact points.

The involvement of a nascent, cycloheximide-sensitive protein in steroidogenic regulation is well-documented (see chapter 1). However, a different 18KDa protein, which exhibits the binding properties of the MBR, has been identified, purified and sequenced from rat adrenal mitochondria (185). cDNA encoding this protein, when expressed in a transformed human kidney cell line, resulted in increased binding of MBR ligands (186). This protein has five potential transmembrane spanning segments which could allow the receptor to be embedded in both inner and outer mitochondrial membranes at contact points which would facilitate cholesterol transport. Whatever the mechanism of mitochondrial cholesterol transport, benzodiazepines have been demonstrated to stimulate pregnenolone synthesis in cholesterol-loaded adrenocortical mitochondria (114,123) and this effect has been attributed to an effect on cholesterol transport via the MBR, rather than any direct alterations in P-450_{scc} activity.

These effects on cholesterol transport prompted the present study of the effects of MBR ligands in cultured cells treated with HDL-cholesterol. In addition to diazepam, the effects of 4-chlorodiazepam (RO5-4864) and an isoquinoline carboxamide, PK11195, were also examined. Both have higher affinity for the MBR than diazepam (122).

Diazepam, RO5-4864 and, in particular, PK11195 all increased the conversion of HDL-cholesterol to aldosterone (Figs.5.6, 5.7 and 5.8) with potencies similar to their relative abilities to increase basal 20α-progesterone synthesis in Y-1 adrenocortical cells [ie-PK11195 > RO5-4864 > diazepam] (122). When the drugs were present in

combination their stimulatory effects were not additive, suggesting that all three drugs bind to the same receptor. Research done on kidney indicates that the MBR has at least two different binding conformations or has separate, mutually exclusive binding sites for benzodiazepines and isoquinoline carboxamides (187).

MBR ligands are suggested to exert their effects by mimicing the actions of endogenous ligands for this receptor (123). Proteins which inhibit diazepam binding to central receptors have been discocvered in a variety of tissues and have been termed endozepines or diazepam-binding inhibitors (DBI) (see chapter 1). Endozepines stimulated pregnenolone formation in cholesterol-loaded mitochondria from mouse Y-1 and MA-10 cells (114). DBI and DBI-like peptides are found in high concentrations in the adrenal cortex and one such protein, Des-[Gly-Ile]-DBI, has been isolated from bovine ZF/R cells (123). Endozepines have been shown to stimulate pregnenolone formation by adrenocortical mitochondria in a manner similar to diazepam (123), suggesting that both ligands may operate through the same receptor, the MBR.

Aldosterone biosynthesis involves the transport of two precursors, cholesterol and DOC, to steroidogenic enzymes located in the inner mitochondrial membrane. Although little is known about mitochondrial cholesterol transport, even less is known about the mechanisms of mitochondrial DOC supply. Diazepam significantly increased conversion of DOC to aldosterone ($p<0.01$) over a concentration range (10^{-8}-10^{-5}mol/l) which was appropriate for a reaction involving the MBR (Fig.5.9). RO5-4864 had similar effects, increasing aldosterone synthesis 1.8 fold at a concentration of 1.8μmol/l (Fig.5.10). This raises the possibility that the stimulatory effects of benzodiazepines on aldosterone synthesis, from added HDL-cholesterol and from added DOC, may both be mediated by a similar mechanism involving the

MBR. If MBR ligands can increase steroid synthesis by activating mitochondrial cholesterol translocation (181), then it is tempting to speculate that these drugs may act in a similar manner to facilitate mitochondrial DOC transport. There is no direct evidence to support this conjecture but a multi-functional role for the MBR has been suggested (121). However, not all experimental evidence supports this hypothesis. PK11195, the ligand which has highest affinity for MBR (122) and which has the most marked effect on aldosterone synthesised from HDL-cholesterol (Fig.5.7), had no effect on aldosterone synthesised from added DOC (Fig.5.10). This suggests that the positive effects of RO5-4864 and diazepam on the conversion of DOC to aldosterone cannot be mediated through the MBR and that both drugs increase conversion of DOC to aldosterone by some other mechanism.

Recent immunofluorescence studies of adrenocortical cells have indicated that benzodiazepine receptors are not associated exclusively with the mitochondrion. Peripheral binding sites have been identified in plasma membranes of both glomerulosa and fasiculata/reticularis cells (188). Similarly, peripheral type plasma membrane receptors have been identified in heart and in synaptosomes (176,177). These have been linked to calcium channel function. In guinea-pig myocytes , both RO5-4864 and diazepam inhibited the depolarisation induced uptake of Ca^{2+} (128,189). As PK11195 blocked the effects of these benzodiazepines, the idea that PK11195 is an antagonist of the peripheral benzodiazepine receptor has become established. In adrenocortical cells, this study and others have noted that $[Ca^{2+}]_o$ uptake is inhibited by midazolam, diazepam and other benzodiazepines (190). Assuming that PK11195 is an antagonist of diazepam-inhibited $[Ca^{2+}]_o$ uptake, this plasma membrane effect may explain the differences between the steroidogenic effects of RO5-4864 and PK11195.

Although it is generally accepted that elevated $[Ca]_i$ increases the activities of both the early and late pathways of aldosterone synthesis (145), there is also evidence to suggest that Ca^{2+} is not an absolute requirement for late pathway activity. For example, purified 11ß-hydroxylase can catalyse formation of aldosterone from DOC in the absence of added Ca^{2+} (19). Recently, it has been reported that a Ca^{2+} concentration of 2.5μmol/l inhibited the conversion of DOC to aldosterone by isolated adrenocortical mitochondria (191). Using adrenocortical mitochondria prepared as per chapter 2, I too have observed similar effects at much higher Ca^{2+} concentrations. Addition of Ca^{2+} in the presence of 1μmol/l corticosterone reduced aldosterone production from 20.7 ± 0.38 to 14.2 ± 0.98 ng/mg protein ($p<0.01$). Cortisol synthesis from added deoxycortisol (1μmol/l) was similarly reduced from 4.7 ± 0.22 to 3.8 ± 0.2 μg/mg protein ($p<0.01$). Given the high concentrations of Ca^{2+} used in these incubations (350μmol/l), it is perhaps unrealistic to assume that changes in $[Ca^{2+}]_i$ in intact cells would influence mitochondrial DOC uptake or conversion of corticosterone to aldosterone in the same way. Nevertheless the hypothesis, that $[Ca^{2+}]_i$ inhibits DOC to aldosterone conversion whilst also inhibiting signal transduction processes which stimulate steroidogenesis, would explain some of the present experiments with RO5-4864, diazepam and PK11195. Diazepam and RO5-4864, in intact cells, could stimulate aldosterone synthesis either through the MBR or, by lowering $[Ca^{2+}]_i$, promoting conversion of DOC to aldosterone. PK11195 would also stimulate steroidogenesis through the MBR but would block RO5-4864 and diazepam effects on DOC conversion. Since PK11195 is only antagonistic at the plasma membrane in the presence of an agonist such as diazepam, it would not inhibit DOC conversion in the absence of diazepam. A third factor to be borne in mind is that at high concentrations, RO5-4864 and PK11195 may competitively

inhibit steroidogenesis by blocking one or more enzyme steps. This might apply particularly at concentrations in excess of 0.1mM, the range at which 17α-hydroxylase and 21 hydroxylase inhibition by midazolam and diazepam becomes apparent (see chapter 4).

To clarify which of these three possible processes are involved, it is important in future studies to establish clear dose-response relationships, for each of the biosynthetic steps involved, to investigate at the same time the effects of these drugs on cellular calcium metabolism and lastly to clarify the different pharmacological properties of the benzodiazepine receptor ligands tested. It is significant that midazolam lowers $[Ca^{2+}]_i$, but may not bind to the MBR yet does bind to central receptors. PK11195 stimulates steroidogenesis through the MBR but is an antagonist of the plasma membrane receptor. Diazepam and RO5-4864 are agonists of the MBR and of the peripheral plasma membrane receptor.

In summary, low concentrations of diazepam can increase basal and hormone-stimulated aldosterone synthesis in cultured ZG cells. The drug presumably mimics the effects of DBI and DBI-like peptides (the proposed endogenous ligands for the MBR) and stimulates intra-mitochondrial cholesterol transport. Two high-affinity MBR ligands, RO5-4864 and PK11195, also increased aldosterone synthesis in cholesterol-loaded glomerulosa cells. In addition, diazepam and RO5-4864 but not PK11195 increased aldosterone synthesised in glomerulosa cells from exogenous DOC. Since PK11195 was ineffective, it seems unlikely that the positive effects of diazepam and RO5-4864 on DOC conversion to aldosterone were MBR-mediated. The identification of benzodiazepine receptors in adrenocortical cell membranes and the observed effects of benzodiazepines on Ca^{2+} influx, indicate that the actions of RO5-4664, diazepam and PK11195 on conversion of DOC to aldosterone may be

associated with Ca^{2+}-channel function (Fig.5.11).

