REGULATION OF HEPATIC STEROID METABOLISM BY PROTEIN KINASE C.

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SUMMARY.

The oxidative metabolism of both drugs and steroids occurs mainly as a consequence of utilising the hepatic monooxygenase system (Kuntzman et al, 1964). It has been shown that the activities of various hepatic enzymes are not constant, but are subject to regulation by the endocrine system, and also by hormones. The main classes of hormones which have been implicated in the regulation of hepatic metabolism are; (a) androgens, as extensively reviewed by Skett & Gustafsson (1979) and Gustafsson and coworkers (1980); (b) the pituitary hormones, including prolactin (Colby et al, 1974) and growth hormone (Colby et al, 1974; Mode et al, 1981); and (c) the pancreatic hormones, particularly insulin (Kato & Gillette, 1965; Kato et al, 1970).

As the different hormonal pathways within the body are subject to complex interactions, and since some hormones have different secretion profiles in male and female animals, the precise mechanism by which hormones act to regulate the activity of hepatic enzymes has not yet been fully elucidated.

One mechanism by which hormones can produce their intracellular effects is by stimulating the hydrolysis of membrane-bound phospholipids. The consequence of this hydrolysis is that two intracellular second messengers are produced, inositol-1,4,5-trisphosphate, which causes an elevation in intracellular free calcium levels, and 1,2-diacylglycerol, which interacts with phosphatidylserine and calcium to activate the phosphorylating enzyme, protein

kinase C. Activation of protein kinase C by receptor-mediated inositol phospholipid hydrolysis, relays information across the cell membrane to regulate a variety of intracellular processes, and hormonal activation of protein kinase C in the liver has been reported. Vasopressin is known to affect hepatic enzymes, such as glycogen synthetase (Blackmore et al, 1986a; Ahmad et al, 1984) and glycogen phosphorylase (Barritt et al, 1988), and vasopressin also has general effects upon hepatic events, especially α_1-mediated adrenergic effects (Gárciá-Sáinz et al, 1986). Angiotensin II has also been reported to have hepatic effects mediated through activation of protein kinase C (Gárciá-Sáinz et al, 1986; Gárciá-Sáinz & Hernandez-Sotomayor, 1987). Recently, some actions of insulin have also been attributed to protein kinase C activation (Acevedo-Duncan et al, 1989; Cooper et al, 1987; Gomez et al, 1988), although evidence implicating hepatic effects of insulin through protein kinsae C are still contradictory.

Recently, it has been demonstrated that, in a reconstituted microsomal membrane system, protein kinase C, and protein kinase A, are capable of phosphorylating cytochrome P450, the terminal oxidase in hepatic mono-oxygenases (Pyerin et al, 1983; Pyerin et al, 1987).

It seemed possible, therefore, that stimulation of inositol phospholipid hydrolysis, and therefore of protein kinase C and subsequent phosphorylation of cytochrome P450, or other proteinaceous components, may be one mechanism by which hepatic monooxygenase activity could be regulated by

hormones.

The proposed aim of the present study was to try to determine whether or not activation of protein kinase C in the rat liver could influence the activity of hepatic monooxygenases, and to try to elucidate the underlying mechanism of any effect which was observed. This study was conducted using isolated rat hepatocytes in an attempt to avoid complex hormonal interactions, and to try to represent a more physiological situation than is often encountered during in vitro studies.

To activate protein kinase C directly, tumor-promoting phorbol esters and synthethic diacylglycerols were used. Phorbol esters are reported to act solely through protein kinase C (Niedel et al, 1983; Castagna et al, 1982) and they are convenient probes of protein kinase C activity in isolated cell systems. Hormonal activation of protein kinase C, via stimulation of inositol phospholipid hydrolysis, was investigated with vasopressin and angiotensin II, and the role of elevating intracellular calcium was investigated by the use of the ionophore A23187. The effect of activating protein kinase C upon the metabolism of the endogenous steroid 4-androstene-3,17-dione was ascertained after various times of incubation with the different compounds.

It was found that activation of protein kinase C with all of the compounds, except angiotensin II, produced a time-dependent inhibition in the activity of all of the enzymes metabolising 4-androstene-3,17-dione. This inhibition of enzymes activity was not sex-dependent, males

and female were both affected to the same extent. With vasopressin, a biphasic pattern of enzymes inhibition was observed, an effect which implies a dependency on diacylglycerol initially, with the later effect being more dependent upon calcium.

Angiotensin II failed to show any inhibition of enzyme activity, and in contrast showed a stimulation of enzyme activity. Angiotensin II is known to affect both protein kinase C and protein kinase A and the stimulation seen with angiotensin II is possibly a reflection of some complex, but poorly understood, interaction between the two cascade systems.

Inhibition of enzyme activity appeared to be solely dependent upon the activation of protein kinase C. Using kinase inhibitors, as originally described by Kase and his group (Kase et al, 1986; Kase et al, 1987), we determined that only the specific protein kinase C inhibitor, K252b, was capable of preventing inhibition of enzyme activity in the presence of phorbol esters. The involvement of protein kinase C was further implicated by the results of the protein kinase C translocation assay, a recognised method for implying protein kinase C in a biological phenomenon. We found that protein kinase C could be translocated from the cytosol to the plasma membrane in the presence of phorbol esters, which are reported to have protein kinase C as their intracellular receptor (Niedel et al, 1983).

By using the protein synthesis inhibitor cycloheximide, it became further apparent that the inhibition of enzyme activity also had a requirement for protein synthesis.

The possibility that the effects of protein kinase C upon enzyme activity were due to the phosphorylation of cytochrome P450, as had been reported previously in in vitro studies (Pyerin et al, 1987), was also examined. We were unable to detect any incorporation of ^{32}P into either immunoprecipitated cytochrome P4502c (P450IIC11), a constitutive enzyme thought to be responsible for up to 90% of hydroxylation of 4-androstene-3,17-dione at the 16α position (Waxman, 1984), immunoprecipitated cytochrome P450PB3a (P450IIB1), the major phenobarbital-inducible enzyme, immunoprecipitated cytochrome P450MC-1b (P450IA1), the major 3-methylcholantherene-inducible isozyme, or immunoprecipitated cytochrome P450UT-1 (P450IIA1), the steroid 7α-hydroxylase, following incubation with 50nM 4β-phorbol-12-myristate-13-acetate. This implies that phosphorylation of cytochrome P450 may not be a physiological effect of activting protein kinase C.

Further investigation into the role of the NADPH-cytochrome P450 enzyme system in protein kinase C-dependent effects revealed that, the inhibition of steroid metabolising enzyme activity by protein kinase C was not dependent upon either the availability or rate of loss of the cofactor NADPH, or the activity of cytochrome c (P450) reductase.

Further phosphorylation studies failed to reveal any specific protein which may have been responsible for the actions of protein kinase C upon enzyme activity.

In conclusion, the results of this study revealed that activation of the phosphorylating enzyme, protein kinase C,

in the isolated rat hepatocyte caused a sex-independent, time-dependent inhibition of the activity of the enzymes metabolising the steroid substrate, 4-androstene-3,17-dione. This effect was apparently independent of the phosphorylation of cytochrome P450, and independent of any effect on a specific component of the cytochrome P450 enzyme system. The precise mechanism of action of protein kinase C, although not fully elucidated in this study, appears to have a requirement for gene expression and de novo protein synthesis. We propose that inositol phospholipid hydrolysis and activation of protein kinase C may be one mechanism through which the activity of hepatic monooxygenases are regulated.

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ABBREVIATIONS.

ACTH	;	Adrenocortocotropin.
ATP	;	Adenosine 5'-Triphosphate.
Cyclic AMP	;	Cyclic adenosine 3', 5'-monophosphate.
DAG	;	1,2-Diacylglycerol.
DOG	;	1,2-Dioctanoylglycerol.
DNA	;	Deoxyribonucleic acid.
EDTA	;	(Ethylenediaminetetraacetic acid).
EGTA	;	(Ethleneglycol-bis(β-aminoethylether) N,N,N',N'- tetraacetic acid).
GH	;	Growth hormone.
IP_3	;	Inositol-1,4,5-trisphosphate.
MOPS	;	(3-(N-morpholino)propanesulfonic acid).
NADH	;	Reduced nicotinamide adenine dinucleotide.
NADPH	;	Reduced nicotinamide adenine dinucleotide phosphate.
PI	;	Phosphatidyl inositol.
PIP	;	Phosphatidyl inositol-4-phosphate.
PIP_2	;	Phosphatidyl inositol-4,5-bisphosphate.
4αP	;	4α-Phorbol.
4αPDD	;	4α-Phorbol-12,13-didecanoate.
PMA	;	4β-Phorbol-12-myristate-13-acetate.
PKA	;	Protein kinase A; Cyclic AMP-dependent protein kinase.
PKC	;	Protein kinase C; Calcium-dependent, phospholipid-dependent protein kinase.
RNA	;	Ribonucleic Acid.

INTRODUCTION

Introduction.

1.1 Historical Background

1.1(a) Discovery of hepatic metabolism.

During the last century, it became apparent that foreign chemicals which were taken into the body of an animal were subsequently excreted in the urine and faeces. The first metabolic pathway to be elucidated in mammals was the formation of hippuric acid, which was found to be the glycine conjugate of ingested benzoic acid and was excreted from the body in the urine (Keller, 1842).

The early ideas about the detoxification of compounds were proposed by Baumann (1876), although the concept of detoxifying a parent compound to produce less harmful metabolites was not developed for another twenty years (Lang 1894; Neumeister, 1895).

It became apparent that the body possessed a variety of protective measures which it could employ to aid the entrance of foreign compounds (xenobiotics) into the systemic circulation, and once these compounds had entered the systemic circulation, hepatic metabolism came into play in order to dispose of the xenobiotics from the body.

The original work upon metabolism was concerned with the metabolism of chemicals and ingested drugs, and extensive work by Williams (1959; 1967) led to the determination of detailed pathways of the routes of metabolism for many different xenobiotics.

Eventually, it was discovered that hormones such as testosterone, could also be metabolised in the liver (Conney & Klutch, 1963) and it was postulated that steroid

metabolism and xenobiotic metabolism could be performed by the same enzymes within the body (Kuntzman et al, 1964).

Metabolism of xenobiotics in the body can be broadly described by two phases, as described by Williams (1959). The first type is Phase 1 metabolism and this is principally concerned with the oxidation, reduction, hydration or hydrolysis of a parent compound. After Phase 1 metabolism, Phase 2 metabolism subsequently occurs and the principle of a Phase 2 reaction is that metabolites are combined with hydrophobic endogenous compounds, in a process commonly referred to as conjugation. Conjugation of a compound greatly facilitates the excretion of the compound in the urine, as it makes the compound more water soluble. Phase 1 and 2 reactions can occur simultaneously, the function of Phase 1 metabolism being to transform a parent compound into a form which can be readily conjugated by Phase 2 (Caldwell, 1982). Phase 1 and 2 processes, although they have clearly distinct functions, are part of one overall process and it is important that they are considered in this context.

Phase 2 conjugation reactions will not be considered in this thesis, although they have been extensively reviewed (Williams, 1967; Caldwell, 1982).

1.1(b) Discovery of hepatic microsomal metabolism.

In the 1940s, intensive study into hepatic drug metabolism began, when it was realised that rat liver homogenates could biotransform compounds which had carcinogenic properties. Mueller and Miller (1949) observed

that the reductive cleavage of the carcinogenic dye 4-dimethylaminoazobenzene yielded dimethylphenyldiamine and aniline as products in a microsomal fraction of liver homogenates. The same group later showed (Mueller & Miller, 1953) that in order for hepatic microsomal systems to function optimally, molecular oxygen, reduced nicotinamide adenine dinucleotide phosphate (NADPH) and reduced nicotinamide adenine nucleotide (NADH) had to be present.

Several years later, Brodie and coworkers (1955) characterized a virtually identical system to that of Mueller and Miller, in rabbit microsomes. This system had identical requirements for oxygen, NADPH and NADH in order to act optimally and through a variety of metabolic pathways, the system could perform a range of metabolic reactions.

The requirement of microsomal drug metabolising enzymes for both molecular oxygen and a reducing agent (NADPH) were enigmatical, until it was shown independently (Mason, 1957; Hayaishi, 1964) that certain oxidases transfer one atom of molecular oxygen to the substrate to form a hydroxylated product, while the other oxygen atom undergoes two equivalent reductions to form a molecule of water. Mason (1957) designated the enzymes the mixed function oxidases, while Hayaishi (1964) referred to the enzymes as the mono-oxygenases. Posner and his group (1961), by using radiolabelled molecular oxygen, proved that the oxygen utilised in metabolic pathways was derived from molecular oxygen and not cellular water.

1.1(c) Discovery of cytochrome P450.

A reduced pigment in rat hepatic microsomes, that had an absorption band with a maximum wavelength at 450nm after binding to carbon monoxide, was first identified independently by both Klingenberg and Garfinkel in 1958. This pigment was further characterized as a P450 hemoprotein in 1964 by Omura and Sato. They presented evidence that the cytochrome contained an integral heavy metal, which was iron (1964a), and after solubilisation, purification and studies as to the properties of this cytochrome (1964b), they named the pigment cytochrome P450 (pigment 450). The role of cytochrome P450 in microsomal mixed function oxidase systems was also established at this time (Cooper et al, 1965) and over the next twenty years it became apparent that multiple forms of cytochrome P450 can exist in mammals and other species.

Although first discovered in hepatic microsomes and associated with catabolic processes, cytochromes P450 also exist elsewhere in the body and cytochromes P450 located in the adrenal gland are vital in the biosynthesis of steroids. These include two microsomal enzymes, steroid 17α-hydroxylase and steroid 21-hydroxylase, and two mitochondrial cytochromes P450, the cholesterol side chain cleavage enzyme and steroid 11β-hydroxylase. The biology, enzymology and regulation of adrenal enzymes has been extensively reviewed (Fevold, 1983; Hall, 1985).

1.2 Cytochrome P450 and hepatic monooxygenases.

1.2(a) Spectrophotometric studies.

Omura and Sato (1964b) detailed the study of the molecular extinction coefficient of the reduced carbon monoxide difference spectrum of cytochrome P450 and this is considered to be the most accurate index of the cytochrome P450 content in microsomes of a variety of partially purified preparations.

In this study it was revealed that there are several unusual optical properties of cytochrome P450;

(i) Carbon monoxide produces an atypical shift of the Soret peak of the reduced cytochrome P450 to higher wavelengths with almost no change in the visible region.

(ii) Reduction of cytochrome P450 elicits only a small spectral change.

(iii) Ethyl isocyanide causes an unusual splitting of the Soret peaks at 430nm and 455nm.

(iv) Type I and Type II spectra can be produced by a variety of substrates, such as hexobarbitone (Type I) and aniline (Type II).

The changes in Type I and Type II binding for cytochrome P450 are interpreted as changes in the ligand field of the haem iron which is present in cytochrome P450.

The haem prosthetic group of cytochrome P450 is

generally accepted to be ferriprotoporphyrin IX (Maines and Anders, 1973) and one distinct difference between cytochrome P450 and other haem containing proteins, such as haemoglobin and myoglobin, is that the haem of cytochrome P450 is not covalently bound to the protein (Hall, 1987). Four of the six ligands of the iron of cytochrome P450 are known to be coordinated with the pyrrole nitrogen (Gibson & Tamburini, 1984), whilst the fifth ligand is thought to be a mercaptide link, probably involving thiolate (Mason, 1965; Waterman & Mason, 1972). The nature of the sixth ligand of the haem is unclear at the present time, although histyl imidazole (Dus, 1976) and oxygen in the form of water, (Griffin & Peterson, 1975) have both been implicated.

As well as combining with carbon monoxide and absorbing at 450nm, the haem of cytochrome P450 also has a natural absorption spectrum, which is produced in the absence of carbon monoxide. This absorption is dependent upon the arrangement of electrons in the d-orbital of the iron. At any one given instant, the electrons of iron within the d orbitals in a single molecule of cytochrome P450 exists entirely in a low spin (S = 1/2) or entirely in a high spin state (S = 5/2). In a low spin state, one electron is unpaired (S = 1/2) whilst in a high spin state 5 electrons are unpaired (S = 5/2). The time spent in each spin state is determined by a dynamic equilibrium between the two states and is established by the conformation of the protein associated with the haem in the presence of a substrate. For example, with Type I substrates, the substrate perturbs the

protein by binding to a site on the hydrophobic portion of the protein, near to the iron. This causes a shift in the dynamic equilibrium into the high spin state. Type II substrates combine with the iron molecule itself and cause a shift in the equilibrium towards the low spin state. Confusion arises in spectral studies with some substrates which are hydroxylated by cytochrome P450, such as benzene and barbitone, but do not produce a binding spectra (Imai & Sato, 1967); as there is no absorption spectra, these substrates cannot be classified under the Type I and Type II headings. In the absence of any substrate, the equilibrium between the two spin states is in favour of a low spin configuration of electrons and this produces a major absorption peak at 420nm which is referred to as the Soret peak (Hall, 1987).

1.2(b) NADPH-Cytochrome P450 reductase.

The equation summarising cytochrome P450-dependent biotransformation reactions is shown below;

$$RH + O_2 + H^+ + NADPH \xrightarrow{\text{cyt P450}} NADP^+ + H_2O + ROH$$

The molecular oxygen binds to the haem component of cytochrome P450, which is present in its oxidised ferric form, and upon oxidation of the substrate the haem iron is reduced to its ferrous form. Reoxidisation completes the cycle.

The electrons in the above equation are transferred from NADPH to cytochrome P450, in order to reduce the

cytochrome. The electron transfer is not a direct pathway and the electrons pass through a flavoprotein electron carrier and from there to cytochrome P450. The electron carrier is NADPH-cytochrome P450 reductase and its ability to function as an electron carrier is due to the composition of this enzyme. The reductase contains two separate flavin moeities, which are flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) (Iyanagi & Mason, 1973), making NADPH-cytochrome P450 reductase rather unusual because most other flavoproteins contain only FMN or FAD. The pathway by which NADPH-cytochrome P450 reductase acts to transfer electrons is summarised below;

$$\underset{\text{NADPH Cyt.P450 Red.}}{\text{NADPH} + H^+ \longrightarrow (\text{FAD} \longrightarrow \text{FMN}) \longrightarrow \text{Cytochrome P450}}$$

The need for an intermediary electron transfer flavoprotein is apparent when it is considered that (NADPH and H^+) is a 2 electron donor system, and cytochrome P450 can only accept one electron at a time. NADPH-cytochrome P450 reductase is thought to act as a "transducer" of reducing equivalents by accepting the electrons from NADPH and transferring them sequentially to cytochrome P450.

It has been discovered that within the microsomal membrane, there is an excess of cytochrome P450 molecules compared to the number of molecules of NADPH-cytochrome P450 reductase. For each molecule of reductase there are between 20 and 30 molecules of cytochrome P450, implying that NADPH-cytochrome P450 reductase can act as the electron carrier for numerous molecules of cytochrome P450.

There have been two theories proposed for the spatial arrangement of cytochrome P450 and the reductase within the cell membrane. One theory proposes that there is a "rigid arrangement" of the two compounds with the reductase surrounded by a cluster of cytochrome P450 molecules in a structurally organised manner, whereas the "fluid " theory proposes that the reductase and the P450 molecules are surrounded by a sheath of phospholipids and this permits free movement through the membrane of each component. Evidence for each theory has been reported (Peterson et al, 1971; Yang et al, 1977).

1.2(c) The sequence of events in cytochrome P450-dependent metabolism.

The central feature of the cytochrome P450 catalytic cycle is the ability of the haem iron to undergo cyclic oxidation and reduction reactions by the pathway summarised in figure 1. These changes in the state of the haem occur in close association to substrate binding and oxygen activation. The molecular details of the cytochrome P450-dependent hydroxylation reactions are briefly summarised below into the seven major stages.

Stage 1 - Substrate binding.