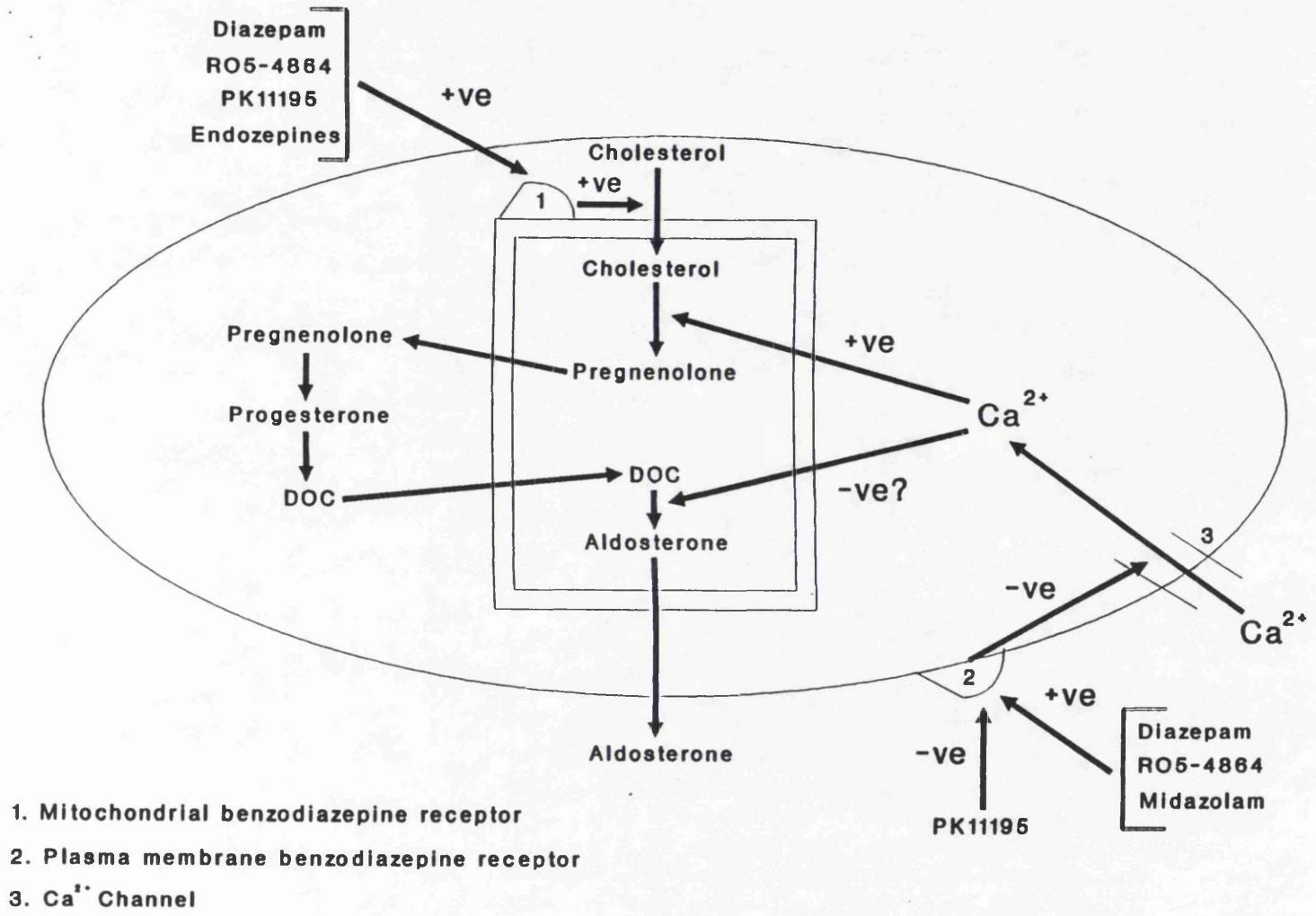


Fig.5.11

Possible mechanisms of action of benzodiazepines and PK11195 on mitochondrial steroid biosynthesis.

CHAPTER 6.

GENERAL DISCUSSION.

The present study has considered three inter-related elements of steroidogenic control:

(i) The supply of cholesterol.

(ii) The role of Ca^{2+}.

(iii) The activity of biosynthetic enzymes.

These control elements have been studied using several agents which reportedly act specifically at particular control sites. I have demonstrated that it is possible for some of these agents to affect steroidogenesis by acting at more than one of these steroidogenic control elements (see fig 6.1). Investigators should be aware of these mixed functions and caution should be exercised when interpreting results of any study with the following agents where steroid output is used as an analytical end-point:

(a) **Dantrolene**: Dantrolene inhibits Ca^{2+} release from the sarcoplasmic reticulum (129) and has been used to inhibit release of intracellular calcium in bovine zona glomerulosa cells (141,142). This property has obvious uses when studying the role of intracelluar calcium metabolism in signal-response coupling in A-II-stimulated aldosterone synthesis.

I have demonstrated that at concentrations which are required to inhibit A-II-mediated release of intracellular calcium, dantrolene also inhibits steroidogenic enzymes. The rate-limiting cholesterol side-chain cleavage reaction is particularly dantrolene-sensitive. This mitochondrial effect is calcium-independent and probably involves direct competitive inhibition between drug and substrate

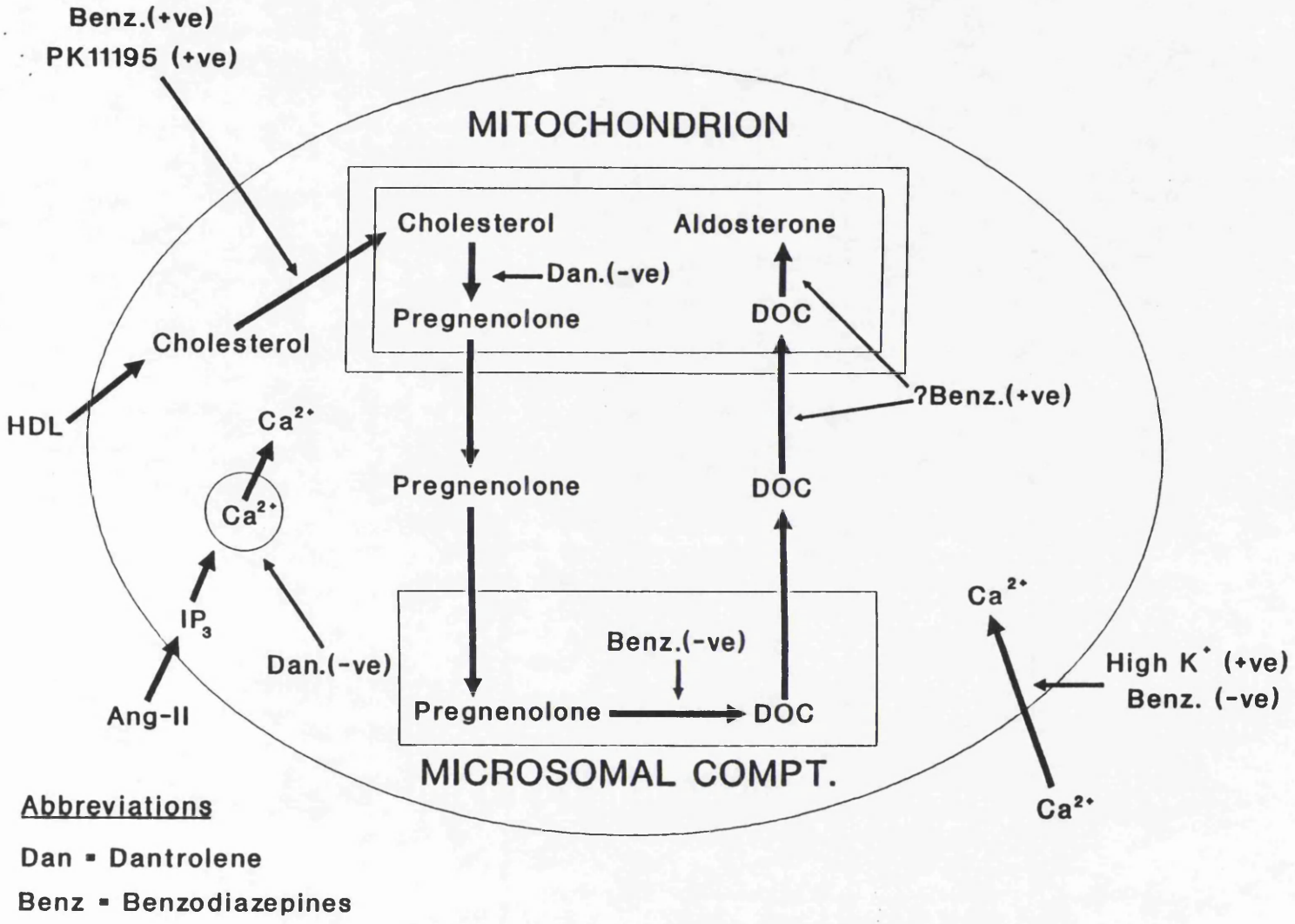


Fig.6.1

A summary of the effects of benzodiazepines, PK11195, HDL and dantrolene on the biosynthesis of aldosterone by the adrenal glomerulosa cell.

(b) **High Density Lipoproteins**

The main physiological role of HDL is the "reverse transport" of cholesterol from peripheral tissues to the liver for catabolism and excretion from the body (100). However, HDL has been shown, in vivo and in vitro, to deliver cholesterol to steroidogenic cells (100,103). I have demonstrated that HDL subfractions, HDL-2 and HDL-3, are readily usable sources of substrate for isolated and cultured adrenocortical cells. Both subfractions increase basal and A-II-stimulated steroid synthesis. However, there is a difference in the responsiveness of freshly isolated zona glomerulosa and zona fasiculata/reticularis cells. The aldosterone response to HDL is lost at high concentrations of A-II while the cortisol response is not. One explanation might lie in the relative dependency of aldosterone and cortisol synthesis on Ca^{2+}. Part of the cardioprotective effect of HDL may be due to its inhibitory effects on agonist-induced increases in platelet ionised calcium (Simpson and Graham, unpublished data; 159,160). My studies, limited as they were, demonstrated no significant acute effect of HDL-2 or HDL-3 on either ^{45}Ca uptake or intracellular free calcium in zona glomerulosa cells. Under steady state conditions, calcium content was lowered by HDL. The added responsiveness of isolated zona fasiculata/reticularis cells to HDL probably reflects a greater ability, when compared to zona glomerulosa cells, to utilise cholesterol derived from lipoproteins (104).

(c) **Benzodiazepines**

There are at least two types of benzodiazepine receptor (113,116): central-type receptors and peripheral-type receptors.

Central receptors are found throughout the CNS and are associated with the GABA-gated chloride ion channel. The therapeutic effects of benzodiazepines as

tranquillisers and sedatives are mediated via the central receptor

Peripheral receptors have been identified in many tissues outside the CNS but are present at highest density in the adrenal cortex where they have been localised to the outer mitochondrial membrane (118,119). They are often refered to as mitochondrial benzodiazepine receptors (MBR). MBR may play a specialised role in steroidogenic control (120). MBR ligands are thought to increase steroidogenesis by mimicing the effects of endogenous proteins, called diazepam binding inhibitors or endozepines, which have been isolated from steroidogenic tissues (122,123).

I have demonstrated that benzodiazepines, can both stimulate and inhibit steroidogenesis. These effects depend on concentration and vary between drugs. For example, at high concentrations (>μmol/l), diazepam and midazolam inhibit microsomal hydroxylation. In addition, each of these drugs has a prefered site of action; diazepam preferentially inhibits 17α-hydroxylase whereas midazolam predominantly affects 21-hydroxylase activity. Both of these drugs can also inhibit K^+-stimulated aldosterone synthesis at lower concentrations (10-20 fold lower) than that required to elicit their direct effects on microsomal hydroxylation. Furthermore, I demonstrated that benzodiazepines can inhibit depolarisation-induced uptake of $[Ca^{2+}]_o$ in zona glomerulosa cells. Similar effects have been reported in other tissues (128,177,178). Midazolam also inhibited basal calcium uptake more markedly in zona glomerulosa than in zona fasiculata/reticularis cells. Stimulation of aldosterone synthesis by K^+ is wholly dependent upon the influx of $[Ca^{2+}]_o$ (84). As benzodiazepines can block this mechanism, it provides an explanation for the added sensitivity of K^+-stimulated aldosterone synthesis to these drugs.

At low concentrations (>nmol/l) diazepam increased basal and hormone-stimulated (A-II and ACTH) aldosterone synthesis in cultured zona glomerulosa cells. The

effect on basal synthesis was not just an acute effect and could be induced by pre-exposure to the drug. Diazepam's action on hormone-stimulated synthesis was most marked at sub-maximal concentrations of A-II and ACTH, suggesting the involvement of a hormone-induced factor which acts at the same site as diazepam to affect steroid synthesis. Diazepam has known affinity for the MBR and benzodiazepines are proposed to stimulate steroidogenesis by mimicing the effects of endogenous ligands for this receptor. The proteins which compete for diazapam binding sites have been named diazepam binding inhibitors inhibitors (DBI's) or alternatively endozepines (114,123) and have been identified in the CNS and, at high concentrations, in the adrenal cortex (124). These proteins are proposed to increase the rate of intra-mitochondrial cholesterol translocation through action at the MBR (114). In support of this theory, I have demonstrated that diazepam and RO5-4864 (4-chlorodiazepam) increase aldosterone synthesis in cultured zona glomerulosa cells which have been cholesterol-loaded by incubation with HDL subfractions. Both diazepam and RO5-4864 can also increase DOC-stimulated late pathway activity. If the MBR influences one type of mitochondrial transport mechanism, cholesterol translocation, then it is interesting to speculate that it may also facilitate transport of another steroid precursor which requires acccess to the inner mitochondrial membrane; DOC. The adrenal MBR has also been reported as having a direct role in the transport of porphyrins, which are necessary for the function of steroid hydroxylase enzymes (121). Thus it is possible that the MBR may form part of a multi-faceted regulatory mechanism that controls not only transport of steroid intermediates but may also affect uptake of other components essential for steroidogenesis.

PK11195 is not a benzodiazepine but is an isoquinoline carboxamide. This

compound can also increase aldosterone synthesis stimulated by both HDL-2 and HDL-3. PK11195 has high affinity for the MBR and so presumably exerts its effects on steroid synthesis by increasing intra-mitochondrial cholesterol transport in a manner similar to benzodiazepines. However, the drug has no effect on conversion of DOC to aldosterone. This makes it less likely that the stimulatory effects of diazepam and RO5-4864 on late pathway activity are mediated through the MBR. They probably involve interaction with cell membrane benzodiazepine receptors which are associated with Ca^{2+}-channel function.

In summary, my studies with benzodiazepines have demonstrated that they can influence aldosterone synthesis through three different mechanisms.

(i) ≥nmol/l: Aldosterone synthesis is stimulated, probably via the MBR, by increasing the rate of cholesterol transport from the outer to the inner mitochondrial membrane.

(ii) >nmol/l: Potassium-stimulated aldosterone synthesis is inhibited by a reduction in uptake of $[Ca^{2+}]_o$. Whether this is mediated via central or peripheral receptors remains to be ascertained. Interestingly, others have recently reported that peripheral benzodiazepines can inhibit both K^+ and A-II-stimulated aldosterone synthesis in isolated bovine glomerulosa cells by inhibiting influx of $[Ca^{2+}]_o$ into the cell (191).

(iii) >μmol/l: Aldosterone and cortisol synthesis are inhibited. The results indicate that midazolam and diazepam are competitive inhibitors of microsomal hydroxylases in steroidogenic tissue.

Thus the net effect of benzodiazepines on aldosterone synthesis will depend upon the relative effect of the drug on each of these mechanisms. This in turn depends upon the identity of the individual drug and on its concentration.

REFERENCES

(1) White, A., Handler, P. and Smith, E.L. In Principles of Biochemistry (Fourth Edition, 1968). Pub. M°Graw-Hill, London.

(2) Junquiera, L.C., Carniero, J. and Long, J.A. "Adrenals, islets of Langerhans, thyroid, parathyroid and pineal body". In Basic Histology (Fifth Edition, 1986). Pub. Lange, New York.

(3) Chattoraj, S.C. "Endocrine Function". In Fundamentals of Clinical Chemistry (Second Edition, 1976). Pub. W.C. Saunders.

(4) Simpson ER: Cholesterol side-chain cleavage, cytochrome-P450 and the control of steroidogenesis. Mol Cell Endocrinol (1979) 13 213-227.

(5) Hiwatashi A and Ichikawa Y: Physiochemical properties of reduced nicotinamide adenine nucleotide phosphate cytochrome P450 reductase from bovine adrenocortical microsomes. Biochim et Biophys Acta (1979) 580 44-63.