This stage is well documented and it involves drug binding to the oxidised (ferric) form of cytochrome P450. The way in which substrates bind to cytochrome P450 was characterised in the late 1960s by Schenkman into three main categories, Type I, Type II and modified Type II (Schenkman

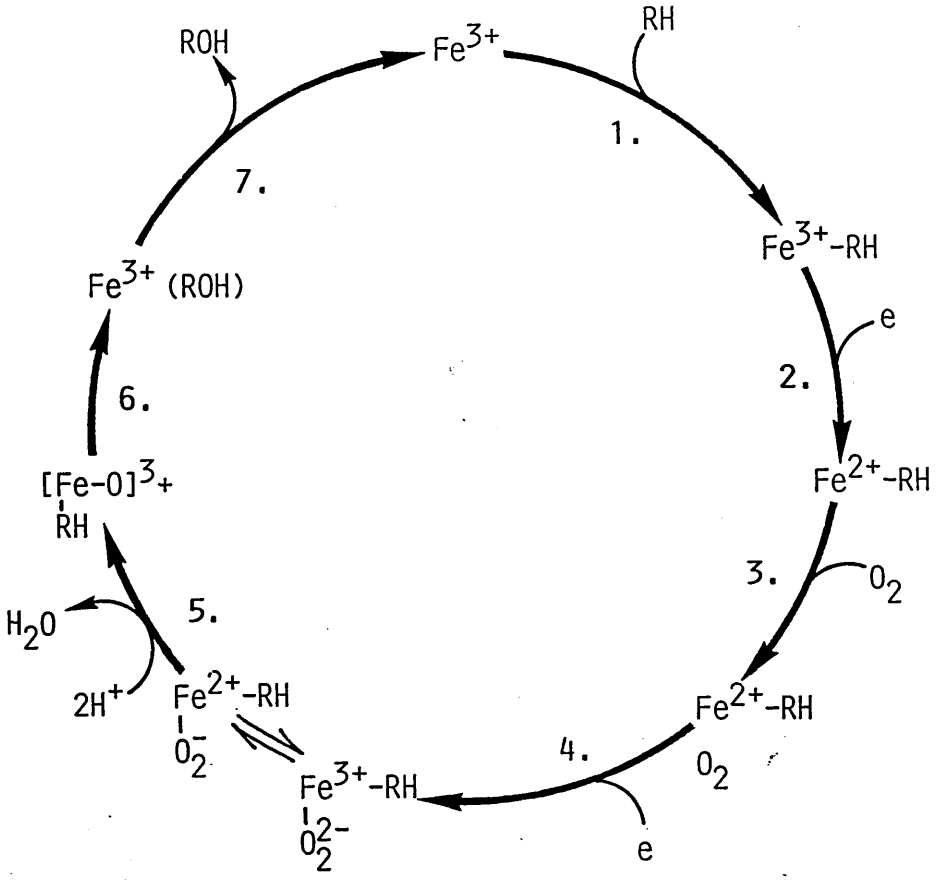


Figure 1:
The catalytic cycle of cytochrome P450.

et al, 1967) and the mode of binding of each substrate determines which category of substrate a drug belongs to. Each substrate causes a characteristic spectral perturbation of the haemoprotein of the cytochrome P450 molecule and each type of substrate has its own characteristic binding site.

Type I substrates bind to the polypeptide chain of the apoprotein and change the conformation of the protein and hence the ligation of the haem prosthetic group (White & Coon, 1980). This conformational change of the protein shifts the spin state of the cytochrome to high spin and results in a characteristic spectral change (Type I spectral change). The Type I spectra is characterized by a maximum absorption at 390nm and a minimum absorption at 420nm in the difference spectrum. The increase in absorbance at 390nm reflects the conversion to a high spin state upon binding of the substrate.

Type II substrates are generally nitrogenous bases and it is thought that they ligate to the haem iron of cytochrome P450 which produces a conformational change to the low-spin, 6 co-ordinated state (Schenkman et al, 1967). The spectral pattern produced by Type II substrates has an absorbance minimum at 395nm and a maximum at 430nm.

The final type of substrate are modified Type II substrates (reverse Type I) and it is thought that these substrates act by displacement of an endogenous component which is usually bound to cytochrome P450 at the Type I binding site, or by binding to the sixth coordinate position on the haem molecule (Peterson et al, 1971). Modified Type II substrates produce a spectral change which is maximum at

420nm and minimum at 390nm, that is the exact reverse of the spectra produced by Type I substrates.

It is generally assumed that Type I substrates (e.g. hexobarbitone and natural steroids) act in a more physiological manner than the Type II substrates (e.g. aniline) and Type I substrates are, therefore, more widely used to investigate the regulatory control of cytochrome P450.

Stage 2 - Electron transfer.

Stage 2 involves the transfer of the first electron from NADPH-cytochrome P450 reductase to cytochrome P450, a process which results in a conversion of cytochrome P450 from an oxidised ferric form to a reduced ferrous form.

The high spin / low spin equilibrium of cytochrome P450 may well be an important factor in mediating the first electron transfer step and it appears that the fully reduced state of the low potential flavin protein acts as the electron donating species.

Stage 3 - Binding of oxygen.

Once the iron of cytochrome P450 has been reduced to its ferrous form, molecular oxygen then binds to the binary ferrous cytochrome P450-substrate complex at the sixth coordinate position of the haem. The binding of a substrate molecule in stage 1 displaces the sixth coordinate ligand and thus facilitates the binding of molecular oxygen (Wolf et al, 1988).

Stage 4 - Electron insertion and oxygen "activation".

Insertion of the second electron causes the oxygen to become a reactive species and an internal rearrangement of the electrons then reoxidises the iron to its ferric form. Although extensive work has been performed, the exact nature of this reactive oxygen species and the precise mechanism of activation of the species remains poorly understood. White and Coon (1980) have reviewed this area of cytochrome P450-dependent reactions and they have tried to resolve some of the debate as to the nature of the reactive oxygen species.

Stages 5-7 in the catalytic cycle of cytochrome P450 are still a subject of debate and although the events below are known to occur, the precise mechanisms remain to be elucidated. White and Coon have again proposed theories for each stage (1980).

Stage 5 - Cleavage of oxygen.

The activated oxygen species is cleaved to produce two separate atoms of oxygen. One combines with two hydrogen ions to yield water, whilst the other oxygen remains attached to the iron molecule.

Stage 6.

A hydrogen atom is removed from the carbon atom on the substrate. The hydrogen atom is combined with the oxygen to form a hydroxyl radical and as a consequence of removing hydrogen, a carbon radical is left on the substrate. These

two radicals recombine and the oxygen is thus transferred from the haem to the substrate to produce a hydroxylated product.

Stage 7 - Dissociation of product.

The hydroxylated product of stage 6 dissociates from the active site and this leaves haem in its ferric form, ready to repeat the cycle with the next molecule of substrate.

1.2(d) Other Phase I biotransformation pathways.

In section 1.2(c) hydroxylation of a substrate was used as an example to illustrate a Phase I reaction mediated by cytochrome P450. Cytochrome P450 is also involved in a variety of other oxidative metabolic reactions such as dealkylation, deamination, sulfoxidation and oxidation. Most of these reactions proceed via a step which involves intermediate hydroxylation, and the hydroxylated intermediate then degrades to yield the final product. Although the final product is different for each type of reaction, all the reactions proceed through the the same initial pathway as illustrated in figure 1 (Brodie et al, 1958).

1.2(e) The involvement of lipid in drug metabolism.

In 1968, Lu and Coon discovered that in order for maximal fatty acid hydroxylation to occur, a heat stable, organic solvent-extractable factor was necessary. The same group later showed that lipid was required for the

hydroxylation of carcinogens, steroids and other oxidative metabolic processes (Lu et al, 1969). The identity of this phospholipid was reported to be phosphatidylcholine (Strobel et al, 1970) and the involvement of phospholipid in microsomal hydroxylation reactions was further implied by other groups (Chaplin & Mannering, 1970; Eling & Di Augustine, 1971). These studies found that upon removal of approximately 70% of the total phospholipid content of microsomes, the metabolism of type I compounds was markedly reduced, whilst the binding of the type I substrate to the cytochrome P450 complex was completely prevented. Type II compounds, however, were found to have their binding and metabolism virtually unaffected by the removal of lipid.

In a repeat of the above experiments, Cater and his group (1972) suggested that the phospholipid dependence of hydroxylation reactions was due to the phospholipids relieving the inhibition caused by detergents, which were used to solubilise the protein.

In an attempt to resolve some of the controversy associated with the role of lipids in drug metabolism, Vore et al (1974) employed the use of lyophilised microsomes, which were more stable to organic extraction techniques and although the lipid is removed from them, the enzymes which remain within the microsomes are catalytically active. Their study showed that removal of phospholipid (both phosphatidylcholine and phosphatidylethanolamine) caused a 10-25% loss of cytochrome P450 and NADPH-cytochrome P450 reductase. The enzymatic activity of these two enzymes, however, was reduced to 30-50% of control activities but

upon addition of a total lipid extract, or synthetic phosphatidylcholine, the extracted microsomes expressed complete activity.

These studies imply that phosphatidylcholine is an essential component in the mediation of drug metabolism. Although the precise mechanism of action of the phospholipid remains to be elucidated, it appears that the lipid factor may be essential for the transfer of electrons between NADPH and cytochrome P450 (Strobel et al, 1970).

1.2(f) Cytochrome b_5.

Although NADPH is the preferred source of reducing equivalents for cytochrome P450-linked mixed function oxidations, addition of NADH to an NADPH-containing medium has long been known to enhance the effect of NADPH (Conney et al, 1957). It was not until 1971, however, that Hildebrandt and Estabrook deduced that cytochrome b_5 was a component of the hepatic cytochrome P450 monooxygenase system and that cytochrome b_5 was responsible for the ability of NADH to stimulate cytochrome P450 monooxygenase activity. Cytochrome b_5 is thought to be the carrier through which the second electron is transferred from NADH to cytochrome P450. Although NADPH via NADPH-cytochrome P450 reductase can also supply the second electron to cytochrome P450, the pathway through cytochrome b_5 is thought to act in preference to the cytochrome P450 reductase pathway, with NADH being the principal donor of the second electron in some systems (Imai & Sato, 1977). It has been shown that NADH alone is unable to support

oxidative metabolism (Correia & Mannering, 1973).

1.3 Multiplicity of cytochrome P450.

1.3(a) Discovery of multiple forms of cytochrome P450.

In 1959, Conney and workers observed that benzo-a-pyrene administration resulted in an increase in the metabolism of benzo-a-pyrene and zoxazolamine, a decrease in the metabolism of pethidine and benadryl and little effect upon the metabolism of chlorpromazine. Later work with the classical inducers phenobarbital and 3-methylcholantherene (3-MC) led to the discovery that enzymes could be induced which had differing substrate specificities and also different carbon monoxide absorption spectra (Kuntzman, 1969; Sladek & Mannering, 1969). Multiplicity of cytochrome P450 has also been proposed on the basis of different rates of turnover for a variety of cytochrome P450-dependent enzymes (Levin et al, 1974; Bradshaw et al, 1978)

1.3(b) Nomenclature of cytochrome P450.

One of the characteristics of cytochrome P450 research has been the confusing nature of the nomenclature of different forms of cytochrome P450. Every laboratory involved in cytochrome P450 purification has its own system of nomenclature and consequently, one cytochrome P450 can have a vast array of different names, all of which represent the same enzyme. The isolation and sequencing of many cytochrome P450 proteins, cDNAs and genes has facilitated the development of a cytochrome P450 classification system,

which is based on primary amino acid sequence alignment data (Nebert et al, 1987). In this system, the cytochromes P450 are grouped into a gene superfamily that is further subdivided into gene families and finally into gene subfamilies. To date, 13 gene families have been identified. There are problems associated with a cytochrome P450 nomenclature system based solely on sequence similarities, as expected due to the complexity of the cytochromes P450, their polymorphic nature and the fact that they are rapidly diverging.

The basics of the nomenclature system are as follows. The gene families are designated by Roman numerals, beginning with I. Gaps are left in the family numbers to allow for the addition of newly discovered gene families. The primary hepatic drug metabolising enzymes currently comprise four gene families, designated I to IV. The extrahepatic cytochromes P450 involved in steroid biosynthesis, fall into families XVII (steroid 17α-hydroxylase), XIX (P450 aromatase) and XXI (steroid 21-hydroxylase) (Nebert et al, 1987). The mitochondrial, yeast and bacterial P450 gene families are referred to as XI, LI/LII and CI/CII respectively. Subfamilies are denoted with sequential capital letters and genes within a subfamily are indicated with sequential Arabic numbers.

For instance, there are eight subfamilies in the P450II gene family, designated A through H, and the next subfamily discovered would be called I. The individual genes are usually numbered depending on the order in which the sequence data was published. Once a cytochrome P450

sequence has been determined, if a clear orthologous counterpart cannot be identified from another species which has been sequenced previously, then the cytochrome P450 is assigned the next consecutive number.

Table 1 lists some of the major rat cytochromes P450 cloned and sequenced to date, along with their trivial names. Table 1 is only an example and is by no means exhaustive or complete.

A brief summary of each cytochrome P450 family is given below.

(i) The P450I gene family.

Two cytochrome P450 genes have so far been examined in mice (Gonzalez et al, 1985), rats (Hines et al, 1985), rabbits (Okino et al, 1985) and humans (Jaiswal et al, 1985).

IA1 and IA2 genes appear to be ubiquitous in mammals and, for the most part, these enzymes have similar catalytic activities in several mammalian species. On the basis of the evolutionary conservation of IA1 and IA2 genes in mammals, and the ubiquitous nature of IA1, it has been tentatively proposed that these enzymes may be crucial for survival and may play a key role in the metabolism of critical endogenous substances. The role of IA1 in carcinogenesis has been reviewed (Gelboin, 1980).

(ii) The P450IIA gene subfamily.

The IIA1 and IIA2 genes are highly homologous, IIA2 shares 93% of the nucleotide sequence and 88% of the deduced

amino acid sequence of IIA1 cDNA. These genes are regulated differently, however, during development and after administration of the carcinogen 3-MC.

IIA1 hydroxylates testosterone and other steroids at the 7α position (Matsunaga et al, 1988), with a minor metabolite resulting from hydroxylation at the 6α position. IIA2, in contrast, exhibits a high level of testosterone 15α hydroxylase activity and only about 5% of the total hydroxylated metabolites are due to hydroxylation at the 7α position.

FAMILY/SUBFAMILY	TRIVIAL NAME	COMMENTS
P450IA1	Man P_1, rat c, rabbit form 6.	Major 3-MC-inducible isozyme
P450IA2	Man P_3, rat d, rabbit form 4.	Major isosafrole-inducible isozyme.Also 3-MC.
P450IIA1	Rat a	Steroid 7α-hydroxylase.
P450IIB2	Rat e	Phenobarbitone-indicible.
P450IIC6	RatPB-1, PB-C, k	Phenobarbitone-inducible, Hormone-independent.
P450IIC7	Rat f	Cross-reactive with rat b, constitutive isozyme.
P450IIC11	Rat h, M-1, 2c, 16α	Steroid 16α-hydroxylase.
P450IIC12	Rat i, 2d, 15β	Steroid 15β-hydroxylase.
P450IIC13	Rat g	Constitutive isozyme.Male-specific,strain-dependent
P450IID1	Rat db1	Debrisoquine-4-hydroxylase.
P450IID2	Rat db2	
P450IIE1	Rat j	Ethanol-inducible.
P450IIIA1	Rat PCN1	Pregnenolone 16α-carbonitrile inducible.
P450IIIA2	Rat PCN2	
P450IVA1	Rat LAw	Clofibrate-inducible, lauric acid w-hydroxylase.
P450IVA2	Rat IVA2	

Table 1; Nomenclature of some of the common rat cytochromes P450. From Gonzalez, 1989.

(iii) The P450IIB gene subfamily.

The cDNAs for the P450b (P450IIB1) and P450e (P450IIB2) in rats were among the first to be isolated completely and sequenced, because they are strongly induced by phenobarbital and also specific polyclonal antibodies were available for these two isozymes. Both genes contain nine exons and only 40 nucleotide differences exist between the two mRNAs (Mizukami et al, 1983).

It has been known for several years that the rat IIB2 subfamily contains multiple genes and sequence data and published restriction maps suggest the presence of greater than six genes with considerable nucleotide similarities (Mizukami et al, 1983). It is unclear, however, whether all of these genes are expressed in the rat.

A third rat IIB gene was isolated from liver, designated IIB3 and has an amino acid sequence 77% similar to IIB1 and IIB2 (Labbe et al, 1988). IIB3 is constitutively expressed as a minor form in male and female rat liver and is not induced by phenobarbital.

(iv) The P450IIC gene subfamily.

The members of this subfamily of cytochromes P450 are, in most cases constitutively expressed. The IIC cytochromes P450 are also under developmental and sex-specific regulation and a few can be induced by phenobarbital.

Two members of the rat IIC family, designated P450 PB-1 (Waxman & Walsh, 1984) and P450f (Ryan et al, 1984) have been isolated and protein sequences determined. PB-1 (also known as PB-C) is developmentally regulated in males and

females (Waxman et al, 1985). P450f is also developmentally controlled in a manner similar to PB-1 (Bandiera et al, 1986) and has a broad specificity and low catalytic activity toward a number of substrates.

The cytochrome P450 IIC11 designated P450h (Ryan et al, 1984) metabolises testosterone at the 16α, 2α and 17 positions. The cytochrome P450IIC11 is a male-specific gene and appears to be localised solely in the liver, as no mRNA of P450IIC11 was found in adult male lung, kidney or testes (Yoshioka et al, 1987).

(v) The P450IID gene subfamily.

The debrisoquine-4-hydroxylase P450, named db1 (IID1) has been purified from rat (Larry et al, 1984) and humans (Distlerath et al, 1985). This enzyme is noted for its polymorphic expression in humans and rats, and antibodies and cDNA probes have been used to study the molecular basis of the human and rat drug oxidation defect.

A second cDNA, db2 (IID2) has also been isolated and found to share a 78% cDNA-deduced amino acid homology with IID1. The IID subfamily has a unique lineage within the P450II family, caused as a consequence of the different evolution between humans and rodents, and the different dietary requirements of the two species.

(vi) The P450IIE gene subfamily.

A distinct ethanol-inducible form of P450, named IIE1, was first identified in rabbits (Koop & Coon, 1984) and later in rats (Patten et al, 1986) and humans (Wrighton et

al, 1987). This enzyme can metabolise substrates such as ethanol, acetone, acetoacetate, diethyl ether, halothane, benzene and pyridine. The activity of IIE1 towards acetone oxidation suggests that IIE1 is involved in the pathway of gluconeogenesis during the fasting state.

The rat, rabbit and human IIE1 cDNAs have been isolated and sequenced and they are about 80% homologous in their deduced amino acid sequences.

(vii) The P450 IIH gene subfamily.

This is a chicken cytochrome P450 isozyme and was originally designated to the rat IIC6 subfamily. The precise sequence of this isozyme is still under investigation.

(viii) The P450III gene family.

The major cytochromes P450 in this family can be induced by the synthetic steroid pregnenolone 16α-carbonitrile (Lu et al, 1972) and they have been shown to be unique from other cytochromes P450. The enzymes in the P450III family have catalytic activities for ethylmorphine N-demethylation, erythromycin demethylation and triacetyloleandomycin metabolism (Wrighton et al, 1985) and testosterone 6β-hydroxylation (Waxman et al, 1985).

Most of the enzyme activity data associated with rat and human cytochromes P450 in the P450III family have been inferred from activity immunoinhibition data and data correlating an activity with levels of immunodetectable protein on Western blots. The reason for this is that

reconstitution of enzyme activity from purified preparations of P450III enzymes has not been successful and as a consequence, the structure of the P450III genes from rats and humans have not been published as yet.

(ix) The P450IV gene family.