(6) Seybertz DW, Lancaster JR Jr., Lambeth JD and Kamin H: Participation of the membrane in side-chain cleavage of cholesterol. J. Biol. Chem. (1980) 254 12088-12098.

(7) Hanukoglu I, Spitzberg V, Bumpus JA, Dus KH and Jefcoate CR: Adrenal mitochondria cytochrome P450scc. J Biol Chem (1981) 256 4321-4328.

(8) Simpson, E.R. and Estabrook, R.W.: Mitochondral malic enzyme. The source of reduced nicotinamide adenine dinucleotide phosphate for steroid hydroxylation in bovine adrenal cortex mitochondria. Arch. Biochem. Biophys. (1969) 129 384-395.

(9) Aupetit B, Accarie C, Emeric N, Vonarx V and Legrand JC: The final steps of aldosterone biosynthesis require reducing power. It is not a dehydrogenation. Biochim Biophys Acta (1983) 834 324-330.

(10) Murray M and Reidy GF. Selectivity in the inhibition of mammalian cytochromes P450 by chemical agents. Pharmacological Reviews (1990) 42 85-101.

(11) Kominami S, Ogawa N, Morimune R, De-Ying H and Takamori S: The role of cytochrome B5 in adrenal microsomal steroidogenesis. J Steroid Biochem Mol Biol (1992) 42 (1) 57-64.

(12) Ulick S: Diagnosis and nomenclature of disorders of the terminal portion of the aldosterone biosynthesic pathway. J Clin Endocrinol Metab (1976) 43 92-96.

(13) Müller J, Shnid C, Bini-Schnetzler, M. and Lauber M: Two forms of cytochrome P450 in rat zona glomerulosa cells. Endocrine Res. (1991) 17 165-184.

(14) Kawando T, Mitsuuchi Y, Toda K, Yokoyama Y, Miyahara K, Miura S, Ohnishi T, Idikawa T, Nakao K, Imura H, Ulick S and Shizuta Y: Role of 18 hydroxylase and IIß hydroxylase in biosynthesis of mineralocorticoids and glucocorticoids. Proc Natl Acad Sci (USA), (1992) 89 1458-1462.

(15) Müller J: Aldosterone biosynthesis in bovine, rat and human adrenal: commonalities and challenges. Mol Cell Endocrinol (1991) 78 C119-124.

(16) Ogishima T, Suzuki H, Hata J, Mitami F and Ishimura Y: Zone specific expression of aldosterone synthase cytochrome P450 and cytochrome P45011ß in rat adrenal cortex. Histochemical basis for functional zonation. Endocrinology (1992) 130 2971-2977.

(17) Lauber M and Müller J: Purification and characterisation of two forms of rat adrenal cytochrome P450IIß: functional and structural aspects. Arch Biochem Biophys (1989) 274 109-119

(18) Watanuki, M., Tilley, B.E. and Hall, P.F. Purification and properties of cytochrome P-450 (11ß and 18 hydroxylase) from bovine adrenocortical mitochondria. Biochim. et Biophys. Acta (1977) 483 236-247.

(19) Hall PF, Yanagibashi K and Kobayashi Y: Synthesis of aldosterone by mitochondria and homogenous IIß hydroxylase from beef and pig. Endocr. Res (1991) 17 135-149.

(20) Hornsby PJ: Steroid xerobiotic effects on the adrenal cortex: mediation by oxidative and other mechanisms. Free Radical Biology and Medicine (1989) 6 103-115.

(21) Cozza EN, Foecking MF and Gomez-Sanchez CE: The binding of cortisol to adrenal mitochondria. J Steroid Biochem. (1990) 35 511-514.

(22) Ohnishi T, Wada A, Nonaka Y, Sugiyama T, Yamono T and Okamoto M. Effect of calmodulin and aldosterone synthesis by a cytochrome P450IIß reconstituted system from bovine adrenocortical mitochondria. J Biochem (1986) 100 1065-1076.

(23) Ohnishi, T., Wada, A., Nonaka, Y., Okamoto, M. and Yamano, H. Effect of phospholipid on aldosterone biosynthesis by a cytochrome P-450 (11) beta-reconstituted system. Biochem. Int. (1984) 715-723.

(24) Yanagibashi K, Kobayashi Y and Hall PF. Ascorbate as a source of reducing equivalents for the synthesis of aldosterone. Biochem Biophys Res Comm (1990) 170 1256-1262.

(25) Raff H and Kolandaruish S. Effects of O_2 on aldosterone release from bovine adrenocortical cells in vitro. Endocrinology (1990) 127 682-687.

(26) Brickner RC, Jankowski B and Raff H. Conversion of corticosterone to aldosterone is the site of oxygen sensitivity in the bovine zona glomerulosa. Endocrinology (1992) 130 88-92.

(27) Vinson GP, Whitehouse BJ and Hinson J. In the Adrenal Cortex. Edited by Mac. E. Hedley, Prentice Hall, New Jersey (1992).

(28) Rosenberg ME, Chinielewski D and Hostetter TH: Effects of dietary protein on rat renin and angiotensinogen gene expression. J Clin Invest (1990) 85 1144-1149.

(29) Fukamizu A, Takahashi S, Seo MS, Tada M, Tanimoto K, Uehera S and Murakami K: Structure and expression of the human angiotensinogen gene. J Biol Chem (1990) 265 7576-7582.

(30) Clauser, E., Gaillard, I., Wei, L. and Corvol, P. Regulation of angiotensinogen gene. Am. J. Hypertension (1989) 2 403-410.

(31) Lindpainter K and Ganten D. The cardiac renin-angiotensin system. Circulation Research (1991) 68 905-921.

(32) Myers, L.M. and Steele, M.K. The brain renin-angiotensin system and prolactin secretion in the male rat. Endocrinology (1991) 129 1744-1748.

(33) Ryan JW: Renin-like enzyme in the adrenal gland. Science (1967) 158 1589-1590.

(34) Auerbach R, Alby L, Grieves J, Lindgren, C., Joseph, J., Morrissey, L.W., Sidky, A., Tu, M. and Watt, S.L. Monoclonal antibody against angiotensin converting enzyme: its use as a marker for murine, bovine and human endothelial cells. Proc Nat Acad Sci (USA) (1982) 79 7891-7895.

(35) Kifor I, Moore TJ, Fallo F, Sperling E, Chiou CY, Menachary A and Williams GH: K+-stimulated aldosterone release from superficial adrenal capsules and enzymatically dispersed cells of the zona glomerulosa. Endocrinology (1991) 129 823-831.

(36) Kifor I, Moore TJ, Fallo F, Sperling E, Menachary A, Chiou CY and Williams GH: The effect of Na+ intake on AII content of the rat adrenal gland. Endocrinology (1991) 128 1277 -1284.

(37) Dzau VJ. Tissue renin-angiotensin systems: Physiologic and pharmacologic implications. Circulation (1988) 77 (Suppl I) I1-I3.

(38) M°Kenna, T.J., Island, D.P., Nicholson, W.E. and Liddle, G.H. Angiotensin stimulates both early and late pathways in aldosterone biosynthesis in isolated bovine glomerulosa cells. J. Steroid Biochem. (1978) 9 967-972.

(39) Barrett, P.Q., Bollag, W.B., Isales, C.M., M°Carthey, R.T. and Rasmussen, H. Role of calcium in angiotenisin-II mediated aldosterone secretion. Endocrine Reviews (1989) 10 496-518.

(40) Spät, A., Enyedi, P., Hajnoczky, G. and Hunyady, L. Generation of calcium signal in adrenal glomerulosa cells. Experimental Physiology (1991) 76 859-885.

(41) Berridge, M.J. and Irvine, R.F. Inositol Phosphates and cell signalling. Nature (1989) 341 197-205.

(42) Hunyady,L., Baukal, A.J., Bor,M., Ely, J.A. and Catt, K.J. Regulation of 1,2 diacylglycerol production by angiotensin-II in bovine adrenal glomerulosa cells. Endocrinology (1990) 126 1001-1008.

(43) Exton, J.H. Signalling through phosphatidylcholine breakdown. J. Biol. Chem. (1990) 265 1-4.

(44) Berridge, M.J. and Irvine, R.F. Inositol trisphosphate, a novel second messenger in cellular signal transduction. Nature (1984) 312 315-321.

(45) Bell, R.L., Kennerly, D.A., Stanford, N. and Majerus, P.W. Diglyceride lipase; a pathway for arachidonate release from human platelets. Proc. Nat. Acad. Sci. (USA) (1979) 76 3238-3241.