A clofibrate-inducible lauric acid w-hydroxylase, named P450IVA1, was first purified from rats (Tamburini et al, 1984). This enzyme is very specific for fatty acid hydroxylations and has no detectable activity toward other substrates such as benzphetamine, ethoxyresorufin and testosterone. Cytochrome P450IVA1 can also be induced by other hypolipidaemic agents and is active in the oxidation of arachidonic acid.

1.4 Conserved domains in cytochrome P450 proteins.

1.4(a) Signal sequence and halt-transfer sequence for membrane insertion.

The membrane environment is highly suitable for cytochromes P450 because many cytochromes P450 substrates are hydrophobic and dissolve in the membrane lipid bilayers. The membrane also allows interactions with other cytochromes P450 and the electron donor enzyme NADPH-cytochrome P450 reductase, for the microsomal enzyme. Highly specific cellular mechanisms allow the insertion of the cytochromes P450 and other intrinsic membrane proteins into the lipid bilayer. The microsomal cytochromes P450 are inserted by the signal sequence recognition particle and docking protein, reviewed by Sabatini et al (1982). Early studies

with secretory and plasma membrane proteins established the participation of amino-terminal hydrophobic segments called "signal sequences", which direct the insertion of the proteins into the membrane or the lumen of the endoplasmic reticulum. The signal sequence is cleaved in nearly all secretory products and most membrane proteins during their translation and membrane insertion.

The cytochromes P450 and other components of the microsomal mixed function oxidase system were found to be synthesised primarily on membrane-bound polyribosomes (Gonzalez & Kasper, 1981). This binding and insertion into the membrane are mediated by the hydrophobic amino-terminal sequence of these proteins.

The amino terminus of all eukaryote cytochromes P450 contains an abundance of hydrophobic residues. An acidic residue that is near the initiation methionine is followed by about 14 to 20 residues of hydrophobic amino acids and then by several basic residues. It was suggested that the combined hydrophobic and basic segment probably serves as the "halt- transfer" signal (Sabatini et al, 1982). It appears that the cytochromes P450 are anchored to the membranes via their amino terminal and that the bulk of the enzyme is exposed to the cytoplasmic side of the endoplasmic reticulum (Finidori et al, 1987). This orientation is also supported by immunological studies in which membrane topology was investigated using antibodies to defined polypeptides located throughout the IIB1 primary sequences.

The mitochondrial cytochromes P450 are synthesized and transferred into membranes by a mechanism that is quite

distinct from the insertion process for the microsomal enzymes. They are synthesized on membrane-free polysomes and released into the cytoplasm with an amino terminal peptide that is subsequently cleaved upon insertion into the mitochondria (Schatz, 1979). The length of the cleavable extrapeptides ranges from 37 to 56 amino acids and it was suggested that a conserved portion of this peptide, notably a periodic arginine and lysine distribution, serves to transport the cytochromes P450 across the outer membrane and into the mitochondrial compartment where they are then bound to the inner mitochondrial membrane. Microsomal and mitochondrial cytochromes P450 have, therefore, evolved distinct sequences common to some other cellular proteins that are required for their insertion into specific intracellular compartments.

1.4(b) Haem-binding, cysteine-containing peptide.

An excellent summary of the experiments leading to the conclusive identification of the fifth ligand to the haem at the active site of all the cytochromes P450 has been published (Gotoh & Fujii-Kuriyama, 1989). Briefly, early studies established that the thiolate of cysteine was involved in binding and two conserved cysteine-containing regions, designated HR1 and HR2, near the amino terminal half and the C-terminal half of the cytochrome P450, respectively, were identified as being highly conserved when mammalian and bacterial sequences were compared. Further evidence suggesting the C-terminal cysteine as the fifth ligand bound to the haem iron was provided by comparisons of

other cytochrome P450 sequences. The crystal structure of the bacterial $P450_{cam}$ unequivocally established this cysteine as the haem-binding thiolate ligand (Poulos et al, 1986). This conserved sequence effectively serves as a fingerprint for a cytochrome P450 protein.

1.4(c) Cytochrome P450 membrane association models and predictions.

The topological association of a cytochrome P450 molecule with the membrane has been predicted by comparative sequence analysis, including hydropathy plots of multiple mammalian cytochrome P450 proteins and extrapolations from the crystal structure of the soluble bacterial cytochrome $P450_{cam}$. Several structural or biochemical features are probably common to the mammalian microsomal cytochromes P450 and are taken into consideration in the design of models: (a) cytochromes P450 are anchored to the lipid bilayer by a hydrophobic amino terminus; (b) they interact with NADPH-cytochrome P450 reductase, an enzyme clearly exposed to the cytoplasmic side of the face of the membrane; (c) they react with many hydrophobic substrates and many of the products of cytochrome P450-mediated reactions are then substrates for other microsomal enzymes, such as UDP-glucuronyl transferase; and (d) the haem is either parallel or at a slight angle to the membrane surface.

1.5 Regulation of cytochrome P450 gene expression.

1.5(a) Inducible genes.

1.5(a)(i) Phenobarbital-inducible cytochrome P450 genes.

The phenobarbital induction response was discovered more that 20 years ago. Administration of this drug resulted in a dramatic proliferation of liver endoplasmic reticulum membrane (Remmer & Merker, 1963) and an increase in the enzymes of the mixed function oxidase system (Orrenius et al, 1965). In 1975, Hutterer and his group reported that, as well as inducing cytochrome P450, phenobarbital also increased the size of the liver, the total microsomal protein content and the phospholipid content of the liver, implying that the effects of phenobarbital are not confined to an effect on cytochrome P450 monooxygenases but that there is a general proliferative effect upon the liver with phenobarbital.

The phenobarbital induction response provokes an increase in several mRNAs, including those coding for epoxide hydratase, NADPH-cytochrome P450 reductase (Hardwick et al,1983), UDP-glucuronyl transferase (Mackenzie, 1986) and cytochrome IIB1 and IIB2 (Atchison & Adesnik, 1983; Hardwick et al, 1983). Phenobarbital-induced mRNA accumulation is thought to be due to an increase in gene transcription rate (Atchison & Adesnik, 1983).

The IIB1 and IIB2 genes are coordinately regulated in the liver by phenobarbital, but as these two mRNAs exhibit 97% nucleotide sequence similarity, oligonucleotide probes specific for the IIB1 and IIB2 genes have been used for quantitation (Omiecinski et al, 1985). The IIB2 gene is

constitutively expressed, while IIB1 is transcriptionally inactive in untreated rats. Phenobarbital administration results in the induction of both mRNAs, IIB1 mRNA being more significantly induced than IIB2.

The expression of certain genes in the IIC family of rabbit and rat, and the rat IIA1 and IIA2 genes can also be induced by phenobarbital. Therefore, regulatory elements for phenobarbital inducibility appear to be conserved in many cytochrome P450 genes.

The cytochrome P450IIC7 (P450f) gene is subject to developmental inducibility with this gene becoming transcriptionally activated when rats reach puberty, and it is not significantly induced by phenobarbital when fully expressed in adult rats (Gonzalez et al, 1986a). A rat cDNA designated 1GC1, that shares 98% of the deduced amino acid sequence of cytochrome P450IIC7 (P450f), and a high level of the amino acid homology between 1GC1 and cytochrome P450IIC7 (P450f) suggests that these cytochromes P450 may be allelic variants. The cytochrome P450IIC7 (P450f) and 1GC1 genes appear to be regulated differently, however. An oligonucleotide for 1GC1 revealed that this gene is activated by phenobarbital in immature rats (Barroso et al, 1988). In contrast, only a slight increase in cytochrome P450IIC7 (P450f) protein was seen in 4 week old phenobarbital-treated rats (Bandiera et al, 1986).

Phenobarbital also induces two genes in the rat P450III family (Gonzalez et al, 1986b). Both the constitutively expressed male specific cytochrome P450IIIA2 (PCN-2) gene and the cytochrome P450IIIA1 (PCN-1) gene, a gene that is

not expressed in either male or female untreated rats, are inducible by phenobarbital. Only cytochrome P450IIIA1 (PCN-1) is induced by steroids, suggesting that these genes have overlapping regulatory controls for steroid inducibility and constitutive expression.

Data regarding the role of specific phenobarbital receptors or cis-acting DNA sequence elements in this induction process are lacking, possibly due to the low affinity of phenobarbital or its extensive metabolism by liver enzymes.

1.5(a)(ii) Steroid-inducible cytochrome P450 genes.

The rat cytochrome P450IIIA1 (PCN-1) gene is induced by glucocorticoids such as dexamethasone and pregnenolone 16α-carbonitrile. This induction is primarily the result of an activation of cytochrome P450IIIA1 (PCN-1) transcription (Simmons et al, 1987) and an increase in mRNA (Gonzalez et al, 1986b).

Of interest is the possible involvement of the glucocorticoid receptor (Govinda et al, 1985) which controls a variety of genes in the liver. Regulation of cytochrome P450IIIA1, however, seems to be distinct from the control of classical glucocorticoid-regulated genes, such as tyrosine aminotransferase. Cytochrome P450IIIA1 (PCN-1) induction requires about a 10-fold higher level of steroid than is needed to induce tyrosine aminotransferase and certain steroids that readily induce cytochrome P450IIIA1 (PCN-1) fail to induce tyrosine aminotransferase (Schuetz et al, 1984; Schuetz & Guzelian, 1984). These studies suggest that either cytochrome P450IIIA1 (PCN-1) gene activation may depend on other factors or that differences exist between these genes in their affinity for receptor-ligand binding to a cis-acting DNA control element.

1.5(a)(iii) Hypolipidaemic drug-inducible cytochrome P450 genes.

The hypolipidaemic drug, clofibrate, induces a battery of enzymes, including the peroxisomal enzymes enoyl CoA: hydratase-3-hydroxyacyl-CoA dehydrogenase, fatty acyl CoA oxidase, 3-ketoacyl-CoA thiolase (Hashimoto, 1982) and the

microsomal cytochrome P450IVA1 (Tamburini et al, 1984). Many other hypolipidaemic agents, including aprofibrate and nafenopin also induce these enzymes. The net result of their induction is an increase in fatty acid w-oxidation. The cytochrome P450IVA1, enoyl-CoA: hydratase-3-hydroxy acyl CoA dehydrogenase and fatty acyl CoA oxidase genes are transcriptionally activated by clofibrate administration. This activation is rapid and occurs within one hour of administration (Hardwick et al, 1987).

A second cytochrome P450 cDNA, designated cytochrome P450IVA2, has recently been isolated from a clofibrate-induced liver library and the cytochrome P450IVA2 mRNA is markedly induced by clofibrate. A clofibrate receptor has recently been purified and it has a molecular weight of 70,000, and exists as a dimer of 140,000 (Lalwani et al, 1987).

1.5(a)(iv) Effect of haem on cytochrome P450 induction.

A relatively neglected area of study in recent years has been the effect of haem on cytochrome P450 induction. In order that cytochrome P450 holoenzyme can accumulate, an adequate haem pool must be available to accomodate the newly synthesized apoprotein. The rate-limiting enzyme in haem biosynthesis, 5-aminolevulinate synthetase (ALV-5), is readily inducible by compounds such as phenobarbital (Granick, 1966) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Poland & Glover, 1973). This induction is due to transcriptional activation of the ALV-5 gene and a popular hypothesis is that the effect of these inducers is to

indirectly decrease the haem pool by increasing the amount of apo-P450 (May et al, 1986). The induced apo-P450 incorporates haem from the cellular haem pool and the resultant decrease in haem levels then derepresses the ALV-5 gene. This metabolic end product control could be due to haem-modulated gene repression. Other evidence suggests that the level of haem mediates transcription of cytochrome P450 genes (Bhat & Padmanaban, 1988). It has been reported that inhibition of haem synthesis or stimulation of haem degradation blocks the induction of IIB2 mRNA by phenobarbital.

1.5(B) Regulation of constitutively expressed cytochrome P450 genes.

1.5(b)(i) Transcriptional regulation during development.

One of the most interesting regulatory phenomena is the activation of cytochrome P450 gene transcription during development. At least four modes of constitutively expressed cytochrome P450 gene regulation have been described: (a) activation of expression immediately after birth; (b) activation of expression at the onset of puberty; (c) activation of expression in males or females referred to as "sex-imprinted" expression; and (d) specific suppression of gene activity in males or females at the onset of puberty.

Elevated levels of both the ethanol-inducible IIE1 and the IID1 and IID2 mRNAs have been detected immediately after birth in rats (Gonzalez et al, 1987; Song et al, 1986). Elevation of IIE1 mRNA was coincident with transcriptional

activation of the IIE1 gene and begins within a few hours after birth. mRNA levels peak at the age of 6 days.

The mechanism governing this regulation is unknown; however, changes in the methylation state of the IIE1 gene correlated with transcription (Umeno et al, 1988). Using DNA restriction endonucleases that are sensitive to cytosine methylation, certain cytosine residues upstream of the IIE1 transcription start site were found to be demethylated within 24 hours of birth. Further demethylation of cytosines, downstream of the start site, was detected at 1 week and 10 weeks of age. It is not known whether this demethylation is a result of transcriptional activation of the IIE1 gene or if demethylation causes gene expression.

Two genes in the rat P450IIC family, cytochrome P450IIC6 (PB-1) and cytochrome P450IIC7 (P450f), are transcriptionally activated at the onset of puberty (Gonzalez et al, 1986). The cytochrome P450IIC7 (P450f) mRNA reaches a steady-state level in adult females that is about 2-fold higher than in adult males. The mechanism by which the cytochrome P450IIC6 (PB-1) and cytochrome P450IIC7 (P450f) genes are activated is not fully elucidated.

1.5(b)(ii) Sex imprinting and gene expression.

Sex specific cytochromes P450 have been purified from rats (Cheng & Schenkman, 1982; Matsumoto et al, 1986; Morgan et al, 1986). Best studied are the male-specific and female-specific cytochrome P450 forms which are commonly designated cytochrome P450IIC11 (P450h) and cytochrome P450IIC12 (P450i), respectively (Ryan et al, 1982).

Cytochrome P450IIC11 (P450h) is expressed only in adult male rats and this expression is dependent on androgen exposure both during the neonatal period and adulthood. Castration of neonates abolishes cytochrome P450IIC11 expression in adults, while castration of adults only partially decreases expression of the enzyme. This partial effect is eliminated if rats are supplemented with testosterone during adulthood, directly demonstrating a role for adult testosterone (Waxman et al, 1985). Similarly, testosterone injection to castrated rats during the first three days of life also results in significant cytochrome P450IIC11 (P450h) expression at adulthood, consistent with the proposal that this enzyme is one of the hepatic proteins that is subjected to neonatal "imprinting" or programming by androgen exposure (Einarsson et al, 1973; Gustafsson et al, 1983a).

The cDNA and gene sequences of cytochrome P450IIC11 (P450h) have been determined and a specific oligonucleotide probe has been used to quantitate cytochrome P450IIC11 (P450h) mRNA during development (Morishima et al, 1987). These studies established that the cytochrome P450IIC11 (P450h) mRNA is exclusively expressed in liver of adult male rats and absent in female liver and in several extrahepatic tissues in males and females. The increase in cytochrome P450IIC11 (P450h) probably results from the activation of gene transcription.

The rat cytochrome P450IIA2 gene is also male specific (Matsunaga et al, 1988). Expression of cytochrome P450IIA2 mRNA is undetectable in females and is activated in males at the onset of puberty. The cytochrome P450IIA1 gene, on the

other hand, is expressed in adolescent males and females and is specifically suppressed only in males at puberty (Nagata et al, 1987). Studies on the cytochrome P450IIA2 protein revealed that its expression in males is programmed by neonatal androgen exposure (Waxman et al, 1988).

The role of the pituitary hormones in the regulation of sex-specific cytochromes P450 has been extensively investigated (Kato et al, 1986; Morgan et al, 1986; Waxman et al, 1988a). Adult male rats have pulsatile secretion of pituitary growth hormone, while adult females have more constant levels, and these patterns are determined by both neonatal androgen exposure and adult testosterone levels (Jansson et al, 1985a). Hypophysectomy reduces expression of cytochrome P450IIC11 (P450h) and this can be reversed by periodic injections of growth hormone (Kato et al, 1986; Morgan et al, 1986). Cytochrome P450IIA2 expression in males, on the other hand, is not dependent upon pulsatile growth hormone secretion, unlike cytochrome P450IIC11 (P450h) (Waxman et al, 1988a). Growth hormone secretion is also involved in the regulation of the female specific cytochrome P450IIC12 (P450i). Continuous infusion of growth hormone reestablishes levels of the female-specific cytochrome P450IIC12 (P450i) in hypophysectomised females (Kato et al, 1986) and this results from a specific increase in cytochrome P450IIC12 (P450i) mRNA levels (Mode et al, 1987). In some cases, growth hormone can suppress cytochrome P450 expression, as will be discussed below.

1.5(b)(iii) Sex-specific gene suppression.

The sex-specific expression of certain male and female cytochrome P450s results from the suppression of gene expression. For instance, the expression of cytochrome P450IIIA2 (PCN-2) mRNA is specifically decreased in female rats when they reach puberty (Gonzalez et al, 1986b). This results in a loss of constitutively expressed testosterone 6β-hydroxylase in the female rat. Expression of cytochrome P450IIA1 mRNA is also decreased in males at puberty (Matsunaga et al, 1988; Nagata et al, 1987), resulting in a decline in IIA1 protein and its associated testosterone 7α-hydroxylase activity.

Growth hormone also appears to play a role in the suppression of cytochrome P450 gene expression. Hypophysectomy resulted in an increase in cytochrome P450IIIA2 (PCN-2) and testosterone 6β-hydroxylase activity, while high levels of growth hormone, administered either by intermittent injection or infusion, decreased levels of this enzyme in adult male rats (Kato et al, 1986; Waxman et al, 1985; Waxman et al, 1988a). These data suggest that the developmental increase in cytochrome P450IIIA2 (PCN-2) expression is the result of continuously high levels of growth hormone as are found in adult females. Expression of the enzyme in immature males and females could be the result of low levels of growth hormone secretion. It was also suggested that suppression of cytochrome P450IIIA2 (PCN-2) expression in females could be the result of decreased circulating corticosteroid (Waxman, 1988).

1.5(b)(iv) Developmental regulation of cytochrome P450 induction.

The induction of proteins of the P450I and P450IIB gene families is also regulated during development. TCDD induces expression of the IA1 gene as early as 10 days of gestational age in the mouse (Tuteja et al, 1985).

Growth hormone has also been shown to have a role in regulation of rat IIE1 (Williams & Simonet, 1988). Hypophysectomy of male and female rats resulted in a 5-fold and 10-fold, respectively, increase in IIE1 content. This increase was partially repressed when these rats were treated with growth hormone. This is another example of suppression of cytochrome P450 expression by growth hormone that needs to be further examined at the gene level.

Hepatic induction of the rat IIB1 and IIB2 mRNAs by phenobarbital is first apparent 2 days prior to birth (day 22 of gestation) and the ratio of inducible levels of these mRNAs varied as a function of age (Giachelli & Omiecinski, 1987). Induction of other cytochrome P450 genes, such as the steroid and clofibrate-inducible genes, has not been investigated in the developing rat. In general, most cytochromes P450 are not expressed or induced prior to birth. The cellular mechanisms controlling inducible, prenatal cytochrome P450 expression are currently unknown.

1.5(c) Posttranscriptional regulation of cytochrome P450.

Cytochrome P450IA2 is regulated to a large extent posttranscriptionally (Pasco et al, 1988). After comparing transcription rates and mRNA levels, it was concluded that

IA2 mRNA is markedly stabilised in liver and extrahepatic tissue by TCDD administration. IA1, however, is regulated primarily at the transcriptional level.

When rats are treated with dexamethasone, mRNAs for IIB1/IIB2 and the NADPH-cytochrome P450 reductase are specifically stabilised in the absence of transcription (Simmons et al, 1987). However, the cytochrome P450IIIA1 (PCN-1) gene is transcriptionally activated and induction of cytochrome P450IIIA1 (PCN-1) protein is directly proportional to mRNA levels after steroid administration in rats or primary hepatocytes (Watkins et al, 1986). The antibiotic, triacetyloleandomycin (TAO), caused an increase in the levels of a cytochrome P450 that is indistinguishable from the dexamethasone-induced enzyme cytochrome P450IIIA1 (PCN-1); however, this increase occurred in the absence of an increase in mRNA in rat hepatocytes cultured in vitro. In contrast, dexamethasone causes an increase in cytochrome P450IIIA1 and P450IIIA2 (PCN-1; PCN-2) mRNA in these cultures. Both agents increase the mRNA(s) in intact animals. It has been suggested that TAO and dexamethasone have different mechanisms of action, possibly with TAO stabilising mRNA and dexamethasone increase transcription.

Induction of a cytochrome P450III protein occurs in the rat as well as in primary cultures of hepatocytes (Watkins et al, 1986). Protein radiolabelling experiments confirmed that TAO induction of cytochrome P450p (cytochrome P450IIIA1/P450IIIA2) is due in part to a decrease in cytochrome P450 degradation, while the degradation rates of other cytochromes P450 was not affected by TAO. It has been

postulated that this effect results from binding of TAO or its metabolite to the cytochrome P450III protein. It has been found that TAO binds to the enzyme in vivo and this binding disrupts catalytic activity and inactivates the enzyme, suggesting that the long-held generalization that inactive proteins are more rapidly degraded may not apply to cytochrome P450III (Watkins et al, 1986)

One of the most profound examples of posttranscriptional regulation is seen with the IIE1 enzyme. This cytochrome P450 is induced under a variety of conditions, including ethanol and acetone treatment, fasting and streptozotocin- or alloxan- induced diabetes. Administration of small compounds such as acetone and ethanol to rats causes a rapid induction of IIE1 protein without affecting levels of IIE1 mRNA (Song et al, 1986). This suggests a posttranscriptional regulation of IIE1 at either the translational level or by stabilization of the protein against degradation. It has been implied that the binding of inducers to IIE1 results in a protection of the enzyme against degradation (Eliasson et al, 1988).

1.6 Steroid metabolism.

Endogenous and exogenous steroids are metabolised by complex enzyme systems which are both cytochrome P450-dependent and -independent. In this study, we employed the well characterised steroid substrate, 4-androstene-3,17-dione, and the metabolic routes of this steroid are illustrated in figure 2. Cytochrome P450- dependent monooxygenases hydroxylate 4-androstene-3,17-dione

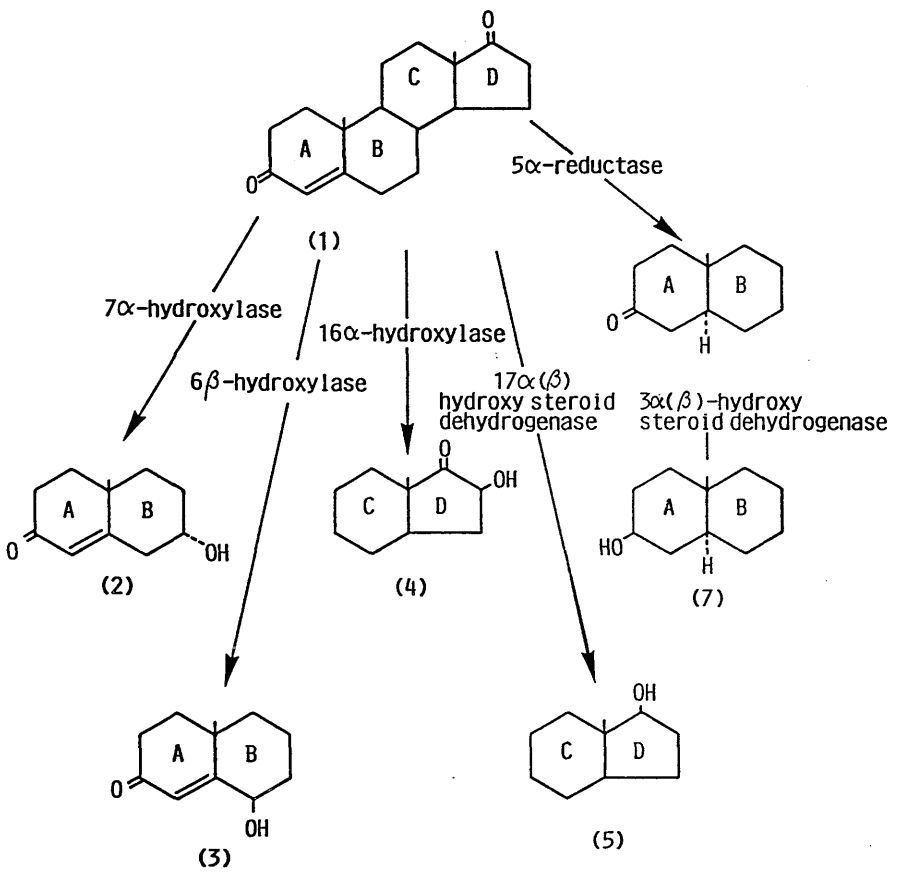


Figure 2:
Metabolic pathway of 4-Androstene-3,17-dione (1).
Shown are; 4-Androstene-7α-OL-3,17-dione (2);
4-Androstene-6β-OL-3,17-dione (3); 4-Androstene-16α-OL-3,17-dione (4);
Testosterone (5); 5α-Androstene-3,17-dione (6); and
5α-Androstene-3α(β)-OL-17one (7).

predominantly at the 7α-, 6β- and 16α-positions.

The male specific cytochrome P450IIC11 (see table 1) has been shown to catalyse 90% of the metabolism of 4-androstene-3,17-dione at the 16α-position (Waxman, 1984) and the same isozyme also hydroxylates testosterone at the 16α-position (Morgan et al, 1985).

Up to 80% of 4-androstene-3,17-dione hydroxylation at the 6β-position is thought to be catalysed by cytochrome P4502a (Waxman et al, 1985), although direct assessment of the activity of the purified enzyme has not yet been determined.

Cytochrome P450IIA1 is responsible for 7α-hydroxylation of 4-androstene-3,17-dione (table 1) (Waxman et al, 1987) and it therefore appears that in the uninduced liver, 4-androstene-3,17-dione metabolism is catalysed by three separate and selective cytochrome P450-dependent isozymes.

5α-Reductase is another 4-androstene-3,17-dione metabolising enzyme and, although it is a membrane-bound microsomal enzyme, it does not contain cytochrome P450 as an integral component. It has been shown, however, that 5α-reductase exists as part of a multicomponent system which possesses many similarities to the cytochrome P450 monooxygenase system. The electron transport chain is composed of three enzymes; steroid 5α-reductase, coenzyme Q_{10} and NADPH-cytochrome oxidoreductase. In this system, coenzyme Q_{10} is a cytochrome and electrons are passed from NADPH to coenzyme Q_{10} by NADPH-cytochrome oxidoreductase and thence from coenzyme Q_{10} to the steroid substrate by 5α-reductase, where they participate in the reduction of the

double bond (figure 2) (Golf & Graef, 1978).

The final enzymes involved in the metabolism of 4-androstene-3,17-dione are 17α/β and 3α/β hydroxysteroid dehydrogenase, both of which are microsomal, although details of their mode of action remain to be fully elucidated.

1.7 Hormonal regulation of hepatic metabolism.

Hepatic metabolism of both drugs and steroids can be performed by the same types of enzymes (Kuntzman et al, 1964) and it has been found that the activities of various hepatic enzymes are not constant, but they can be regulated by the endocrine system and also by hormones known to have *in vivo* effects. Three main types of hormones are known to exert effects upon hepatic metabolising enzymes and each type will be briefly discussed below. Most reports regarding hormonal control of hepatic metabolism concentrate on sexually differentiated enzymes and metabolic control, and this section will also consider the control of hepatic metabolism in this context.

1.7(a) Androgens.

In 1958, Yates and his coworkers showed that castration of male rats caused an increase in the activity of the steroid metabolising enzyme 5α-reductase to levels of activity normally associated with a female profile of metabolising activity. Upon treatment of the castrates with testosterone, the effect upon 5α-reductase was reversed. This implied that the activity of 5α-reductase in male rats

was subject to androgenic control. The controlling influence of androgens upon drug metabolism has been reported for a variety of substrates, including aniline (Quinn et al, 1958), hexobarbitone (Quinn et al, 1958) and ethylmorphine (Castro & Gillette, 1967; Davies et al, 1968). Androgenic control of phase II metabolism reactions has also been reported, as reviewed by Skett (1987).

More extensive study, predominantly by Gustafsson and his group, determined that androgens appear to work through a complex pathway divided into two levels. A comprehensive review of this work has been published by Skett & Gustafsson (1979) and Gustafsson et al (1980). The main findings of this work, were that androgens can imprint the brain of a rat in the neonatal period (such that neonatally castrated male rats show a completely female pattern of metabolism) and secondly, androgens can maintain the activity of some enzymes in the adult period by an action via the pituitary gland. Imprinting appears to affect both enzyme activities and the ability of enzymes to respond to androgens in the adult period. The concept of imprinting with regards to regulating the expression of cytochrome P450 has been previously considered (section 1.5(B)2). Most of the effects of androgens upon enzyme activity are a consequence of imprinting and it appears that androgens have no direct effect on the liver to control drug and steroid metabolism.

1.7(b) Pituitary hormones.

It is widely accepted that the hypothalamo-pituitary axis is the site of control for hepatic drug and steroid metabolism and much work has been done to identify the factors from the pituitary which are involved in this control. Many pituitary hormones have been purified and tested on the liver for their effect on drug and steroid metabolism, including FSH (Gustafsson & Stenberg, 1975), LH (Gustafsson & Stenberg, 1975), prolactin (Colby et al, 1974), growth hormone (Colby et al, 1974; Mode et al, 1981) and ACTH (Colby et al, 1974). All of these hormones were found to have some effect upon the liver, but none of them was obviously the so-called "feminising factor", which had been proposed by Gustafsson and his workers (Gustafsson et al, 1975; Skett, 1978). A review of the attempts to define, isolate and characterise the "feminising factor" has been published (Gustafsson et al, 1980).

It was subsequently found that the "feminising factor" appears to be growth hormone (GH) (Mode et al, 1981 ; Skett & Young, 1982). It was shown that GH, upon infusion into male rats, causes a feminisation in the metabolism of both drugs and steroids (Mode et al, 1981; Skett & Young, 1982). The lack of effect with GH in early experiments was found to be due to the fact that in both male and female rats, mean serum concentrations of GH are very similar. However, it became apparent that GH is secreted differently in males and female and this difference in the pattern of secretion of GH appeared to be important for the maintenance of sex differences in the liver (Mode et al, 1981; Skett & Young,

1982). In male rats, GH is secreted in a pulsatile manner, with large peaks of secretion at 3-4 hour intervals, and in the intervening time period, levels of GH are virtually undetectable. The female rat, in comparison, exhibits a more sustained and constant serum level of GH (Terry et al, 1977; Edén, 1979).

Mode and coworkers (1982) investigated further the role of the secretory pattern of GH and they discovered that treatments which caused a feminisation of hepatic steroid metabolism also altered the secretory pattern of GH to a female type, that is from pulsatile to constant levels. Upon injection of estradiol valerate into male rats, there was a decrease in the 16α-hydroxylation and an increase in the 5α-reduction of the steroid substrate 4-androstene-3,17-dione, an effect characteristic of a feminisation of steroid metabolism. Administration of estradiol valerate was also found to elevate the baseline secretion of GH, and depress the peak height of GH secretion, resulting in a more constant level of GH secretion. Castration of male rats produced the same effect as administration of estradiol valerate.

Somatostatin, the hypothalamic factor which inhibits the release of GH, was also investigated in the control of hepatic steroid metabolism. Norstedt et al (1983) showed that lesioning the periventricular area of the hypothalamus caused a decrease in the levels of somatostatin in the brain and a decrease in 6β- and 16α-hydroxylation in hepatic microsomes (a feminisation). Addition of anti-somatostatin antibodies was found to mimic the effect of lesioning,

implying that the effects on somatostatin and hepatic steroid metabolism are linked. The current idea as to how such an interaction occurs, is that the male pattern of hepatic drug and steroid metabolism is maintained by somatostatin from the hypothalamus, depressing the basal secretion of GH, and that the removal of this factor leads to a more female-type (constant) GH secretory pattern and a subsequent feminisation of hepatic metabolism. Recently, the role of GH in androgenic imprinting of steroid metabolism was investigated by Jansson and workers (1985b). They showed that upon neonatal castration of male rats, there was an appearance of female type GH secretion pattern, together with a feminisation of steroid metabolism, as described above. Neonatal androgen treatment reversed this effect, suggesting that neonatal androgen programs the brain for male type, pulsatile secretion of GH and, thus produces the male pattern of steroid metabolism in the adult period.

The control of sex differences in hepatic drug and steroid metabolism is thought to be as follows: androgens control the liver by their action on the secretory pattern of GH, both by a direct control in the adult period and as a consequence of androgenic imprinting in neonatal life. It is the sex difference in the secretory pattern of GH which appears to influence the rate at which, and the route by which, drugs and steroids are metabolised in the liver.

This "somatogenic" control of hepatic function appears to have some conflicting evidence. Monosodium glutamate (MSG) has been found to affect GH secretion, and drug and steroid metabolism. Administration of MSG to neonates

destroys the arcuate nucleus in the brain and causes an impairment of GH pulsatile secretion (Terry et al, 1981). MSG administered in this way was found, however, to have no effect upon the metabolism of aniline, hexobarbitone or 4-nitrophenol (glucuronidation) (Shapiro et al, 1986), although a marked depression of GH secretion was observed. Such evidence questions the role of GH as the sole hormonal controller of hepatic drug and steroid metabolism.

1.7(c) Pancreatic hormones.

The discrepancies in the "somatogenic" theory of control mentioned above, can possibly be explained by the role of the pancreatic hormones in controlling hepatic function. It is well known that chemically induced or spontaneous diabetes mellitus can markedly affect drug metabolism in the rat liver (Kato & Gillette, 1965; Kato et al, 1970). The effects of diabetes are sex dependent in the rat with male specific enzyme activities being inhibited and some female enzyme activities being enhanced (Kato & Gillette, 1965; Kato et al, 1970; Reinke et al, 1978).

It was proposed (Kato & Gillette, 1965; Reinke et al, 1978) that the effects of diabetes are related to decreases in serum testosterone levels and, indeed, testosterone synthesis and release has been found to be inhibited in rats (Murray et al, 1981). Insulin was thought to stimulate the synthesis and release of testosterone and thus indirectly maintain the male profile of hepatic steroid metabolism. Another report noticed that the effects of diabetes and castration were similar, streptozotocin treatment had little

effect in castrated male rats and androgen administration could partially reverse the effects of diabetes (Skett et al, 1984). However, the same study also reported that at the time of maximum diabetes (3 days) the levels of testosterone in serum were normal, whilst at 21 days diabetes, serum testosterone levels were markedly reduced, but hepatic drug metabolism was normal. The clear distinction between the two effects indicates that they are not linked directly, but there is some correlation between their effects upon hepatic function. It was thought that insulin and testosterone may act through a common mediator to exert their effects upon the liver and this mediator was thought to be GH. GH was proposed as the mediator based on the evidence that diabetes caused a marked change in the GH secretory pattern (Tannenbaum, 1981), and testosterone produced a similar effect (Gustafsson et al, 1983a). Although GH has been proposed as the mediator for insulin and testosterone action upon the liver, the evidence is by no means conclusive.

The current level of knowledge as to the precise control of sex differences in drug and steroid metabolism is confusing, although it does now appear that GH acts as a "feminising" and insulin as a "masculinising" hormone. In vivo experiments are difficult to interpret, as there are many complex interactions between hormones, but reports have suggested that glucagon and IGF-1 (and IGF-2) may also be involved in the control of heptatic drug and steroid metabolism (Hussin & Skett, 1987).

1.8 Hormonal transduction through second messengers.

Two major second messenger systems exist within the cell and they are (a) adenylate cyclase activation and production of cyclic adenosine 3',5'-monophosphate (cyclic AMP) and (b) turnover of inositol phospholipids and the production of the intracellular second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). As a consequence of producing these second messengers, protein kinases are activated, a process which initiates intracellular protein phosphorylation. Protein phosphorylation is thought to be responsible for the ultimate intracellular effect, mediated by these second messenger systems.

1.8(a) Cyclic AMP and cyclic AMP-dependent protein kinase.

Cyclic AMP was first identified in 1960 by Sutherland and Rall, and it was found to be produced intracellulary from adenosine 5'-triphosphate (ATP) by a process which required magnesium ions and activation of the enzyme adenylate cyclase. Adenylate cyclase is a membrane-bound enzyme and originally it was thought to be composed of two components; one was thought to be a receptor for hormones, located on the extracellular membrane surface and the other a catalytic unit, which was situated on the cytosolic side of the membrane. Rodbell and his group (1971) determined that guanosine triphosphate (GTP) was essential for activation of the catalytic subunit of the receptor and the same group showed later (Rodbell et al, 1975) that binding of hormone to its receptor enhanced this interaction.

In 1977, a GTP-binding protein was identified as a

component of adenylate cyclase (Pfeuffer, 1977). Activation of adenylate cyclase requires the interaction of three separate components: the hormone receptor on the outer surface of the cell membrane, the catalytic subunit at the inner surface of the membrane and a GTP-binding protein (N) which mediates the effect of the hormone upon the catalytic subunit of adenylate cyclase (Rodbell, 1980). The GTP-binding components exist within the cell in the resting state as a heterotrimeric complex composed of three subunits, α, β and γ. At least two types of GTP-binding protein are known to exist, one is stimulatory and is referred to as N_s and the other is inhibitory and is known as N_i

The association of stimulatory hormones with their receptors initiates GTP binding to N_s, on its α subunit, and this binding of GTP dissociates the α subunit from the rest of the binding protein complex (β and γ). The α subunit of N_s, still coupled to GTP, then interacts with and activates the catalytic subunit of adenylate cyclase (Gilman, 1984). Activation of adenylate cyclase is terminated by the hydrolysis of GTP to the inactive form guanosine diphosphate (GDP) by the action of an intrinsic GTPase (Jakobs et al, 1984). The mechanism by which N_i mediates inhibitory effects upon adenylate cyclase is less clearly understood, with conflicting reports as to the mechanism of action of N_i which need to be further elucidated before the precise mechanism of this binding protein can be explained in biochemical terms.

The intracellular actions of cyclic AMP are mediated

through the activation of cyclic AMP-dependent protein kinase (PKA), and, as well as activating PKA, an elevation of intracellular cyclic AMP also causes a concomitant activation of cyclic AMP phosphodiesterase (Corbin et al, 1985). Activation of the phosphodiesterase is a regulatory mechanism, which causes the degradation of cyclic AMP to the inactive compound, adenosine 5'-monophosphate (5'-AMP) and the cessation of the intracellular effect. Figure 3 illustrates the sequence of events in the hormonally stimulated generation of cyclic AMP and its subsequent degradation.

PKA is composed of two different subunits; a regulatory (R) subunit and a catalytic (C) subunit. PKA is activated by the dissociation of these two subunits and the subsequent release of a free C subunit (Kumon et al, 1970). PKA is not a single species but exists as a family of closely related enzymes with identical C groups but slightly differing R groups, with regards to amino acid sequences (Robinson-Steiner et al, 1984).

1.8(b) Inositol phospholipid hydrolysis.