(46) Cooper, D.M.F. Bimodal regulation of adenylate cyclase. FEBS Letts. (1982) 138 157-163.

(47) Meyer, T., Wensel, T. and Stryer, L. Kinetics of calcium channel opening by inositol (1,4,5) trisphosphate. Biochemistry (1990) 29 32-37.

(48) O'Neil KT and Degrado WF: How calmodulin binds its targets. T.I.B.S. (1990) 15 59-64.

(49) Meldolesi, J. and Pozzan, T. Pathways of Ca^{2+} influx at the plasma membrane; voltage-, receptor- and second messenger-operated channels. Exp. Cell Research (1987) 171 271-283.

(50) Hosey, M.M. and Lazdunski, M. Calcium channels: molecular pharmacology, structure and regulation. J. Membr. Biol. (1988) 104 81-105.

(51) Matsunaga, H., Yamashita, N., Manuyama, Y., Kojima, I. and Kurokawa, K. Evidence for two distinct voltage-gated calcium channel currents in bovine adrenal glomerulosa cells. Biochem. Biophys. Res. Comm. (1987) 149 1049-1054.

(52) Cohen C.J., M°Carthey, R.T., Barrett, P.Q. and Rasmussen, H. Calcium channels in adrenal glomerulosa cells: K^+ and A-II increase T-type calcium channel current. Proc. Nat. Acad. Sci. (USA) (1988) 85 2412-2416.

(53) Natke, E. and Kabela, E. Electrical responses in cat adrenal cortex: possible relation to aldosterone secretion. Am. J. Physiol. (1979) 237 E158-162.

(54) Shepherd, R.M., Fraser, R., Nichols, D.J. and Kenyon, C.J. Efflux of potassium ions in A-II stimulated bovine adrenicortical cells. J. Endocrinol. (1991) 128 297-303.

(55) Blatz, A.L. and Magleby, K.L. Calcium-activated potassium channels. Trends Neurosci. (1987) 10 463-476.

(56) Peterson, O.H., Findlay, I., Suzuki, K. and Dunne, M.J. Messenger-mediated control of potassium channels in secretory cells. J. Exp. Biol. (1986) 124 33-52.

(57) Tabares, L., Lopez-Barneo, J. and De Miguel, C. Calcium and voltage-activated potassium channels in adrenocortical cell membranes. Biochimica et Biophysica Acta (1985) 814 96-102.

(58) Hajnoczky, G., Csordas, G., Hunyady, L., Kalapos, M.P, Balla, T., Enyedi, P. and Spät, A. Angiotensin-II inhibits the Na^+/K^+ pump in rat zona glomerulosa cells. Endocrinology (1992) 130 1637-1644.

(59) Berridge MJ and Gallione A. Cystolic calcium oscillators. FASEB J. (1988) 2 3074-3082.

(60) Grynkiewicz, G., Poenie, M. and Tsien, R.Y. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. (1985) 260 3440-3450.

(61) Quinn, S.J., Enyedi, P., Tillotson, D.L. and Williams, G.H. Kinetics of cytosolic calcium and aldosterone responses in rat adrenal glomerulosa cells. Endocrinology (1991) 129 2431-2441.

(62) Harper, J.F., Cheung, W.Y., Wallace, R.W., Huang, H.L., Levine, S.L. and Steiner, A.L. Localisation of calmodulin in rat tissues. Proc. Nat. Acad. Sci. (USA) (1980) 77 366-370.

(63) Ganguly, A., Chiou, S., Fineberg, N.S. and Davis, J.S. Greater importance of Ca^{2+}-calmodulin in maintenance of Ang-II and K^+-mediated aldosterone secretion: lesser role of protein kinase C. Biochem. Biophys. Res. Comm. (1992) 182 254-261.

(64) Chauhan VPS, Cauhan A, Deshmukh DS and Brockerhoff H: Lipid activators of protein kinase C. Life Sciences (1990) 47 981-986.

(65) Lang, U. and Vallotton, M.B. Angiotensin-II but not potassium induces subcellular distribution of protein kinase C in bovine adrenal glomerulosa cells. J. Biol. Chem. (1987) 262 8047-8050.

(66) Rasmussen, H. The cycling of calcium as an intracellular messenger. Scientific American (Oct. 1989) 44-51.

(67) Simpson, E.R. and Waters, J. Effect of calcium on pregnenolone formation and cytochrome P-450 in rat adrenal mitochondria. J. Steroid. Biochem. (1975) 6 395-400.

(68) Hanukoglu, I., Privalle, C.T. and Jeffcoate, C.R. Mechanisms of ionic activation of adrenal mitochondria cytochrome P-450$_{scc}$ and 11ß. J. Biol. Chem. (1981) 256 4329-4335.

(69) Pralong, W.F., Hunyady, L., Varnai, P. and Spat, A. Pyridine nucleotide redox state parallels aldosterone production in K^+-stimulated adrenal glomerulosa cells. Proc. Nat. Acad. Sci. (USA) (1992) 89 132-136.

(70) Kramer, R.E. Evidence that angiotensin-II decreases mitochondrial calcium in the glomerulosa cell. Mol. Cell Endocrinol. (1990) 74 87-100.

(71) Fujita, K., Aguilera., G. and Catt, K.J. The role of cAMP in aldosterone production by isolated zona glomerulosa cells. J. Biol. Chem. (1979) 254 8567-8574.

(72) Hanukoglu, I., Feuchtwanger, R. and Hanukoglu, A. Mechanism of corticotropin activation and cAMP induction of mitochondrial cytochrome P-450 enzyme systems in adrenal cortex cells. J. Biol. Chem. (1990) 265 20602-20608.

(73) Tremblay, E. Payet, M.D. and Gallo-Payet, N. Effects of ACTH and A-II on cytosolic calcium in cultured adrenal glomerulosa cells. Cell Calcium (1991) 12 655-673.

(74) Papadopoulos, V., Widmaier, E.P. and Hall, P.F. The role of calmodulin in the responses to ACTH of plasma membranes from adrenal cells. Endocrinology (1990) 126 2465-2473.

(75) Campbell, W.B., Brady, M.J., Rosolowsky, L.J. and Falck, J.R. Metabolism of arachidonic acid by rat adrenal glomerulosa cells. Endocrinology (1991) 128 2183-2194.

(76) Campbell, W.B., Gomez-Sanchez, C.E., Adans, B.V., Schmitz, J.M. and Iskovitz, H.D. Attenuation of angiotensin-II and angiotensin-III induced aldosterone release by prostaglandin synthesis inhibitors. J. Clin. Invest. (1979) 64 1552-1557.

(77) Natarjan, J.L., Natarjan, R., and Stern, N. Specific action of the lipoxygenase pathway in mediating angiotensin-II induced aldosterone synthesis in isolated adrenal glomerulosa cells. J. Clin. Invest. (1987) 80 1763-1780.

(78) Natarjan R., Dunn, W.D., Stern, N. and Nadler, J. Key role of diacylglycerol-mediated 1,2 lipoxygenase product formation in angiotensin-II induced aldosterone synthesis. Mol. Cell. Endocrinol. (1990) 72 73-80.

(79) Tremblay, A., Waterman , M.R., Parker, K.L. and Lehoux, J.G. Regulation of rat adrenal m-RNA and protein levels for cytochrome P-450's and adrenodoxin by dietary Na^+ and K^+ intake. J. Biol. Chem. (1991) 266 2245-2251.

(80) Lauber, M., Boni-Schnetzler, M. and Müller, J. K^+ raises cytochrome P-450 11ß mRNA levels in zona glomerulosa of rat adrenals. Mol. Cell. Endocrinol. (1990) 72 159-166.

(81) Capponi, A.M., Lew, P.D., Jonnot, L. and Vallotton, M.B. Correlation between cytosolic free Ca^{2+} and aldosterone production in bovine adrenal glomerulosa cells: evidence for a difference in the mode of action of A-II and K^+. J. Biol. Chem. (1984) 259 8863-8869.

(82) Kojima, I., Kojima, K. and Rasmussen, H. Characteristics of A-II, K^+ and ACTH induced calcium influx in adrenal glomerulosa cells. J. Biol. Chem. (1985) 260 9171-9176.

(83) Barrett, P.Q., Isales, M., Bollag, W.B. and M°Carthey, R.T. Ca^{2+}-channels and aldosterone secretion. Am. J. Physiol. (1991) 261 F706-719.

(84) Balla, T., Varnai, P. and Spät, A. Effect of high K^+ concentration and dihydropyridine Ca^{2+} channel agonists in cytoplasmic Ca^{2+} and aldosterone production in rat adrenal glomerulosa cells. Endocrinology (1990) 127 815-822.