Receptor-mediated hydrolysis of inositol phospholipids is an accepted mechanism for transducing various signals such as those from certain hormones, neurotransmitters and growth factors into the interior of the cell. In 1953, the response of the phospholipids to the stimulation of cell surface receptors was recognized by Hokin and Hokin, who first showed that, in some excretory tissues, such as the pancreas, acetylcholine induced a rapid incorporation of ^{32}P

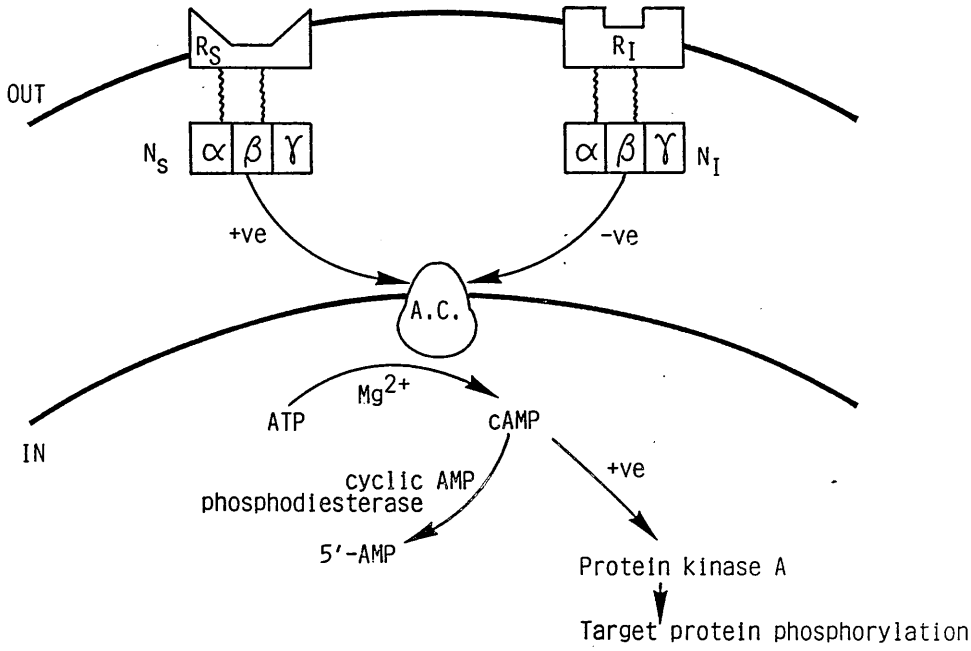


Figure 3:
Sequence of events in hormonal stimulation of cyclic AMP generation and subsequent degradation. Abbreviations: N_S, stimulatory receptor; N_I, inhibitory receptor; AC, adenylate cyclase; ATP, adenosine 5'-triphosphate; cAMP, cyclic adenosine 3', 5'-monophosphate; 5'-AMP, adenosine 5'-monophosphate.

into phosphatidylinositol and phosphatidic acid. It became evident later that this incorporation results from the enhanced breakdown and resynthesis of phospholipids occuring in many stimulated cells. Durell and workers (1969) suggested a potential role of this phospholipid response in receptor function and Michell (1975) subsequently postulated that the phospholipid breakdown may be related to calcium gating.

It was Michell (1975) who reported that hormones were capable of stimulating the breakdown and resynthesis of inositol phospholipids and this led to the concept that turnover of inositol phospholipids could serve as a pathway for signal transduction.

A modification to the theory was proposed in 1981, when Michell and his group reported that the specific hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) by hormonal stimulation was the main mediator of intracellular hormonal communication. PIP_2 can be hydrolysed by two different enzymes (Irvine, 1982): one is a phosphomonoesterase which cleaves off a phosphate from PIP_2 to yield phosphatidyl-inositol-4-monophosphate (PIP) and the other is a phospho-diesterase known as phospholipase C, which is activated by hormonal receptor occupation. Hydrolysis of PIP_2 by phospholipase C yields two products, inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG).

IP_3 is released into the cytoplasm where it functions to mobilise calcium from its intracellular stores, principally from the endoplasmic reticulum. IP_3 is sequentially degraded into inositol-1-phosphate and then

inositol, by a series of phosphatases, and the inositol is then reincorporated into the phosphatidylinositol turnover cycle.

DAG remains within the cell membrane and it functions by activating its specific kinase, protein kinase C (PKC). PKC will be dealt with in greater detail in a separate section (1.9). DAG is metabolised very rapidly into phosphatidic acid by DAG kinase and once again this product re-enters the phosphatidyl inositol biosynthesis pathway. PIP_2 is resynthesized from inositol and phosphatidic acid by consecutive phosphorylation by phosphatidylinositol kinase and phosphatidylinositol-4-phosphate (PIP) kinase (reviewed by Berridge, 1985). The sequence of events in hormonal stimulation of inositol phospholipid hydrolysis and resynthesis are shown in figure 4.

1.9 Protein kinase C and signal transduction.

Protein kinase C (PKC) is an enzyme that is activated by the receptor-mediated hydrolysis of inositol phospholipids, and relays information in the form of a variety of extracellular signals across the cell membrane to regulate many calcium-dependent processes. This enzyme also serves as the receptor for tumor-promoting phorbol esters, and because of this, PKC has attracted the attention of biologists interested in the process of signal transduction and carcinogenesis. PKC is now known to be a large family of proteins with multiple subspecies that have subtle individual characteristics. Biochemical and immunohistochemical studies suggest that PKC subspecies may

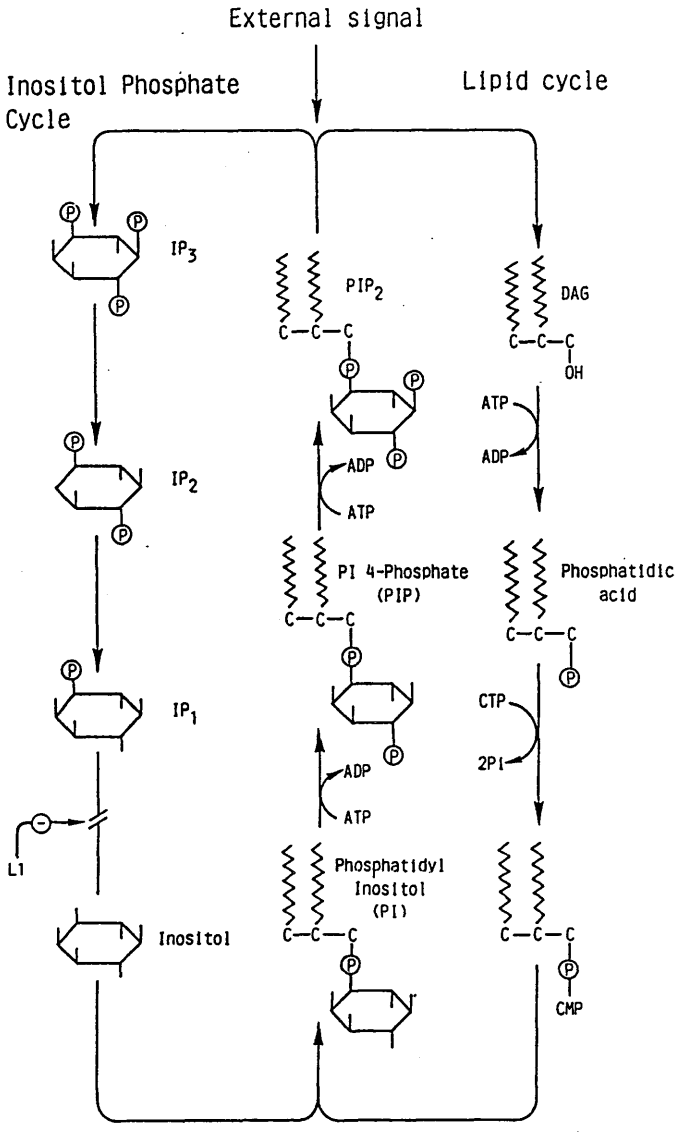


Figure 4:
Inositol phospholipid cycle showing inositol phospholipid hydrolysis and subsequent resynthesis. Abbreviations: DAG, diacylglycerol, PIP_2, phosphatidyl inositol-4,5-bisphosphate; IP_3, inositol-1,4,5-trisphosphate; IP_2, inositol-1,4-bisphosphate; IP, inositol-l-phosphate.

be differently located in particular cell types and with limited intracellular localisations.

1.9(a) Protein kinase C in signal transduction.

When PKC was first detected in 1977, as a proteolytically activated kinase present in many tissues, the enzyme had no obvious role in signal transduction (Inoue et al, 1977). Later, it was shown that PKC was a calcium-activated, phospholipid-dependent enzyme and it was firmly linked to signal transduction by the demonstration that diacylglycerol (DAG), one of the earliest products of inositol phospholipid hydrolysis, is essential for the full activation of PKC (Nishizuka, 1984a). The activation of cellular responses by this route is separate from, and synergistic to, those activated via an increase in intracellular calcium, as shown schematically in figure 5.

The pivotal role of PKC in signal transduction was first demonstrated in the release of serotonin from platelets (Kaibuchi et al, 1983). PKC has also been implicated in the release, secretion and exocytosis of cellular constituents from a variety of endocrine, exocrine and neuronal tissues, as well as in the modulation of membrane conductance and activation of other cellular function as reviewed by Nishizuka (1986). Under appropriate conditions the two routes for signal passage, PKC activation and calcium mobilization, can be opened selectively and independently by the application of a permeable DAG, 1-oleoyl-2-acetylglycerol, for the former, and a calcium ionophore, A23187, for the latter. DAG is normally

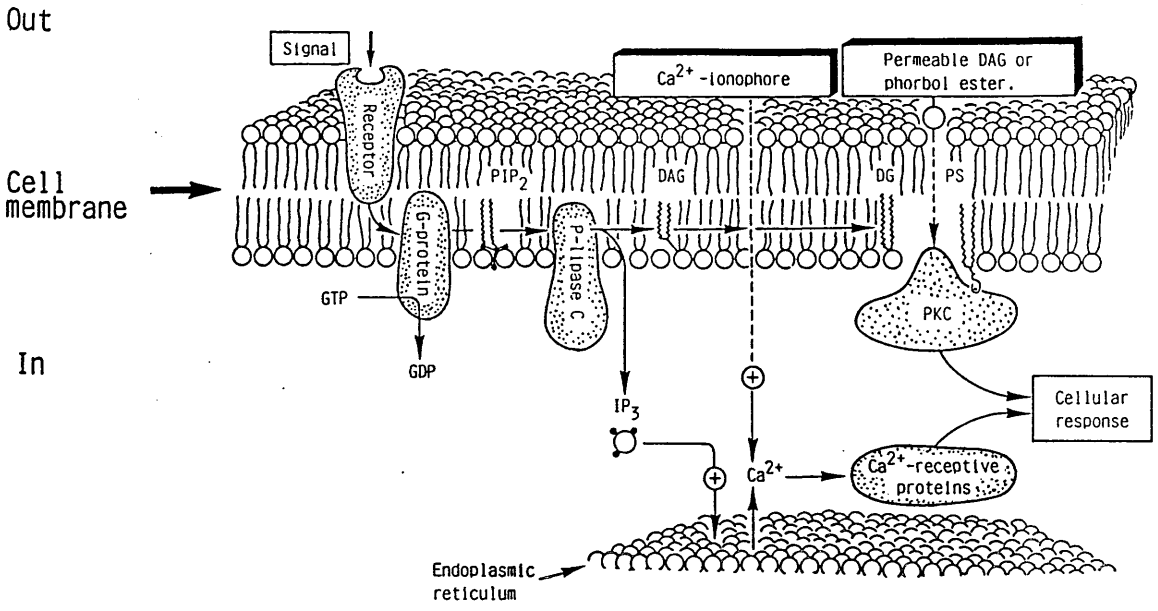


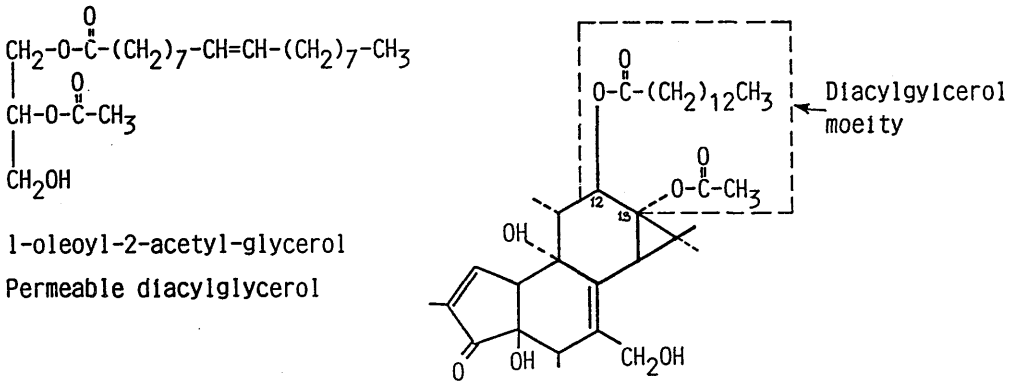
Figure 5:
Schematic representation of the bifurcating pathway of signal transduction involving protein kinase C (PKC) and calcium. Abbreviations; PIP_2, phosphatidyl inositol-4,5-bisphosphate; DAG/DG, diacylglycerol; PS, phosphatidylserine; P-Lipase C, phospholipase C; IP_3, inositol-1,4,5-trisphosphate.

insoluble and cannot be intercalated into the cell membrane. However, if the fatty acyl moiety is replaced by a short chain such as an acetyl group, then the resulting DAG possesses detergent-like properties and is easily dispersed to activate PKC without disruption of the intact cell membrane. It was also noticed that a powerful tumor-promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA), also known as 4β-phorbol-12-myristate-13-acetate (PMA), mimics the action of DAG and activates PKC directly (Castagna et al, 1982).

1.9(b) Protein kinase C as a target of tumor promoter action.

In 1941, Berenblum stated that "croton oil causes a marked augmentation of carcinogenesis when applied at weekly intervals to mice skin in conjunction with a dilute solution of benzpyrene in acetone". The principal constituent of croton oil was later identified as a phorbol ester by Hecker (1967) and Van Duvren (1969). Figure 6 shows the most powerful tumor-promoter, TPA (also called PMA), along with a cell-permeable DAG, 1-oleoyl-2-acetylglycerol (OAG) and it can be seen that tumor-promoters possess a DAG-like structure in their molecule.

In 1982, it was found that TPA could substitute for DAG and activate PKC directly both in vivo and in vitro, and it is now assumed that PKC is the intracellular receptor for tumor-promoting phorbol esters (Castagna et al, 1982; Niedel et al, 1983).



1-oleoyl-2-acetyl-glycerol

Permeable diacylglycerol

12-o-tetradecanoyl phorbol-13-acetate

Phorbol Ester

Figure 6:
The chemical structure of a cell permeable diacylglycerol and a widely used tumor-promoting phorbol ester.

1.9(c) Structural heterogeneity.

Although once considered to be a single entity (Nishizuka, 1984a), recent molecular cloning and enzymological analysis has revealed the existence of multiple subspecies of PKC in a variety of mammalian tissues (Nishizuka, 1988). Initially four cDNA clones which encode for α-, βI-, βII- and γ-subspecies were found in bovine, rat and human brain, and later in human spleen cDNA libraries. The integrated nomenclature used here is the recognised nomenclature for PKC subspecies (Nishizuka, 1988).

Partial genomic analysis has indicated that βI- and βII-subspecies are derived from a single mRNA transcript by alternative splicing. Recently another group of cDNA clones, encoding at least a further three subspecies having σ, ε, and ζ sequences have been isolated from the rat brain library by using a mixturs of α-, βII- and γ-cDNA as probes under low stringency conditions (Ono et al, 1987; Ono et al, 1988a). These subspecies have a common structure closely related to, but clearly distinct from, the above four subspecies.

Figure 7 shows schematically the structures of the PKC subspecies identified to date. They are all composed of a single polypeptide chain, with the group of α-, βI-, βII- and γ-subspecies each having four conserved regions (C_1 to C_4) and five variable regions (V_1 to V_5). The βI- and βII-subspecies differ from each other only in a short range of about 50 amino acid residues at their carboxy-terminal end region V_5 and even in this area, they possess a high degree of sequence homology. Although the second group of

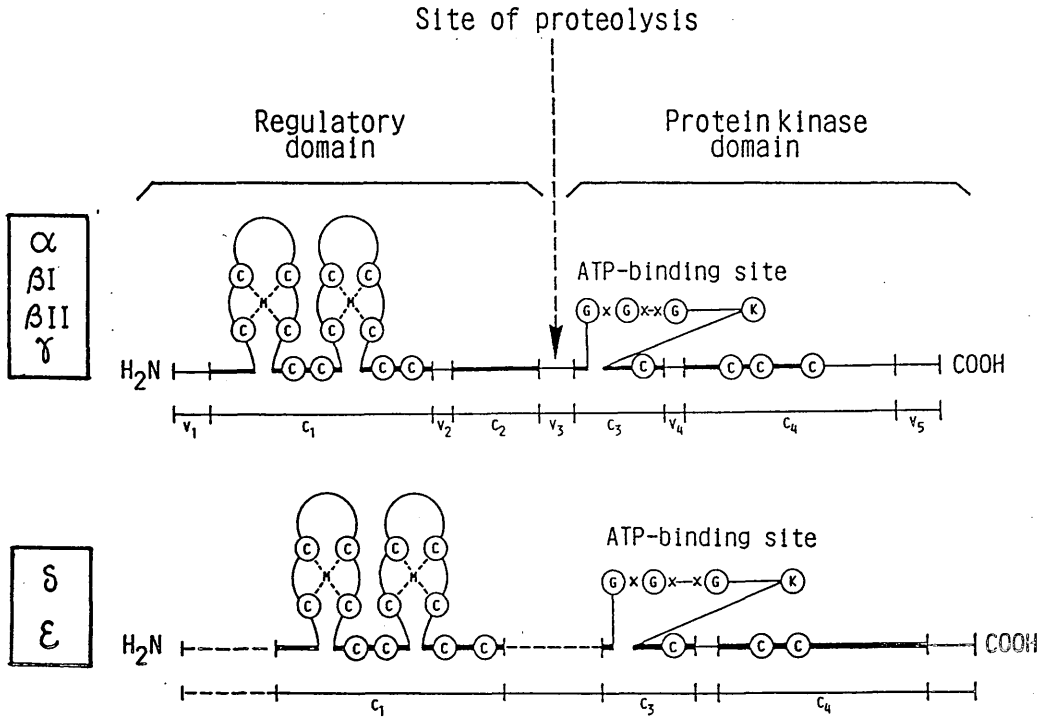


Figure 7:
Common structure of Protein Kinase C subspecies - C,G,K,X and M represent cysteine, glycine, lysine, any amino acid and metal ion respectively.

σ, ε and ζ-subspecies lack the C_2 region, the molecular mass of the enzyme molecules is, nevertheless, similar. The characteristics of these members of the PKC family are summarized in table 2.

	Amino acid Residues	Mol.Wt.	Sub-fraction	Activators	Site
α	672	76,799	type III	PS+DG+Ca^{2+} AA+Ca^{2+}	All body tissues
βI	671	76,790	type II	PS+DG+Ca^{2+}	Some tissues & cells
βII	673	76,933	type II	PS+DG+Ca^{2+}	Many tissues & cells
γ	697	78,366	type I	PS+DG+Ca^{2+} AA	Brain & spine
σ	673	77,517	?	PS+DG+(Ca^{2+})	Many tissues
ε	737	83,474	?	PS+DG+(Ca^{2+})	Brain only ?
ζ	592	67,740	?	PS+(DG+Ca^{2+})	Many tissues.

Table 2; Subspecies of protein kinase C from mammalian tissues.

The conserved region G contains a tandem repeat of a cysteine rich sequence,

$Cys\text{-}X_2\text{-}Cys\text{-}X_{13(14)}\text{-}Cys\text{-}X_2\text{-}Cys\text{-}X_7\text{-}Cys\text{-}X_7\text{-}Cys$

where X represents an amino acid. ζ-PKC contains only one set of this cysteine-rich sequence, thus having a relatively smaller mass than the other PKC species. The cysteine-rich sequence resembles the so-called "zinc-finger" that is found in many metalloproteins which are related to transcriptional regulation. There is no evidence available, however, that PKC binds to DNA directly. The conserved regions of C_1 and C_2 include the regulatory domain of PKC (Lee & Bell, 1986), but as yet the sites involved in Ca^{2+}, DAG and phospholipid binding have not been identified with any certainty. The mechanism of PKC activation is thought to be a complex interaction between all these different components, but it is known that fully activated PKC is a quaternary complex of PKC, DAG, calcium and phospholipid, located at the inner surface of the cell membrane.

The carboxy-terminal half of PKC, containing the regions C_3 and C_4 represents the actual kinase of the enzyme. The conserved region C_3 has an ATP-binding sequence, Gly-X-Gly-X-X-Gly-...-Lys^{21}. The regulatory and protein kinase domains are cleaved by limited proteolysis, catalysed by the calcium-dependent neutral protease calpain, at one or two specific sites in the variable region, V_3.

1.9(d) Individual enzymological characterization.

Characterization of each PKC subspecies is a prerequisite for determining the potential roles of this enzyme family in signal transduction. The variable regions seen in each subspecies may play key roles in governing the individual enzymological characteristics and possibly their specific localisation and function. The diversity of the sequence in these regions allows the separation of PKC into several fractions upon chromatography on hydroxyapatite columns (Huang et al, 1987). To date, three subfractions, type I, II, and III have been identified and shown to correspond to γ-, (βI & βII) and α-subspecies respectively (table 2) (Kikkawa et al, 1987). PKCs with βI and βII sequence show nearly identical kinetic and catalytic properties and can be distinguished from each other only by immunocytochemical properties (Shearman et al, 1988). The subspecies having σ-, ε- and ζ sequence expressed in COS 7 cells can be partially separated upon chromotography (Ono et al, 1988a) but their correspondence to the subfractions chromatographically obtained from tissues remains to be clarified.

The PKC subspecies which have been identified thus far exhibit subtle differences in enzymatic properties. PKC with γ(type I) and α (type III) sequences show much less activation by DAG than the mixture with the βI and βII (type II) sequence. The PKCs with the βI and βII sequence show substantial activity without added calcium in the presence of DAG and phospholipid (Sekiguchi et al, 1988). In addition to the characteristic activation of PKC by DAG, it

has also been reported that cis- and trans-unsaturated fatty acids can activate purified rat brain PKC (Seifert et al, 1988), phosphatidylethanolamine selectively activates type I subspecies in rat brain (Asaoka et al, 1988) and a variety of acyl CoAs and diol-lipids appear to be capable of potentiating activation of PKC in the presence of calcium and phosphatidylserine (Bronfmann et al, 1988; Severin et al, 1988). Different phospholipids and products of arachidonate metabolism appear to play important regulatory functions in controlling PKC activity within the intact cell. A model has recently been proposed for PKC regulation based on a dual control mechanism through which phospholipase C activation and DAG production initially activates PKC and causes a subsequent down-regulation of the kinase, whilst activation of phospholipase A_2 and the production of arachidonic acid and its metabolites causes a sustained activation of PKC (Portilla et al, 1988).

In addition to the well-defined PKC subspecies, structurally undefined enzymes have been obtained from some tissues such as heart and platelets, and these enzymes respond to phospholipid, calcium and DAG in different ways. For instance, one enzyme obtained from human platelets is not sensitive to calcium (Tsukuda et al, 1988) and the exact relation of this kinase to the different subspecies already identified remains unclear.

1.9(e) Limited proteolysis of protein kinase C.

PKC was originally detected as an undefined protein kinase which was present in many tissues and could be activated by limited proteolysis with calpain (Inoue et al, 1977). This hydrolytic cleavage occurs at one or two specific sites in the region V_3, resulting in the release of a catalytically fully active fragment, which subsequently is rapidly removed from the cell (Kishimoto et al, 1989). Calpain I, which is active in the micromolar range of calcium, cleaves PKC preferentially in the presence of phosphatidylserine and DAG or TPA, suggesting that the activated form of PKC is a target for calpain action (Kishimoto et al, 1983). Quantitative analysis shows that calpain has differential activity on the various PKC subspecies; the γ-subspecies (type I) is very susceptible, whereas the α-subspecies (type III) is relatively resistent to proteolysis (Kishimoto et al, 1989).

Although the limited proteolysis of PKC may generate a catalytically fully active fragment, previously called PKM (Kishimoto et al, 1983; Inoue et al, 1977), the physiological significance of this proteolysis has not been unequivocally established. Two alternative possibilities may be considered:

Firstly, this proteolysis may be a process to activate PKC and the resulting protein kinase fragment may play some role in the control of cellular function (Melloni et al, 1986). It is also possible that the regulatory fragment has some role in the control of gene expression, as it contains a DNA-binding motif.

Secondly, in contrast, the limited proteolysis may be a process by which degradation of PKC is initiated, eventually depleting PKC from the cell. Recent reports from different workers (Ballester & Rosen 1985; Ase et al, 1988a) have shown that, in a variety of tissues and cell types, PMA, which induces persistent activation of PKC due to its stable properties, elicits translocation of the enzyme from the soluble fraction to the membrane and its subsequent degradation, termed down-regulation. In fact the catalytically active fragment, PKM, is not always recovered from the cell and is probably degraded further by the action of proteases. It has recently been shown that upon treatment with PMA, some PKC subspecies co-expressed in a single cell such as KM3 or in the HL-60 cell line, disappear at different rates, which may reflect the selectivity of calpain action described above (Ase et al, 1988a). It is more plausible that the limited proteolysis of the PKC molecule by calpain, particulary calpain I, is directly related to the degradation of PKC and that, when activated persistently, various subspecies of PKC are depleted from the cell at different rates due to the differential rate of proteolysis at their variable region, V_3.

1.10 Protein phosphorylation and intracellular activity.

Activation of protein kinases with their respective agonists causes the phosphorylation of intracellular proteins, by employing the terminal phosphate of ATP in the presence of magnesium. Phosphorylation by kinases typically involves seryl & threonyl residues and once phosphorylated,

these proteins undergo a conformational change which subsequently alters the biological activity of the protein. The proteins phosphorylated by PKA have been reviewed by Cohen (1985).

PKC is known to phosphorylate an extensive range of proteins within the cell (Nishizuka, 1986) and several of these proteins are used as intracellular molecular markers to implicate PKC in a biological phenomenon. Two of these markers are an 80kD protein (Erasalimsky et al, 1988; Rodriguez-Pena et al, 1986; Malvoisin et al, 1987) and a 40kD protein (Watson et al, 1988; Kazlauskas & Cooper, 1988; Vila & Weber, 1988).

PKA and PKC frequently phosphorylate the same protein (Kitamura et al, 1988; Jahn et al, 1988) and some of these phosphorylations may be relevant in order to modulate in vivo functioning of these proteins. In 1978, Garrison reported that cyclic AMP analogues increased the phosphorylation state of 12 cytosolic proteins and that 10 of the same proteins were also susceptible to phosphorylation by hormones which could initiate inositol phospholipid hydrolysis. In a later study (Garrison et al, 1984) it was reported that 7 of the 10 proteins which could be stimulated by inositol phospholipid hydrolysis could also be phosphorylated by the addition of the calcium ionophore A23187, whilst the remaining 3 proteins were a target for phosphorylation in the presence of PMA, suggesting an involvement of PKC.

It has been shown that the amino acid sequence in the immediate vicinity of a phosphorylation site is a primary

factor in determining substrate recognition, as reviewed by Krebs and Beavo in their classic article on the regulation of enzyme activity (1979). It is thought that phosphorylation of different proteins or of differing residues on the same protein may be attributable to differential recognition by each kinase.

1.11 Dual action for cellular regulation through protein kinase C transduction.

Several physiological functions have been assigned to PKC, including an involvement in secretion, exocytosis, modulation of ion conductance, interaction and down-regulation of receptors, smooth muscle contraction, gene expression and cell proliferation, as reviewed by Nishizuka (1986). It is attractive to speculate that the different members of the PKC family may each have different roles in the control of cellular function, although little is known at present to allow a discussion of such an action of the PKC subspecies in biochemical terms.

It is now recognised that synergistic interaction between PKC and calcium pathways, as described by Nishizuka (1984a), underlies a variety of cellular responses to external stimuli. In the calcium pathway, inositol-1,4,5-trisphosphate (IP_3) is shown to mobilize calcium from its internal store (Berridge & Irvine, 1984). A large body of evidence has accumulated to indicate that PKC has a dual action, providing positive forward, as well as negative feedback control over various steps of cell signalling processes (Nishizuka, 1986). In short-term responses, a

major role of PKC appears to lie in decreasing calcium concentrations in a manner shown in figure 8. Persistent and high elevations of calcium concentrations within the cell can have deleterious effects and ultimately lead to cell death. The appearance of physiological second messengers, such as DAG, is very rapid and transient in comparison with the more sustained elevation in calcium levels. In biological systems, a positive signal is normally followed by an immediate negative feedback signal. A number of reports have suggested that, in various cell types, PKC has a function to activate the calcium-transport ATPase and Na^{+}/Ca^{2+} exchange protein, both of which remove calcium from the cell cytosol (Nishizuka, 1986). The recent observations that the phorbol ester, PMA, inhibits the frequency of repetitive calcium transients recorded from single cells could be explained by such an inhibitory action of PKC. Although it sounds paradoxical, such a negative feedback role of PKC is not confined to short-term responses, but may be extended to long-term responses such as cell growth and proliferation.

1.12 Interaction between inositol phospholipid hydrolysis and cyclic AMP generating pathways.

It has been reported that elevation of intracellular cyclic AMP can produce an increase in intracellular calcium levels by enhancing calcium influx (Staddon and Hansford, 1986) and this effect appears to be mediated by the phosphorylation of a protein which is situated on or near the calcium gating channels (Poggioli et al, 1986). It is

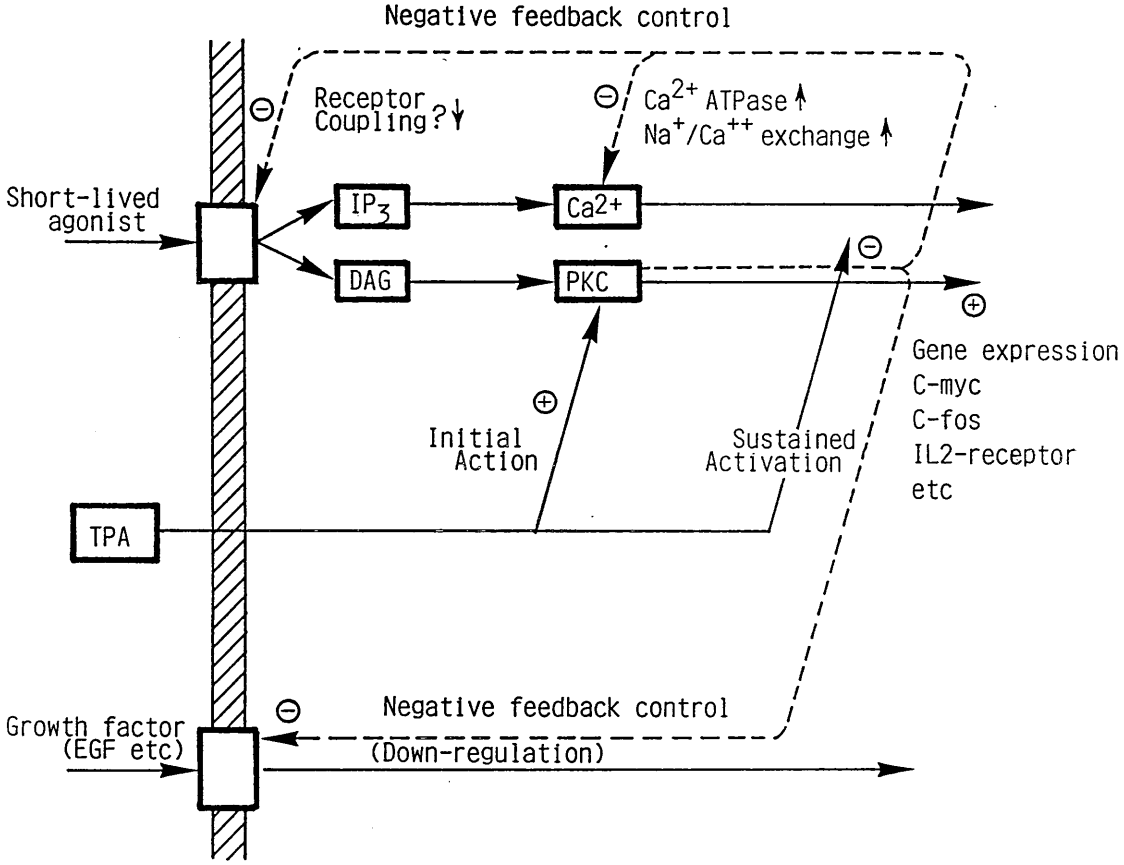


Figure 8:
A model for the dual action of protein kinase C and of tumor-promoting phorbol ester. The scheme emphasizes the negative-feedback role of protein kinase C in cellular regulation.
IP3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; PKC, protein kinase C; TPA, 12-0-tetradecanoyl-13-acetate. (Adapted from Nishizuka, 1988).

now recognised that PKA and PKC can affect each others activity in a variety of cellular functions. For instance PKC appears to be able to directly activate adenylate cyclase in order to promote cyclic AMP accumulation in response to hormonal (glucagon) stimulation (Thams et al, 1988; Ho et al, 1988a). The precise method by which PKC can activate adenylate cyclase is still unclear, but the effect may be due to PKC enhancing the heterotrimeric $\alpha\beta\gamma$ form of N_i resulting from a decreased inhibition of basal adenylate cyclase activity (Choi & Toscano, 1988). It has also been reported that phorbol ester activation of PKC can inhibit the activation of adenylate cyclase (Heyworth et al, 1985) and the hormonal (glucagon) stimulation of cyclic AMP accumulation (Gárciá-Sáinz et al, 1985).

Although interactions between PKA and PKC signal transduction systems are known to exist, the precise physiological relevance and the mechanism of these interactions remain to be fully elucidated.

1.13 Intracellular transduction of hormonal signals through protein kinase C.

A variety of hormones are known to exert some of their intracellular actions through inositol phospholipid hydrolysis and a production of IP_3 and DAG, resulting in an elevated intracellular level of calcium and activation of PKC, respectively. Some hormones appear more selective for one limb of this pathway than the other, for instance some

hormones act to selectively increase the level of calcium, with only a minor effect upon activation of PKC.

1.13(a) Vasopressin.

The hormone vasopresin is known to bind to its membrane-bound receptors in order to simulate inositol phospholipid hydrolysis (Blackmore et al, 1986a; Cabot et al, 1988a). Vasopressin promotes the production of both DAG and IP_3 and can consequently have effects mediated through both activation of PKC and elevation of intracellular calcium (Blackmore et al, 1986a). There is a strong body of evidence implicating PKC as the major effector of vasopressin's intracellular action, particularly in isolated rat hepatocytes.

It has been reported that vasopressin can inhibit α_1-mediated effects in isolated rat hepatocytes (Gárciá-Sáinz et al, 1986) and in producing this effect, vasopressin phosphorylates intracellular proteins in a manner which was consistent with activation of PKC. Gárciá-Sáinz and Hernandez-Sotomayor later reported (1987) that vasopressin was able to inhibit the stimulation of ureogenesis in isolated rat hepatocytes, again through the activation of PKC.

Other hepatic effects of vasopressin involving activation of PKC include inactivation of glycogen synthetase (Blackmore et al, 1986a), anti-ketogenic effects (Nomura et al, 1987) and regulation of hepatic phospholipid biosynthesis, particularly phosphatidylethanolamine and phosphatidylcholine (Tijburg et al, 1987b).

Extrahepatic effects of vasopressin have also been reported to involve PKC including the hydrolysis of glycerophospholipids in a REF 52 rat embryo cell line (Cabot et al, 1988a) and contraction of mesangial cells (Troyer et al, 1988).

It appears, therefore, that activation of PKC as a consequence of vasopressin-stimulated inositol phospholipid hydrolysis is responsible for mediating the majority of the hepatic effects of this hormone, with minor extrahepatic effects.

1.13(b) Angiotensin II.

Angiotensin II is a hormone known to cause profound elevations in intracellular levels of calcium and a consequence of these high calcium levels is that PKC can become activated (Kishimoto et al, 1983). Again, hepatic effects of angiotensin II have been reported; angiotensin II is able to cause protein phosphorylation in a pattern consistent with activation of PKC, in an identical manner to vasopressin (Gárciá-Sáinz et al, 1986). Angiotensin II also inhibits ureogenesis in isolated rat hepatocytes, although the involvement of PKC in this effect is controversial (Gárciá-Sáinz & Hernandez-Sotomayor, 1987).

An extrahepatic effect of angiotensin II has been reported by McAllister and Hornsby (1988) who observed that the hormone could inhibit forskolin-induced increases in the activity of steroid biosynthetic enzymes in adrenocortical cells, apparently through activation of PKC.

Angiotensin II appears to be able to affect both PKA

and PKC in a complex, and poorly understood, manner. The effect seems to be regulated by different G proteins (Bouscarel et al, 1988b) and a complex and highly integrated regulatory system appears to be at work. Hence, although angiotensin II has been reported to act via activation of PKC, studies investigating such an effect of this hormone require careful interpretation because of the interaction between PKA and PKC transduction pathways.

1.13(c) Insulin.

An unexpected hormonal activator of PKC was found to be insulin. Insulin is well known to exert intracellular effects through ribosomal S6 kinase, protease-activated kinase and a cytosolic serine kinase (Blackshear et al, 1987b; Yu et al, 1987), but insulin was also found to be capable of activating PKC (Gomez et al, 1988).

The involvement of PKC in insulin action is controversial, some reports implicating an action through this kinase and others refuting such an action. The controversy arose because insulin does not activate PKC directly, being unable to initiate inositol phospholipid hydrolysis. Activation of PKC by insulin appears to be an indirect effect, with insulin acting to enhance PIP_2 hydrolysis which has been stimulated by α_1 agonists, as a consequence of stimulating de novo synthesis of PI and PIP (Pennington & Martin, 1985).

A physiological role for insulin activating PKC is being intensively studied at present, and hopefully some of the controversy about insulin's actions through this kinase

will soon be resolved.

1.14 A role for protein kinase C in hepatic monooxygenase regulation?

Although PKC has been implicated in a variety of metabolic effects, such as lipogenesis and glucose transport in adipocytes (Allard et al, 1987) and also in hepatocytes (Bijleveld et al, 1988; Chowdhury & Agius, 1987), there is a lack of conclusive evidence implicating PKC in the regulation of hepatic monooxygenases. There is no evidence involving PKC in either the induction or degradation of cytochrome P450.

One report from Pyerin and his colleagues (1987), implied that both PKA and PKC could phosphorylate specific cytochrome P450 isozymes in both rats and rabbits. Some of these isozymes appeared to be more susceptible than others to phosphorylation, and also the kinases seemed to have different selectivities towards the isozymes. This report implied that *in vitro* phosphorylation of cytochrome P450 isozymes was not solely dependent upon one kinase.

Hormones have been reported to be capable of inhibiting hepatic monooxygenases by increasing intracellular levels of cyclic AMP (Berry & Skett, 1988a). Elevation of cyclic AMP levels either directly or indirectly produced the same inhibitory effect upon the metabolism of the steroid substrate, 4-androstene-3,17-dione, and the effect was both time- and concentration-dependent. A role for cyclic AMP in the regulation of monooxygenase activity has been provided by Weiner and his group (1972), where it was reported that

in vivo administration of the cyclic AMP analogue, dibutyrl cyclic AMP, inhibited the metabolism of hexobarbitone. In vivo administration of dibutyrl cyclic AMP has also been reported to inhibit the phenobarbitone-mediated induction of cytochrome P450 (Hutterer et al, 1975). Induction of cytochrome P450 in vivo by 3-methylcholantherene and phenobarbitone (Byus et al, 1976) has also been reported to be accompanied by an increase in intracellular cyclic AMP and activation of PKA. An analagous effect for PKC has not been reported.