(85) Ganguly, A., Chiou, S. and Davis, J.S. Intracellular mediators of potassium-induced aldosterone secretion. Life Sciences (1990) 46 173-180.

(86) Hajnoczky, G., Varnai, P., Bunday, L., Furrago, A. and Spät, A. The role of protein kinase C in control of aldosterone production by rat adrenal glomerulosa cells-Activation of protein kinase C by potassium. Endocrinology (1992) 130 2230-2236.

(87) Kojima, I, Kojima, K. and Rasmussen, H. Intracellular calcium and adenosine 3'5'-cyclic monophosphate as mediators of potassium-induced aldosterone secretion. Biochem. J. (1985) 228 69-76.

(88) Farrago, A., Seprodi, J., and Spät, A. Subcellular distribution of protein kinase C in bovine adrenal glomerulosa cells. Biochem. Biophys. Res. Comm. (1988) 156 628-633.

(89) Atlas, S.A. Atrial Natriuteric Factor: a new hormone of cardiac origin. Recent Prog. Horm. Res. (1987) 42 207-242.

(90) Atarashi, K., Mulrow, P. J,, Franco-Saenz, R., Snujda, R. and Rupp, J. Inhibition of aldosterone secretion by an atrial extract. Science (1984) 224 992-994.

(91) Chartier, L., Schiffrin, E. and Thibault, G. Effect of atrial natriuretic factor (ANF) related peptides on aldosterone secretion by adrenal glomerulosa cells: critical role of the intramolecular disulphide bond. Biochem. Biophys. Res. Comm. (1984) 122 171-174.

(92) Kenyon, C.J. and Jardine, A.G. Atrial natriuretic peptide: water and electrolyte homeostasis. In Baillière's Clinical Endocrinology and Metabolism (Aug.1989) 3 431-450. Edited by P.H. Baylis. Baillière Tindall, London.

(93) Matsuoka, H., Ishi, M., Hirata, Y., Atarashi, K., Sugimoto, T., Kanagawa, K. and Mutsuo, H. Evidence for a lack of role of cGMP in effect of α-lANP in aldosterone inhibition. Am. J. Physiol. (1987) 252 E643-647.

(94) Churchill, P.F. and Kimura, T. Topological studies of cytochromes P-450_{scc} and P-$450_{11\beta}$ in bovine adrenocortical inner mitochondrial membranes. J. Biol. Chem. (1979) 254 10443-10448.

(95) Iida, S., Papadopoulos, V. and Hall, P.F. The influence of exogenous free cholesterol on steroid synthesis in cultured adrenal cells. Endocrinology (1989) 124 2619-2624.

(96) Simpson, E.R. and Waterman, M.R. Regulation of the synthesis of steroidogenic enzymes in adrenocortical cells by ACTH. Ann. Rev. Physiol. (1988) 50 427-440.

(97) Boyd, G.S., M°Namara, B., Suckling, K.E. and Tocher, R. Cholesterol metabolism in the adrenal cortex. J. Steroid Biochem. (1983) 23 345-348.

(98) Stryer, L. In Biochemistry (1981) W.H. Freeman and company, San Francisco. 470-473.

(99) Brown, M.S., Kovanen, P.T and Goldstein, J.L. Receptor-mediated uptake of lipoprotein cholesterol and its utilisation for steroid synthesis in the adrenal cortex. Hormone Res. (1979) 35 215-249.

(100) Johnson, W.F., Mahlberg, F.H., Rothblatt, G.H. and Phillips, M.C. Cholesterol transport between cells and high density lipoproteins. Biochim. et Biophys. Acta (1991) 1085 273-298.

(101) Brinton, E.A., Oran, J.F., Chen, C.H., Albers, J.J.and Bierman, E.L. Binding of HDL to cultured fibroblasts after chemical alteration of apoprotein amino acid residues. J. Biol. Chem. (1986) 261 495-503.

(102) Mahley, R.W., Innerarity, T.L., Weisgraber, K.H., Rall Jr, S.C., Hui, D.Y., Lulazer, A., Boyles, J.K., Taylor, J.M. and Levy-Wilson. Cold Spring Harbor Symposia on quantitative biology (1986) Volume LI 821-828.

(103) Simpson, H.D, Shepherd, R., Shepherd, J., Fraser, R., Lever, A.F. and Kenyon, C.J. Effects of cholesterol and lipoproteins on aldosterone secretion by bovine zona glomeruolsa cells. J. Endocrinol. (1988) 121 125-131.

(104) Toth, I.E., Szalay, K.Sz, Szabo, D. and Pill, J. Effects of cholesterol synthesis inhibitor in presence and absence of HDL on corticosteroidogenesis of isolated zona glomerulosa and fasiculata cells. J. Steroid Biochem. Mol. Biol. (1990) 37 687-691.

(105) Gwynne, J.F. and Mahafee, D. Esterification of cholesterol in HDL decreases its ability to support ACTH-stimulated steroid synthesis by rat adrenocortical cells. J. Biol. Chem. (1987) 262 16349-16356.

(106) Parinaud, J., Perret, B., Ribbes, H., Hugues, C., Pontonnier, G. and Douste-Blazy, L. High density lipoprotein and low density lipoprotein utilisation by human granulosa cells for progesterone synthesis in serum-free culture: respective contributions of free and esterified cholesterol. J. Endocrinol. Metab. (1987) 64 409-417.

(107) Jefcoate, C.R., Dibartolomeos, M.J., Williams, C.A. and M^{c}Namara, B.C. ACTH regulation of cholesterol movement in isolated adrenal cells. J. Steroid Biochem. (1987) 27 721-729.

(108) Privalle, C.T., Crivello, J.F. and Jeffcoate, C.R. Regulation of intra-mitochondrial transfer to side-chain cleavage cytochrome P-450 in rat adrenal gland. Proc. Nat. Acad. Sci. (USA) 80 (1983) 702-706.

(109) Conneely, O.M., Headon, D.R., Olson, C.D., Ungan, F. and Dempsey, M.E. Intramitochondrial movement of adrenal sterol carrier protein with cholesterol in response to corticotropin Proc. Nat. Acad. Sci. (USA) (1984) 81 2970-2974.

(110) Vahouny, G.V., Chanderbhan, R., Noland, B.J., Irwin, D., Dennis, P., Lambeth, J.D. and Scallen, T.J. Sterol Carrier Protein 2: Identification of sterol carrier protein 2 and site of action for mitochondrial cholesterol utilisation. J. Biol. Chem. (1983) 258 11731-11737.

(111) Krueger, R.J. and Orme-Johnson, N.R. Acute adrenocorticotropic hormone stimulation of adrenal corticosteroidogenesis: Discovery of a rapidly induced protein J. Biol. Chem. (1983) 258 10159-10167.

(112) Pederson, R.C and Brownie, A.C. Cholesterol side-chain cleavage in the rat adrenal cortex: isolation of a cycloheximide-sensitive activator peptide. Proc. Nat. Acad. Sci. (USA) (1983) 80 1882-1886.

(113) Pederson, R.C. and Brownie, A.C. Steroidogenic activator polypeptide isolated from a rat Leydig cell tumour. Science (1987) 236 188-190.

(114) Papadopoulos, V., Berkovich, A., Kreuger, K.E, Costa, E. and Guidotti, A. Diazepam-binding inhibitor and its processing products stimulate mitochondrial steroid biosynthesis via an interaction with mitochondrial benzodiazepine receptors. Endocrinology (1991) 129 1481-1488.

(115) Whitwam, J.G. Benzodiazepines. Anaesthesia (1987) 42 1255-1257.

(116) Braestrup, C. and Squires, R.F. Specific benzodiazepine receptors in rat brain characterised by high affinity [^{3}H]diazepam binding. Proc. Nat. Acad. (USA) (1977) 74 3805-3809.

(117) Schwartz, R.D. The GABA receptor-gated ion channel: Biochemical and pharmacological studies of structure and function Biochem. Pharmacol. (1988) 37 3369-3375.

(118) De Souza, E.B., Annholt, R.R.H., Murphy, K.M.M., Snyder, S.H. and Kuhrer, M.J. Peripheral-type benzodiazepine receptors in endocrine organs: Autoradiographic localisation in rat pituitary, adrenal and testis. Endocrinology (1985) 116 567-573.

(119) Annholt, R.R.H., Pederson, P.L., De Souza, E.B. and Snyder, S.H. The peripheral-type benzodiazepine receptor. J. Biol. Chem. (1986) 261 576-583.

(120) Antkiewicz-Michaluk, L., Guidotti, A. and Kreuger, K.E. Molecular characterisation and mitochondrial density of a recognition site for peripheral-type benzodiazepine ligands. Mol. Pharmacol. (1988) 34 272-278.

(121) Verma, A., Nye, J.S. and Snyder, S.H. Porphyrins are endogenous ligands for the mitochondrial (peripheral-type) benzodiazepine receptor. Proc. Nat. Acad. Sci. (USA) (1987) 84 2256-2260.

(122) Muhkin, A.G., Papadopoulos, V., Costa, E and Kreuger, K.E. Mitochondrial benzidiazepine receptors regulate steroid biosynthesis. Proc. Nat. Acad. Sci. (USA) (1989) 86 9813-9816.

(123) Besman, M.J., Yanagibashi, K., Lee, T.D., Kawamura, M., Hall, P.F. and Shively, J.E. Identification of des-[gly-ile]-endozepine as an effector of corticotropin-dependent adrenal steroidogenesis: stimulation of cholesterol delivery is mediated by the peripheral benzodiazepine receptor. Proc. Nat. Acad. (USA) (1989) 86 702-706.

(124) Bovolin, P., Schlichting, J.L., Miyata, M., Ferrarese, C., Guidotti, A. and Alho, H. Distribution and characterisation of diazepam binding inhibitor (DBI) in peripheral tissues of the rat. Regulatory Peptides (1990) 29 267-281.

(125) Mussoti, M., Slobdyanski, E., Konkel, D., Costa, E. and Guidotti, A. Regulation of diazepam binding inhibitor in rat adrenal gland by adrenocorticotropin. Endocrinology (1991) 129 591-596.

(126) Brown, A.S, Hall, P.F., Shoyab, M. and Papadopoulos, V. Endozepine/diazepam binding inhibitorin adrenocortical and leydig cell lines: absence of hormonal regulation. Mol. Cell. Endocrinol. (1992) 83 1-9.

(127) M^{c}Namara, B.C. and Jefcoate, C.R. The role of sterol carrier protein-2 in stimulation of steroidogenesis in rat adrenal mitochondria by adrenal cytosol. Archives Biochem. Biophys. (1989) 15 53-62.

(128) Mestre, M., Carriot, T., Balin, C., Vzam, A., Renalt, C., Dubroeucq, M.C., Gueremy, C., Doble, A. and Le Fur, G. Electrophysiological and pharmacological evidence that peripheral-type benzodiazepine receptors are coupled to calcium channels in the heart. Life Sci. (1985) 36 391-400.

(129) Durant, N.N., Lee, C. and Katz, R.L. The action of dantrolene on transmitter mobilisation at the rat neuromuscular junction. Eur. J. Pharmacol. (1980) 68 403-408.

(130) Lihrmann, I.H., Delarue, C., Homo-Delarche, F., Feuilloley, M., Belanger, A. and Vaudry, H. Effects of TMB-8 and dantrolene on ACTH and AII-induced steroidogenesis by frog interrenal gland; evidence for a role of intracellular calcium in angiotensin action. Cell Calcium (1987) 8 269-282.

(131) Kojima, K., Kojima, I. and Rasmussen, H. Dihydropyridine calcium agonist and antagonist effects on aldosterone secretion. Am. J. Physiol. (1984) 247 E645-650.

(132) Utili, R., Boitnott, J.K. and Zimmermann, H.J. Dantrolene-associated hepatic injury. Gastroenterology (1977) 72 610-616.

(133) Francis, K.T. and Hamrick, M.E. Dantrolene inhibition of the hepatic mixed function oxidase system. Res. Commun. Chem. Path. Pharmac. (1979) 23 69-80.

(134) Roy, S., Francis, K.T., Born, C.K. and Hamrick, M.E. Interaction of dantrolene with the hepatic mixed function oxidase system. Res. Commun. Chem. Path. Pharmac. (1980) 27 507-520.

(135) Francis, K.T. and Hamrick, M.E. Effects of dantrolene on adrenal cortical function. Biochem. Pharmacol. (1980) 29 1669-1672.

(136) Shepherd, R.M. The role of potassium ions in angiotensin II-stimulated aldosterone secretion. Ph.D. Thesis, Univ. of Glasgow (1989).

(137) Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. Protein measurement with the Folin phenol reagent. J. Biol. Chem. (1951) 193 265-275.

(138) Holloway, C.D, Kenyon, C.J., Dowie, L.J., Corrie, J.E.T., Gray, C.E. and Fraser, R. Effects of the benzodiazepines diazepam, des-n-methyldiazepam and midazolam on corticosteroid biosynthesis in bovine adrenocortical cells in vitro; location of site of action. J. Steroid Biochem. (1989) 33 219-225.

(139) Newman. D. The distribution of range in samples from a normal population expressed in terms of an independent estimate of standard deviation. Biometrika (1939) 31 20-30.

(140) Kojima, I.,Kojima, K. and Rasmussen, H. Effects of Ang II and K^+ on Ca efflux and aldosterone production in adrenal glomerulosa cells. Am. J. Physiol. (1985) 248 E36-43.

(141) Kojima, I., Kojima, K. and Rasmussen, H. Role of calcium fluxes in the sustained phase of angiotensin II-mediated aldosterone secretion from adrenal glomerulosa cells. J. Biol. Chem. (1985) 260 9177-9184.

(142) Kojima, I., Kojima, K. and Rasmussen, H. The temporal integration of the aldosterone secretory response to angiotensin occurs via two intracellular pathways. J. Biol. Chem. (1984) 259 14448-14457.

(143) Bird, I.M., Walker, S.W. and Williams, B.C. Agonist stimulation of phosphoinositide metabolism in the adrenal cortex and its relationship to steroidogenesis. J. Mol. Endocr. (1990) 5 191-209.

(144) Vallner, J.J, Honibger, I.L., Stewart, J.T. and Peyton, A.B. Dantrolene and metabolite levels in 6 patients on chronic therapy. Curr. Ther. Res. (1979) 25 79-91.

(145) Schiebinger, R.J., Braley, L.M., Menachery, A.M. and Williams, G.H. Unique calcium dependencies of the activating mechanism of the early and late aldosterone biosynthetic pathways in the rat. J. Endocr. (1986) 110 315-325.

(146) Loose, D.S, Ken, P.B., Hirst, M.A., Marcus, R.A. and Feldman, D. Ketoconazole blocks adrenal steroidogenesis by inhibiting cytochrome P450-dependent enzymes. J. Clin. Invest. (1983) 71 1495-1499.

(147) Lambert, A., Mitchell, R. and Robertson, W.R. Biopotency and site of action of drugs affecting testicular steroidogenesis. J. Endocr. (1987) 113 457-461.

(148) Fraser, R., Holloway, C.D. and Kenyon, C.J. Inhibition of corticosteroid 11ß hydroxylation in bovine zona fasiculata cells by the potassium entry blocker 4-aminopyridine. J. Steroid Biochem. (1986) 24 777-778.

(149) Shepherd, R.M., Fraser, R. and Kenyon, C.J. Membrane permeability to K^+ and the control of aldosterone synthesis: effects of valinomycin and cromakalim in bovine adrenocortical cells. J. Mol. Endocrinol. (1992) 9 165-173.

(150) Havel, R., Eder, H.A. and Bragdon, J.H. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J. Clin. Invest. (1955) 34 1345-1353.

(151) Siedel, J., Hägele, E.Q., Ziegenhorn, J. and Wahlfeld, A.W. Reagent for the enzymatic determination of serum total cholesterol with improved lipolytic efficiency. Clin. Chem. (1983) 29 1075-1080.

(152) Kramer, R.E. Angiotensin II causes sustained elevations in cytosolic calcium in glomerulosa cells. Am. J. Physiol. (1988) 255 E338-346.

(153) Mauger, J.P., Poggioli, J., Guesdon, F. and Claret, M. Noradrenaline, vasopressin and angiotensin II increase Ca^{2+} influx by opening a common pool of Ca^{2+} channels in isolated rat liver cells. Biochem. J. (1984) 221 121-127.

(154) Walker, S.W., Strachan, M.W.J., Nichol, M., Williams, B.C. and Bird, I.M. Dose -dependent effect of Angiotensin-II, acetylcholine and vasopressin in the cytosolic concentration of Ca^{2+} in suspensions of primary cultures of zona fasiculata/reticularis cells from bovine adrenal cortex.
J. Mol. Endocrinol. (1991) 6 197-203.

(155) Rink, T.J. and Pozan, T. Using Quin-2 in cell suspensions Cell Calcium (1985) 6 133-144.

(156) Hanmani, I., Lagendre, C. and Maume, B.F. Induction of corticosteroid biosynthetic pathway by ACTH and HDL in newborn rat adrenocortical cells cultured in serum-free medium. Biochim. et Biophys. Acta (1986) 886 457-467.