1.15 Aims of the present project.

(a) To assess the effects of directly activating protein kinase C upon the hepatic metabolism of the steroid substrate, 4-androstene-3,17-dione, using a variety of agents (tumor-promoting phorbol esters, vasopressin, synthetic diacylglycerol, angiotensin II).

(b) To observe protein kinase C translocation in the presence of tumor-promoting phorbol esters.

(c) To determine the role of the NADPH- cytochrome P450 enzyme system in any protein kinase C-dependent effect which we observed.

(d) To elucidate the role of protein kinase C upon hepatic steroid metabolism by using protein kinase inhibitors.

(e) To elucidate the role of protein synthesis in the actions of protein kinase C upon hepatic steroid metabolism.

(f) To determine if the effects of protein kinase C upon the metabolism of 4-androstene-3,17-dione were due to changes in the phosophorylation state of cytochrome P450 isozymes.

(g) To determine if the effects of protein kinase C upon the metabolism of 4-androstene-3,17-dione were due to changes in the phosphorylation state of specific protein kinase C protein substrates.

It was hoped, therefore, that this study would determine whether or not protein kinase C was involved in the hormonal control of hepatic steroid metabolising enzymes, some of which are monooxygenases, and to try to determine the underlying mechanism of any effect which we observed. It was hoped that this study would provide an insight into the physiological and pharmacological roles of activation of protein kinase C in the regulation of the hepatic biotransformation system.

MATERIALS AND METHODS

MATERIALS AND METHODS.

Methods can be separated into three main sections:

(1) Assessment of protein kinase C activation, directly and indirectly, and subsequent effects on 4-Androstene-3,17-dione metabolism.

(2) Assessment of the NADPH/cytochrome P450 enzyme system.

(3) Assessment of Phorbol-12-myristate-13-acetate effects upon protein phosphorylation.

The methods used in each of these three sections is summarised below.

2.1 ANIMALS.

Mature Wistar rats, bred in the department and weighing 250-300g, were used throughout the study.Males were used in each case, apart from section 2.5 in which female rats were used. The animals were kept in light and temperature controlled conditions (lights on 0700-1900; 19 ± 1°C) and allowed free access to food (CRM Nuts, Labsure,Croydon) and tap water.

2.2 HEPATOCYTE ISOLATION.

Rats were anaesthetised prior to hepatocyte isolation, using halothane (3.5%) in oxygen/nitrous oxide (0.4L/0.8L per minute). The method used was the two-stage collagenase perfusion technique of Seglen (1973). Prior to hepatocyte

isolation approximately 500ml of calcium-free Hanks B.S.S. (section 2.24(a)) was gassed for 30 minutes with 95.5%:5% O_2/CO_2 at 37°C. The temperature of the perfusate was kept at 37°C for the duration of the perfusion process, using an insulated reservoir, and the perfusate gassed continously with O_2/CO_2 as above. After induction of anaesthesia,the animal was dissected open and the hepatic portal vein isolated, cleared of fat and the vein cannulated. After cannulation, the liver was perfused in situ with calcium-free Hanks B.S.S. at a perfusion rate of flow of 80ml per minute for 1 minute to wash out blood from the liver. After puncturing the vena cava, the liver was perfused for a further 10 minutes at a flow rate of 60ml per minute to cause breakdown of the calcium-dependent bonds between the cells. After this time,the perfusate buffer was changed to a buffer containing 60mg of collagenase in 120ml Hank's B.S.S. resupplemented with 4mM of $CaCl_2$.The perfusion was continued for a further 15-20 minutes, during which time the collagenase containing buffer was recycled through the liver. When the liver had been sufficiently digested, as ascertained by visual examination of the liver which showed the actual cells visible below the liver capsule, the entire liver was removed from the animal and after careful removal of the liver capsule, the hepatocytes were dispersed into incubation medium. The hepatocyte suspension was filtered through gauze to separate connective tissue and undigested liver from the hepatocytes and the suspension then centrifuged twice at 500g for 3 minutes in a Damon-IEC DPR 6000 centrifuge to sediment intact cells. The supernatant,

containing excess cell debris, was removed and the isolated cells resuspended in Ham's F-10 culture medium, supplemented with 0.1% Bovine serum albumin and 1000 IU of penicillin/streptomycin per ml (Hussin & Skett, 1987) at a cell density of 3 x 10^7 cells per ml. This method yielded approximately 5 x 10^7 cells per gram wet weight of liver,with a cell viability of greater than 97%, assessed by trypan blue exclusion.

2.3 INCUBATION OF INTACT HEPATOCYTES WITH PROTEIN KINASE C ACTIVATING AGENTS.

Freshly isolated hepatocytes were plated onto sterile 10cm diameter culture dishes (Nunclon, Denmark) at a cell density of 10^7 cells per ml in Ham's F-10 supplemented with 0.1% bovine serum albumin. The dishes were then placed in an incubator set at 5% CO_2, 98% humidity and 37°C for 30 minutes to allow the cells to recover from the isolation process before the experiment commenced. Studies involving incubations of 24 hours, to allow receptor levels to recover prior to study, were maintained in the incubator as stated above. After recovery in the incubator, the cells were transferred to 25 ml conical flasks in a shaking water bath at 37°C, where they were subjected to treatment with the various protein kinase C activating agents. The agents were added to the cells in the conical flasks and left in contact with the cells for periods of time ranging between 5 and 200 minutes. The agents investigated are known to exert either all of their effect via protein kinase C or partially via protein kinase C (Castagna et al, 1982; Niedel et al, 1986;

Nishizuka, 1986). The agents studied were: (a) 4β-Phorbol-12-myristate-13-acetate (PMA; 1nM and 50nM); (b) 4α-Phorbol (4αP; 100nM); (c) 4α-Phorbol-12,13-didecanoate (4αPDD; 100nM); (d) 1,2-Dioctanoylglycerol (DOG; 5μM); (e) Vasopressin (100nM and 100μM); (f) Calcium ionpohore A23187 (100nM); (g) PMA and A23187 (50nM/100nM respectively); and (h) Angiotensin II (100nM). The phorbol esters were dissolved in the solvent dimethyl sulfoxide (DMSO) and DOG was dissolved in acetone. All other compounds were dissolved in glass distilled water. Control cells received drug vehicle alone. Following incubation with the various agents, the cells were removed from the conical flasks and centrifuged at 500g for 3 minutes in a Damon-IEC DPR 6000 centrifuge. After removal of the supernatant and washing the cells in incubation medium (section 2.24(b)) viability was assessed by trypan blue exclusion and was found to be in excess of 90% following each incubation period. The cell suspensions were centrifuged once more under the same conditions as above and finally resuspended in incubation medium for the determination of 4-androstene-3,17-dione metabolising activity.

2.4 DETERMINATION OF 4-ANDROSTENE-3,17-DIONE METABOLISM.

Metabolism of the steroid substrate 4-androstene-3,17-dione is known to occur due to the action of the various enzymes in the liver as shown if figure 2.

Gustafsson and Stenberg (1974) were responsible for elucidating the activities of the various enzymes involved in the metabolism of 4-androstene-3,17-dione and a

modification of their methods of assessing its metabolism were employed here. Approximately 10^7 hepatocytes were suspended in incubation medium (section 2.24(b)) and to the cell suspension was added 500µg (0.1µCi) of (4-^{14}C)-androstene-3,17-dione and the suspension incubated for 30 minutes at 37°C in a shaking water bath.The reaction was stopped by adding 10 ml of Folch (chloroform : methanol 2:1) to each sample, followed by 1ml, 0.9% of NaCl solution to aid extraction of the substrate and its metabolites into the organic layer. The samples were then shaken overnight. The organic layer, once removed, was evaporated to dryness under nitrogen at 45°C and the residue redissolved in 5 drops of chloroform.Samples were then applied to TLC plates (Merck F254) and developed in chloroform/ethyl acetate (4:1) to separate the metabolites and unchanged substrate. Autoradiography of the TLC plates identified the location of each of the bands shown in figure 9 and the amount of ^{14}C-label present in each band (metabolites and substrate) was evaluated by liquid scintillation counting in 5ml Ecoscint (National Diagnostics, New Jersey) in a Packard Tricarb 2000CA counter. The amount of each metabolite, expressed as pmole metabolite formed per minute per 10^6 cells was determined using the formula shown on the following page:

$$\text{pmol metabolite/min/}10^6\text{cells} = S \times \frac{Cm}{Ct} \times \frac{1}{t} \times \frac{1}{n} \times \frac{1}{MW}$$

S = amount of substrate added (µg)

Cm = d.p.m. metabolite

Ct = d.p.m. total

t = incubation time (minutes)

n = number of cells (divided by 10^6)

MW = molecular weight of substrate (280)

It is apparent from figure 9 that there are six main metabolites of androstenedione which can be separated by this method. However, in figure 2 we have shown that 3α/β oxosteroid oxidoreductase does not metabolise the parent compound, but metabolism by this enzyme occurs subsequent to metabolism by 5α-reductase. Hence, for the purpose of this study, only the activities of the enzyme metabolising the parent molecule was evaluated and hence the amount of metabolite produced by subsequent metabolism of 5α-androstane-3,17-dione (as produced by 5α reduction of androstenedione) by 3α/β oxosteroid oxidoreductase was not determined separately but the amount of metabolite produced by this enzyme was added to the amount of unchanged 5α-androstane-3,17-dione to determine the total amount of metabolite produced by the activity of 5α reductase per minute per 10^6 cells.

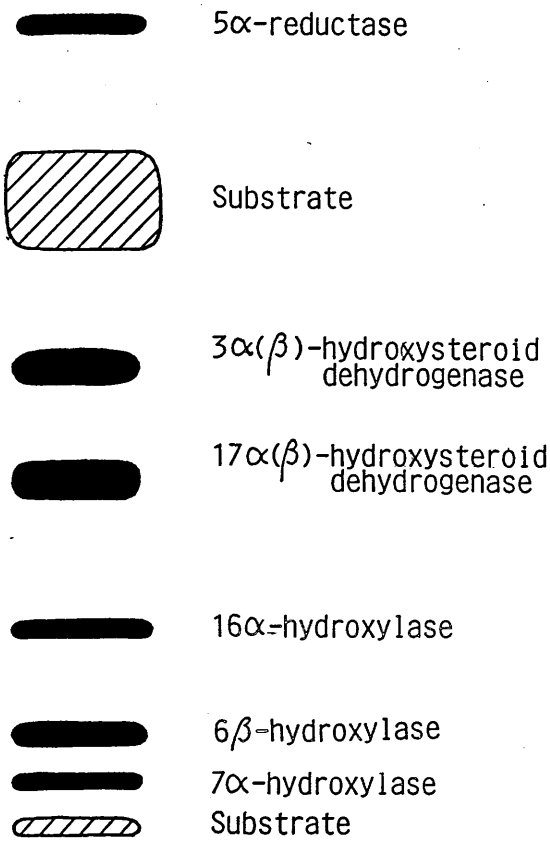


Figure 9:
Diagrammatic representation of the separation of the metabolites of 4-androstene-3,17-dione by one dimensional thin layer chromatography.

2.5 DETERMINATION OF SEX DIFFERENCES IN PHORBOL ESTER-MEDIATED ALTERATION OF 4-ANDROSTENE-3,17-DIONE METABOLISM.

To investigate if the effect of the phorbol esters upon steroid metabolism were dependent on the sex of the animal, the effects of the active phorbol ester PMA and its inactive analogues 4αP and 4αPDD upon the metabolism of 4-androstene--3,17-dione were examined in hepatocytes isolated from female Wistar rats kept under the same conditions as described in section 2.1. Hepatocytes were isolated as described in section 2.2, incubated with PMA (50nM), 4αP (100nM) or 4αPDD (100nM) for 10-120 minutes, carried out as described in section 2.3 and androstenedione metabolising activity was determined as described in section 2.4.

2.6 ELECTROPERMEABILISATION OF HEPATOCYTES.

2.6(a) THEORETICAL ASSESSMENT OF ELECTROPERMEABILISATION PARAMETERS.

Electropermeabilisation of freshly isolated hepatocytes was carried out essentially by the method of Knight and Baker (1982). By this method, a potential difference applied across the plasma membrane of the hepatocyte punctures two pores in the membrane which allows the access of small molecules to the interior of the cell. A diagrammatic representation of the hepatocyte subjected to an electrical field is shown in figure 10 and the potential difference across the membrane at any point (P) around the circumference of the cell (Vp) is given by the equation

$$Vp = CbE\cos\theta.$$

In the equation, b is the cell radius, the angle θ is

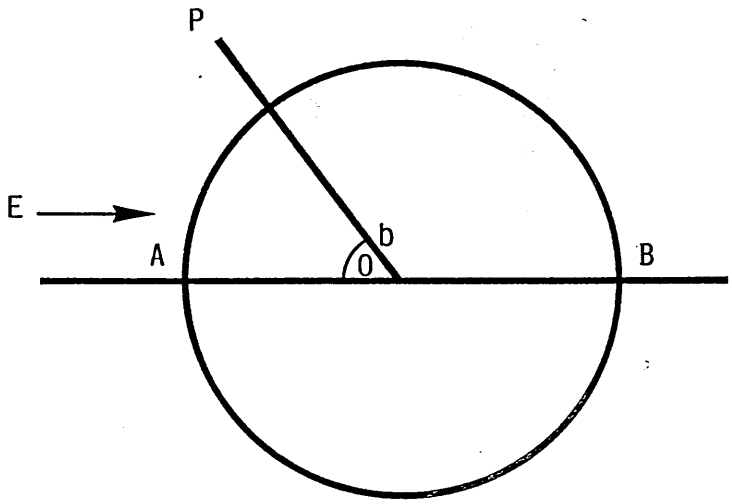


Figure 10:
Diagrammatic representation of a cell of radius b subjected to an electrical field of force E. (Adapted from Knight & Baker, 1982).

as shown in the diagram, E is the strength of the applied electric field and C is a constant dependent on the relative conductivities of the extracellular field, the cytosol and the membrane with relation to cell size and membrane thickness. For ease, the value of C is taken to be 1.5 (Knight & Baker, 1982). By the equation, it can be seen that between points A and B, the potential difference across the membrane of the hepatocyte will be at its greatest and at this point the value of cosθ will be 1. Hence,the equation can be simplified to;

$$Vp = 1.5bE$$

It has been shown that plasma membranes can rupture at voltages in excess of 1V (Zimmerman et al, 1974) and it has been shown that hepatocytes have a radius of approximately 10μm (Knight & Baker, 1982) and so an applied field strength of 1000V/cm would yield a transmembrane potential of 1.5V across the plasma membrane. Cell mitochondria have a radius of approximately 0.5μm (Knight & Baker, 1982) and so an applied field strength of 1000V/cm would yield a transmembrane potential of 75mV. An applied field strength of 1000V/cm is theoretically sufficient to cause rupture of the cell membrane of the hepatocyte without causing damage to other cell organelles.

2.6(b) EXPERIMENTAL ASSESSMENT OF ELECTROPERMEABILISATION PARAMETERS.

The apparatus used to induce cell electropermeabilisation of the hepatocyte is shown in figure 11.

The efficiency of the apparatus to electro-

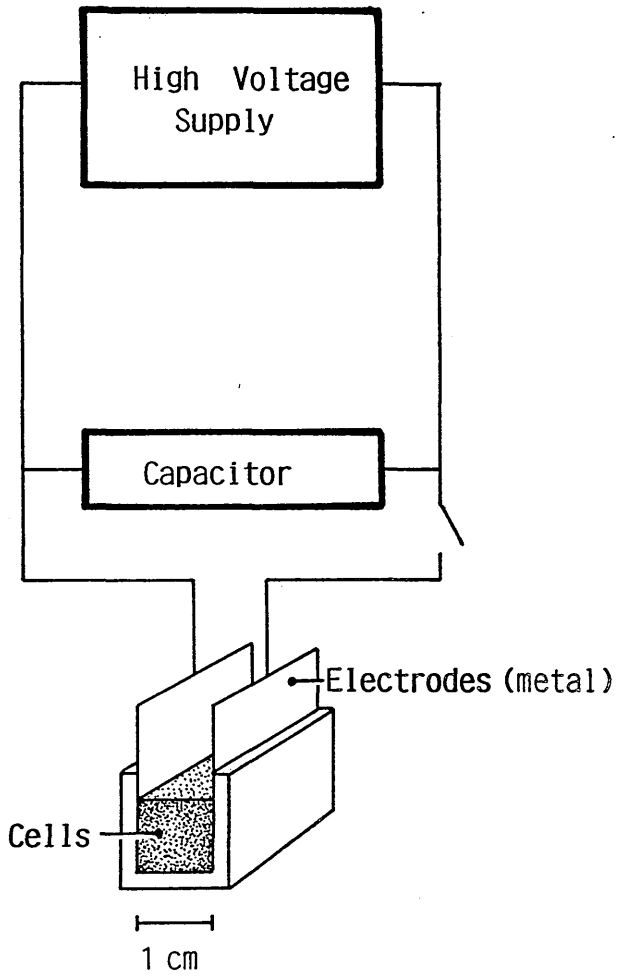


Figure 11:
Representation of the apparatus used to induce electropermeabilisation to hepatocyte suspensions. (Adapted from Knight & Baker, 1982).

permeabilise hepatocytes and to allow access of the small compound, cyclic AMP, was investigated extensively in our laboratory by Dr. Lesley Berry (Ph.D. thesis, 1989). By subjecting the cells to a range of parameters to cause electropermeabilisation (2-10 pulses at 1000-3000V/cm) and then assessing the cells for the degree of access of cyclic AMP, determined by radioimmunoassay it became apparent that 5 pulses at 1000V/cm resulted in an almost 100% permeabilisation of the cell membrane, without excessive cell damage, a value which correlated well with the theoretical assessment of permeabilisation.

As a further confirmation that permeabilisation had occurred, cells were stained with the dye ethydium bromide. Permeabilisation of the cells allows the dye into the cell where it can be seen as a red intracellular marker. Cell viability, as assessed by trypan blue exclusion, was found to be reduced to 75-80% by electropermeabilisation (1000V/cm x 5) but there was no apparent further reduction in cell viability over a three hour incubation period following permeabilisation. The optimum parameters to use for further experiments were chosen to be 5 pulses at 1000V/cm.

2.7 ASSESSMENT OF THE ROLE OF PROTEIN SYNTHESIS IN THE ACTIONS OF PHORBOL ESTERS.

To assess the role of protein synthesis in the effects of PMA (50nM) in electropermeabilised hepatocytes, the translation blocker cycloheximide (10μM) (Peshka, 1971) was added to permeabilised hepatocytes after a 30 minute prein-

cubation period as described in section 2.3. After the initial preincubation with cycloheximide, the cells were resupplemented with cycloheximide to give a final concentration in the incubations of 10μM and then challenged with PMA (50nM). The cells were then incubated for times ranging between 10 and 80 minutes under conditions described previously (section 2.3). 4-Androstene-3,17-dione metabolism was assessed as described in section 2.4.

2.8 ASSESSMENT OF THE ROLE OF PROTEIN KINASES IN THE EFFECTS OF PHORBOL ESTERS.

The role of the different intracellular protein kinases in the effects of PMA in permeabilised hepatocytes were assessed by the use of the following non-selective and selective protein kinase inhibitors: (a) K252a, 20nM ((8R*,9S,11S*)-(-)-9-Hydroxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H,8H,11H-2,7b,11a-triazadibenzo(a,g)cycloocta(c,d,e)trienden-1-one; inhibits all protein kinases) ; (b) K252b, 20nM (the 9-carboxylic acid derivative of K252a; a selective inhibitor of protein kinase C) ; and (c) KT5720, 60nM (the 9-hexoxy-carbonyl derivative of K252a; a selective inhibitor of protein kinase A) (Kase et al, 1986; Nakanishi et al, 1986; Kase et al, 1987). The inhibitors were added to the hepatocytes prior to permeabilisation to give the concentrations listed above. The cells were preincubated with the inhibitors for 30 minutes and then repermeabilised and resupplemented with the inhibitors to maintain the correct active concentrations. The inhibitors were added in DMSO and control cells received

DMSO only. Incubation with PMA (50nM) (section 2.3) and assessment on 4-androstene-3,17-dione metabolism (section 2.4) was as described previously. Preliminary experiments indicated that the inhibitors were unable to penetrate the cell membrane in the intact cell and so the roles of the protein kinase inhibitors on the actions of PMA were assessed in electropermeabilised hepatocytes only.