(157) Tall, A.R. Plasma High density lipoproteins: Metabolism and relationship to atherogenesis. J. Clin. Invest. (1990) 86 379-384.

(158) Desai, K., Bruckdorfer, R., Hutton, R.A., and Owen, J.S. Binding of Apo-E rich high density lipoprotein particles by saturable sites on human blood platelets inhibits agonist-induced platelet aggregation. J. Lipid Research (1989) 30 831-839.

(159) Stimpel, M., Neyses, L., Locher, R., Knorr, M. and Vetter, W. High density lipoproteins-modulators of the calcium channel? J. Hypertension (1983) 3 (Supp.3) S49-51.

(160) Zhou, Q., Jimi, S., Smith, T.L. and Kummerow, F.A. The effect of cholesterol on the accumulation of intracellular calcium. Biochim. et Biophys. Acta (1991) 1085 1-6.

(161) Gallo-Payet, N. and Payet, M.D. Excitation-secretion coupling: involvement of potassium channels in ACTH-stimulated rat adrenocortical cells. J. Endocrinol. (1989) 120 409-421.

(162) Shibita, H., Kojima, I. & Ogata, E. Diazepam inhibits potassium induced aldosterone secretion in adrenal glomerulosa cells. Biochem. Biophys. Res. Comm. (1986) 135 994-999.

(163) Vinson, G.P., Whitehouse, B.J., Teja, R. & Hinson, J.P. Benzodiazepine receptor mediated inhibition of steroidogenesis in rat adrenocortical cells. J. Endocrinol. (1991) 129 (supplement), 284.

(164) Lambert, A, Frost, J., Mitchell, R., Wilson, A.V. & Robertson, W.R. On the site of the anti-adrenal steroidogenic effect of etomidate and megestrol acetate. Clin. Endocrinol. (1984) 21 721-727.

(165) Ayub, M. & Levell, M.J. Inhibition of adrenal steroidogenic enzymes in vitro by imadazole drugs including ketoconazole. J. Steroid Biochem. (1989) 32 515-524.

(166) Kenyon, C.J., Young, J., Gray, C.E. & Fraser, R. Inhibition by etomidate of steroidogenesis in isolated bovine adrenal cells. J. Clin. Endocrinol. Metab. (1984) 58 947-949.

(167) Kenyon, C.J., Fraser, R., Birnie, G.G., Connell, J.M.C. & Lever, A.F. Dose related in vitro effects of ranitidine and cimetidine on basal and ACTH-stimulated steroidogenesis. Gut (1986) 27 1143-1145.

(168) Dowie, L.J., Smith, J.E., MacGilchrist, A,J., Fraser, R., Honour, J.W., Reid, J.L. & Kenyon, C.J. In vivo and in vitro studies of the site of inhibitory action of omeprazole in adrenocortical steroidogenesis. Eur. J. Clin. Pharmacol. (1988) 35 625-629.

(169) Annholt, R.R.H., De Souza, E.B., Kuhan, M.J. & Snyder, S.H. Depletion of peripheral-type benzodiazepine receptors after hypophysectomy in rat adrenal glands and testis. Eur. J. Pharmacol. (1985) 110 41-46.

(170) Foster, R.M.V., Lobo, M., Rasmussen, H. & Marusic, E.T. Calcium: its role in the mechanism of action of angiotensin II and potassium in aldosterone production. Endocrinology (1981) 109 2196-2201.

(171) Müller, J. & Baumann, K. Multifactorial regulation of the final steps of aldosterone synthesis in the rat. J. Steroid Biochem. (1974) 5 795-800.

(172) Braley, L.M., Menachery, A.I., Brown, E.M. & Williams, G.H. The effects of extracellular K^+ and angiotensin II on cytosolic calcium and steroidogenesis in adrenal glomerulosa cells. Biochem. Biophys. Res. Comm. (1984) 123 810-815.

(173) Aguilera, G. & Catt, K.J. Participation of voltage-dependent calcium channels in the regulation of adrenal glomerulosa function by angiotensin II and potassium. Endocrinology (1986) 118 112-118.

(174) Quinn, S.J., Cornwall, M.C. & Williams, G.H. Electrical properties of isolated rat adrenal glomerulosa and fasiculata cells. Endocrinology (1987) 120 903-914.

(175) Spät, A., Balla, T., Cragoe E.J. Jr., Hajnoczky, Gy. & Hunyady, L. Angiotensin-II and potassium activate different calcium entry mechanisms in rat adrenal glomerulosa cells.
J. Endocrinol. (1989) 122 361-370.

(176) Rampe, D. & Triggle, D.J. Benzodiazepines and calcium channel function. T.I.P.S. (1986) 7 461-464.

(177) Rampe, D. & Triggle, D.J. The actions of diazepam and diphenylhydantoin on fast and slow Ca^{2+} uptake processes in guinea pig cerebral cortex synaptosomes. Can. J. Physiol. Pharmacol. (1987) 65 538-543.

(178) Taft, W.C. and Delorenzo, R.J. Micromolar affinity benzodiazepine receptors regulate voltage-sensitive calcium channels in nerve terminal preparations. Proc. Nat. Acad. Sci. (USA) (1984) 81 3118-3122.

(179) Saavo, V. Central-type and peripheral-type benzodiazepine receptors. Ann. of Clin. Res. (1988) 20 348-355.

(180) Amsterdam, A. & Byung, S.S. An inducible functional peripheral benzodiazepine receptor in mitochondria of steroidogenic granulosa cells. Endocrinology (1991) 129 503-510.

(181) Krueger, K.E. & Papodopoulos, V. Peripheral-type benzodiazepine receptors mediate translocation of cholesterol from outer to inner mitochondrial membranes in adrenocortical cells. J. Biol. Chem. (1990) 265 3772-3779.

(182) Papadopoulos, V., Nowzari, F.B. & Krueger, K.E. Hormone-steroidogenesis is coupled to mitochondrial benzodiazepine receptors. Tropic hormone action is inhibited by flunitrazepam. J. Biol. Chem. (1991) 266 3682-3687

(183) Le Fur, G., Vaucher, N., Perrier, M.L., Flamier, A., Benavides, J., Renault, C., Dubroeucq, M.C., Guérémy, C. and Uzan, A. Differentiation between two ligands, [^{3}H]RO5-4864 and [^{3}H]PK11195, for peripheral benzodiazepine binding sites by thermodynamic studies. Life Sci. (1983) 33 449-457.

(184) Olson, J.M.M., Ciliax, B.J., Mancini, W.R. and Young, A.B. Presence of peripheral type binding sites on human erythrocyte membranes. Eur. J. Pharmacol. (1988) 152 47-53.

(185) Antkiewicz-Michaluk, L., Muhkin, A.G., Guidotti, A. and Krueger, K.E. Purification and characterisation of a protein associated with peripheral-type benzodiazepine binding sites.
J. Biol. Chem. (1988) 263 17317-17321

(186) Sprengel, R., Werner, P., Seeburg, P.H., Mukhin, A.G., Santi, M.R., Grayson, D.R., Guidotti, A. and Krueger, K.E.
Molecular cloning and expression of a cDNA encoding a peripheral-type benzidiazepine receptor. J. Biol. Chem. (1989) 264 20415-20421.

(187) Skowronski, R., Beaumont, K. and Funestil, D.D. Modification of the peripheral type benzodiazepine receptor by arachidonate, diethylpyrocarbonate and thiol reagents. Eur. J. Pharmacol. (1987) 143 305-314.

(188) Oke, B.O., Suarezquain, C.A., Riond, J., Ferrara, P. and Papadopoulos, V. Cell surface localisation of the Peripheral-Type Benzodiazepine Receptor (PBR) in adrenal cortex. Mol. Cell. Endocrinol. (1992) 87 (No.1-3) R1-6.

(189) Mestre, M., Carriot, T., Belin, C., Uzan, A., Renault, C., Dubroeucq, M.C., Guérémy, C. and Le Fur, G. Electrophysiological and pharmacological characterisation of peripheral benzodiazepine receptors in a guinea pig heart preparation. Life Sci. (1984) 35 953-962.

(190) Python, C.P., Capponi, A.P. and Vallotton, M.B. Effect of peripheral-type benzodiazepines on cytosolic calcium and aldosterone production in adrenal zona glomerulosa cells. In Ninth International Congress of Endocrinology, Nice. (1992) Abstract no. P.02.31.119, pge. 122.

(191) Capponi et al. Calcium regulation of adrenal steroid secretion. Proceedings of the 9th International Congress of Endocrinology. In press.