2.9 ASSESSMENT OF PROTEIN KINASE C ACTIVATION BY PHORBOL ESTERS.

Measurement of protein kinase C translocation from the cytosol to the plasma membrane is a well-accepted method for determining levels of activation of this kinase by various agonists (Gopalakrishna et al, 1986; Niedel & Blackshear, 1986). As the kinase is the proposed intracellular receptor for the phorbol esters (Niedel et al, 1983; Nishizuka, 1984a) techniques now exist which determine protein kinase C levels in both the soluble and particulate fractions and this can provide information as to the degree of protein kinase C activation in the presence of known activators of this enzyme. The experimental details of the assay used in this study are described in detail by Pelech et al (1986) and the advantage of this procedure over other assays is that it avoids homogenization and fractionation of the cells and thus minimizes the influence of factors that could potentially interfere with a quantitative analysis of the subcellular distribution of protein kinase C. Isolated intact hepatocytes (as described in section 2.2) were incubated with either PMA (100nM, 10nM, 5nM or 0.1nM) or

DMSO as described in section 2.3.

(a) Preparation of extracts: After centrifugation, the cells were washed twice with 1ml of ice-cold phosphate buffered saline (100mM, pH 7.4) . The soluble extract was produced by incubating the cells at 0°C for 5 minutes with 0.2ml of buffer A (0.5mg of digitonin/ml, 20mM MOPS, pH 7.2, 10mM EGTA and 5mM EDTA). The cell remnants, after removal of the soluble extract, were solubilized in 0.2ml of buffer A plus 0.5% Triton X to obtain the particulate extract. The extracts were used immediately after they were prepared.

(b) Enzyme assay: The cellular extracts were incubated for 10 minutes at 30°C in the absence or presence of 7.5µg of trypsin/ml and 0.15mg of lima bean trypsin inihibitor added subsequently to terminate trypsinolysis. All kinase assays contained 20nM KT5720 (see section 2.9), 50µM (γ^{32}P)ATP, 75mM magnesium chloride, 1mg histone H1/ml and 10µl of the soluble or particulate extract in a final volume of 25µl. The kinase assays commenced upon the addition of (γ^{32}P)ATP and were of 15 minutes duration at 30°C. At the conclusion of the reaction period, 20µl aliquots were spotted onto 1.5cm² pieces of Whatman P81 phosphocellulose paper and washed five times for at least 2 minutes per wash in a solution of 10ml of phosphoric acid/L of H_2O. The wet filter papers were transferred into scintillation vials containing 5ml Ecoscint and analyzed for radioactivity in a Packard Tricarb 2000CA counter. The results are expressed as fmole ATP/minute/10^6 cells.

2.10 ASSESSMENT OF THE ROLE OF COFACTORS IN PHORBOL ESTER-MEDIATED ALTERATIONS IN 4-ANDROSTENE-3,17-DIONE METABOLISM.

To investigate if the effects of PMA are mediated by changes in the levels of cofactors associated with the cytochrome P450-linked enzymes, the metabolism of 4-androstene-3,17-dione was examined in hepatocytes which had been homogenized and then supplemented with excess cofactors following incubation with PMA (incubation with PMA as described in section 2.3). Before resuspending in incubation medium and before assaying with 4-androstene-3,17-dione, the washed hepatocytes were resuspended in a small volume of incubation medium and homogenized using a Potter-Elvejhem homogeniser with a teflon pestle (2500 r.p.m. for 5 strokes). The homogenates were then made up to 3ml volume with incubation medium, to give a cell density of approximately 10^7 homogenized cells in each 3ml aliquot. To each homogenate aliquot was added 0.8mg NADP and 4mg isocitric acid in 100µl manganese chloride (6mg/100ml) followed by 10µl of isocitrate dehydrodgenase to start the reaction. The samples were then preincubated at 37°C for 2 minutes to allow the generation of NADPH in the homogenates. 500µg (0.1µCi) of 4-androstene-3,17-dione was then added to each sample and the metabolism of 4-androstene-3,17-dione was assayed as described in section 2.4

2.11 FURTHER ASSESSMENT OF THE ROLE OF COFACTORS IN PHORBOL ESTER-MEDIATED EFFECTS IN HEPATOCYTES.

As well as assessing cofactor availability (section 2.10) cofactor degradation was also studied. Intact hepatocytes were treated with PMA (50nM) or DMSO as in section 2.3. The centrifuged cells were resuspended in a small volume of incubation medium and homogenised using the Potter-Elvejhem homogeniser (2500 r.p.m. for 5 strokes) and the homogenate resuspended in incubation medium to give a cell density of 3 X 10^7 homogenate cells per ml. 3ml aliquots of homogenate suspension were added to cuvettes and to the test cuvette was added 2mg of NADPH. The contents were mixed rapidly and read against a reference cuvette, which contained homogenate only, in a Shimadzu UV/VIS spectrophotometer at 340nm. The rate of disappearance of NADPH from the homogenate aliquot was determined using a rate assay programme and the rate of disappearance of NADPH was expressed as µmole NADPH per minute per 10^6 cells.

2.12 ASSESSMENT OF NADPH CYTOCHROME C(P450) REDUCTASE ACTIVITY IN THE PRESENCE OF PMA.

NADPH cytochrome c (P450) reductase activity was routinely measured by the method of Philips and Langdon (1962). This assay makes use of the increase in absorbance at 550nm of the artificial electron acceptor, cytochrome c, when reduced. For this assay, three solutions were prepared; Solution A which contained 9.75mg potassium cyanide, 366mg nicotinamide dissolved in 100ml Tris buffer (0.05M at pH7.4) containing 1mM EDTA; Solution B which

contained cytochrome c, 3.68mg/ml in distilled water and solution C which contained NADPH, 5mg/ml in distilled water. Hepatocytes were incubated as described in section 2.3 with PMA (50nM)/DMSO and centrifuged cells homogenised with the Potter-Elvejhem homogeniser (2500 r.p.m. for 5 strokes) then made up to 3ml aliquots in incubation medium at a cell density of 3 X 10^7 cells. The procedure for the assay was; 2ml of solution A and 0.1ml of solution C were incubated in a test-tube for 8 minutes at room temperature. After this time, 0.5ml of solution B was added and incubated for a further 2 minutes at room temperature. 0.5ml of cell homogenate was added to the solution mix, the contents mixed rapidly and then added to a cuvette. The change in absorbance at 550nm was recorded against a blank which had the same contents as above, except the addition of solution C was replaced by the addition of 0.1ml of distilled water. The concentration of NADPH cytochrome c was determined using the molar extinction coefficient (19.1 X 10^3). The activity of the reductase is expressed as µmole cytochrome c (P450) reduced per minute per 10^6 cells.

2.13 ISOLATION OF HEPATOCYTES IN PHOSPHATE-FREE MEDIUM.

Hepatocytes were isolated by the two stage collagenase perfusion technique as described in section 2.2. The protocol was modified slightly in that phosphate-free, calcium-free Hanks B.S.S. (section 2.24(c)) was used for the isolation procedure instead of calcium-free Hanks B.S.S. as used in section 2.2. Again viability was assessed by trypan blue exclusion and found to be greater than 98%.

Following isolation, the hepatocytes were suspended in phosphate-free incubation medium (section 2.24(d)) supplemented with 0.1% bovine serum albumin at a cell density of 2 X 10^7 cells per ml. The medium was found to maintain cells in a satisfactory condition for up to 4 hours, a time period well in excess of the incubation times of our studies.

2.14 PREINCUBATION OF HEPATOCYTES WITH (^{32}P)ORTHOPHOSPHATE.

Intact hepatocytes were labelled up with (^{32}P)orthophosphate for 90 minutes at 37°C, at a concentration of 10µCi per 10^6 cells. The incubations were performed in 25ml conical flasks in a water bath maintained at 37°C. The cell suspension was aspirated with 95% O_2 / 5%CO_2 to maintain the atmosphere and to prevent cell clumping.

2.15 INCUBATION OF (^{32}P) LABELLED HEPATOCYTES WITH PMA.

After preincubation with (^{32}P)orthophosphate, cells were centrifuged at 500g for 3 minutes in a Damon-IEC DPR 6000 centrifuge, the supernatant was removed and cells washed with phosphate-free incubation medium (+ 0.1% bovine serum albumin). This process was repeated three times to remove excess and unbound (^{32}P) orthophosphate from the cells. The final suspension of the cells was in

phosphate-free incubation medium (+ 0.1% bovine serum albumin) at a cell density of 5 X 10^6 cells per ml. Incubations were performed in 100µl aliquots of this cell suspension (5 X 10^5 cells per incubation).

PMA was added in a 5µl volume to the 100µl aliquot to give a final concentration of 50nM and control cells received 5µl of drug vehicle alone. Incubations were conducted at 37°C for times ranging between 10 and 60 minutes.

2.16 PREPARATION OF SOLUBILISED CELL SAMPLES. Following incubation with PMA (50nM), 400µl of sample solvent (bromophenol blue,0.01% w/v, sodium dodecyl sulphate, 4.6% w/v, "Tris" base, 20% w/v, mercaptoethanol, 10% w/v and glycerol, 15%w/v, in distilled water , pH 6.8) was added to each 100µl aliquot sample and the samples were then boiled in a 100°C water bath for 5 minutes to ensure solubilisation of cell proteins. The solubilised samples were allowed to cool to room temperature and the samples either used directly or stored at -20°C.

2.17 ISOLATION OF SPECIFIC CYTOCHROME P450 ISOZYMES FOLLOWING INCUBATION WITH ^{32}P-LABELLED ORTHOPHOSPHATE. After preincubation with (^{32}P)orthophosphate as in section 2.14, the cells were spun at 500g for 3 minute in a Damon-IEC DPR 6000 centrifuge and the supernatant removed. The cells were resuspended in phosphate-free incubation medium and then treated with PMA(50nM)/DMSO for 60 minutes as described in section 2.15. After centrifugation, the

cells were resuspended in 2ml of 1.8% sodium cholate solution and the cells dispersed by passage through a syringe and needle (26g). The dispersed sample was then made up to 10ml with sodium cholate (1.8% w/v) in nitrocellulose ultracentrifuge tubes, the tubes capped and centrifuged at 105,000g for 1 hour at 4°C in a Beckman L8-M ultracentrifuge. 500µl aliquots of a 1:1000 dilution of specific cytochrome P450 antibodies were then added to 1ml aliquots of ultracentrifuged supernatant (10^6 cells). The following specific P450 antibodies were investigated:

Trivial Name	P450 Family
* αPB2c	P450II C11
* αPB3a	P450II B1
* MC-1b	P450I A1
* UT 1	P450II A1

From nomenclature by Pyerin et al (1987) and Gonzalez (1989)

The antibodies were raised in rabbits against purified rat cytochrome P450 isozymes. The antibodies were made up in phosphate-free incubation medium supplemented with 0.1% bovine serum albumin. After addition of the antibodies, the samples were incubated on ice for 30 minutes and then centrifuged at 25,000g for 30 minutes at 4°C in a Beckman L8-M ultracentrifuge. The supernatant was removed from each sample and the immunoprecipitate was washed and dissolved in 50µl of sample solvent, boiled and cooled as in section 2.16.

2.18 FURTHER ASSESSMENT OF SPECIFIC CYTOCHROME P450 ISOZYMES.

To further assess phosphorylation of specific cytochrome P450 isozymes, the experiments described in section 2.13 - 2.17 were repeated, with modifications. After collagenase perfusion, the hepatocytes were isolated into phosphate-free incubation medium, as in section 2.13, but the incubation medium was supplemented with 20mM β-glycerophosphate, a phosphatase inhibitor. After isolation, hepatocytes were treated as described in sections 2.14 and 2.17 to yield the specific isozyme precipitates. The specific isozyme precipitates were then added to 5ml Ecoscint scintillation fluid and the degree of phosphate incorporated into the precipitates was determined by counting the precipitates in a Packard Tricarb 2000 CA liquid scintillation counter.

2.19 ASSESSMENT OF HAEM CONTENT.

Antibody-antigen precipitates to the specific isozymes αPB2c, αMC-1b, UT-1 and αPB3a were obtained as described in section 2.15 and 2.17. The resulting precipitates were dissolved in 5.3ml of 0.1M NaOH and 0.7ml of pyridine and the resulting mixture left to stand at room temperature for 30 minutes. After this time, 3ml samples were taken and placed in cuvettes and baseline readings obtained. The samples were then reduced by the addition of 5mg of sodium dithionite and the test sample bubbled for 30 seconds with carbon monoxide. The reduced CO spectrum was then measured in a Shimadzu UV/VIS spectrophotometer between the

wavelengths of 500 and 600nm, based on the method of Omura ans Sato (1964b). The change in absorbance (Δ Abs) between 557 and 575nm was measured and nmoles haem produced determined.

2.20 PREINCUBATION OF HEPATOCYTES WITH (γ-^{32}P)ADENOSINE-5-TRIPHOSPHATE (ATP).

Intact hepatocytes were isolated as in section 2.13, into phosphate-free incubation medium supplemented with 20mM β-glycerophosphate and 0.1% bovine serum albumin, at a cell density of 2 X 10^7 cells per ml. The intact cells were electropermeabilised (1000V for 5 pulses) and the permeabilised cells were then incubated at 0°C for 15 minutes in the presence of 100µCi of (γ^{32}P)ATP.

2.21 INCUBATION OF (γ^{32}P)ATP-LABELLED HEPATOCYTES WITH PMA.

After preincubation with (γ^{32}P)ATP as in section 2.20, the hepatocytes were divided into 100µl aliquots (approximately 5 X 10^5 cells per incubation). To each 100µl aliquot was added 5µl of DMSO or 5µl of PMA (final concentration 50nM per incubation), and all incubations were then performed at 37°C for time periods ranging between 10 and 60 minutes. After the respective incubation time, cell samples were solubilised as described in section 2.16.

2.22 ASSESSMENT OF THE ACTION OF PROTEIN KINASE INHIBITORS IN (γ^{32}P)ATP-LABELLED HEPATOCYTES.

Hepatocytes were isolated as in section 2.20. After labelling with (γ^{32}P)ATP, 100µl aliquots of labelled cells were incubated at 37°C for 30 minutes with the specific protein kinase C inhibitor, K252b (section 2.8), which was added in a 5 µl volume to give a concentration of 20nM. Control cells received drug vehicle alone (DMSO) and were also incubated at 37°C for 30 minutes. Following treatment with K252b, 5µl of PMA (50nM final concentration) or 5µl DMSO were added to the aliquots and the samples further incubated for between 10 and 60 minutes at 37°C.

Solubilised samples were prepared as in section 2.16.

2.23 SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS PAGE) OF SOLUBILISED CELL SAMPLES AND DETECTION OF PHOSPHOPROTEINS.

The solubilised cell samples were resolved into their constituent proteins on 7.5% polyacrylamide slab gels, as described by Smith (1984). Samples generated from whole cell proteins were applied to the gels in 20µl aliquots (equivalent to 2 X 10^4 cells, 12-15µg protein) to each sample well. Samples generated from immunoprecipitated cytochrome P450 isozymes were applied in the full 50µl volume (extract from 10^6 cells) to the sample well. The samples were subjected to electrophoresis towards the anode at a constant voltage of 150V for 3.5 hours until the bromophenol blue marker in the sample solvent had reached the bottom of the gel. The gels were then fixed and stained

for 1.5 hours at room temperature in 0.1% w/v coomasie brilliant blue R-250 in methanol/glacial acetic acid/water (5:1:1). Gels were destained for 24 hours at room temperature in methanol/glacial acetic acid/water (8:1:1) with several changes of destaining solution. Destained gels were placed on absorbent paper, covered in non porous plastic film (Cling Wrap) and then dried under vacuum at a temperature of 60°C on a Bio-Rad Model 224 Gel slab dryer for 3 hours. Dried gels were then autoradiographed at -70°C for 4 weeks ((^{32}P)orthophosphate-labelled samples) or for 10 days ($(\gamma^{32}P)$ATP-labelled samples). The degree of incorporation of ^{32}P into whole cell proteins or immunoprecipitated samples was assessed by visual analysis of X-ray film after developing.

2.24 BUFFERS AND PHYSIOLOGICAL SOLUTIONS.

(A) CALCIUM-FREE HANKS BALANCED SALT SOLUTION.

KCL	200mg
KH_2PO_4 (anhydrous)	30mg
NaCl	4000mg (4g)
Na_2HPO_4 (anhydrous)	24mg
$NaHCO_3$ (anhydrous)	1050mg (1.05g)

In glass distilled water to 1 litre, pH 7.4.

(b) INCUBATION MEDIUM.

Glucose	1000mg (1g)
$MgSO_4.7H_2O$	100mg
$MgCl_2.6H_2O$	100mg
$CaCl_2$	185mg

In 100ml of Hanks B.S.S. and made up to 1 litre.

(c) CALCIUM-FREE, PHOSPHATE-FREE HANKS BALANCED SALT SOLUTION.

KCl	200mg
K_2SO_4 (anhydrous)	30mg
NaCl	4000mg (4g)
Na_2SO_4 (anhydrous)	24mg
$NaHCO_3$ (anhydrous)	1050mg (1.05g)

In glass distilled water to 1 litre.

(d) PHOSPHATE-FREE INCUBATION MEDIUM.

As in 2.24(b) but made up in 100ml of calcium-free, phosphate-free Hanks B.S.S. and made up to 1 litre with distilled water.

2.25 CHEMICALS.

Chemicals and reagents are listed according to the manufacturer.

Sigma Chemicals Ltd., Poole, Dorset, U.K.

4β-Phorbol-12-myristate-13-acetate

4α-Phorbol

4α-Phorbol-12,13-didecanoate

1,2-Dioctanoylglycerol

4-Androstene-3,17-dione (Unlabelled)

Ionophore A23187

Angiotensin II

Vasopressin

Cycloheximide

"Tris" Base

Bromophenol Blue

Bis-acrylamide

Acrylamide

Sodium dodecyl sulphate

Coomasie Brilliant Blue R-250

β-Glycerophosphate

Nicotinamide

Cytochrome c

Digitonin

MOPS (3-(N-Morpholino)propanesulfonic acid)

EDTA (Ethylenediaminetetraacetic acid)

EGTA (Ethylene Glycol-bis(β-aminoethyl Ether)N,N,N',N'-Tetraacectic acid)

Trypsin

Trypsin inhibitor (Lima bean)

Histone H1

BCL Ltd., Lewes, East-Sussex, U.K.

Collagenase

Bovine serum albumin (Fraktion V)

NADP

NADPH

Isocitrate dehydrodgenase

Isocitric acid

Amersham International plc., Aylesbury, Bucks., UK.

(4-^{14}C)Androstene-3,17-dione

(γ^{32}P)Adenosine triphosphate (Triethylene ammonium salt)

Gibco BRL Ltd., Paisley, Scotland, UK.

Hams F10

Penicillin/Streptomycin solution

Kodak, France

X Ray film (X-Omat S)

Developer (D-19)

Fixer (FX-40)

Miscellaneous

(^{32}P)Orthophosphate was supplied by the Radionuclide Dispensary, Western Infirmary, Glasgow, U.K.

K-252a,K-252b & KT5720:These were kindly donated by Dr H. Kase, Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan.

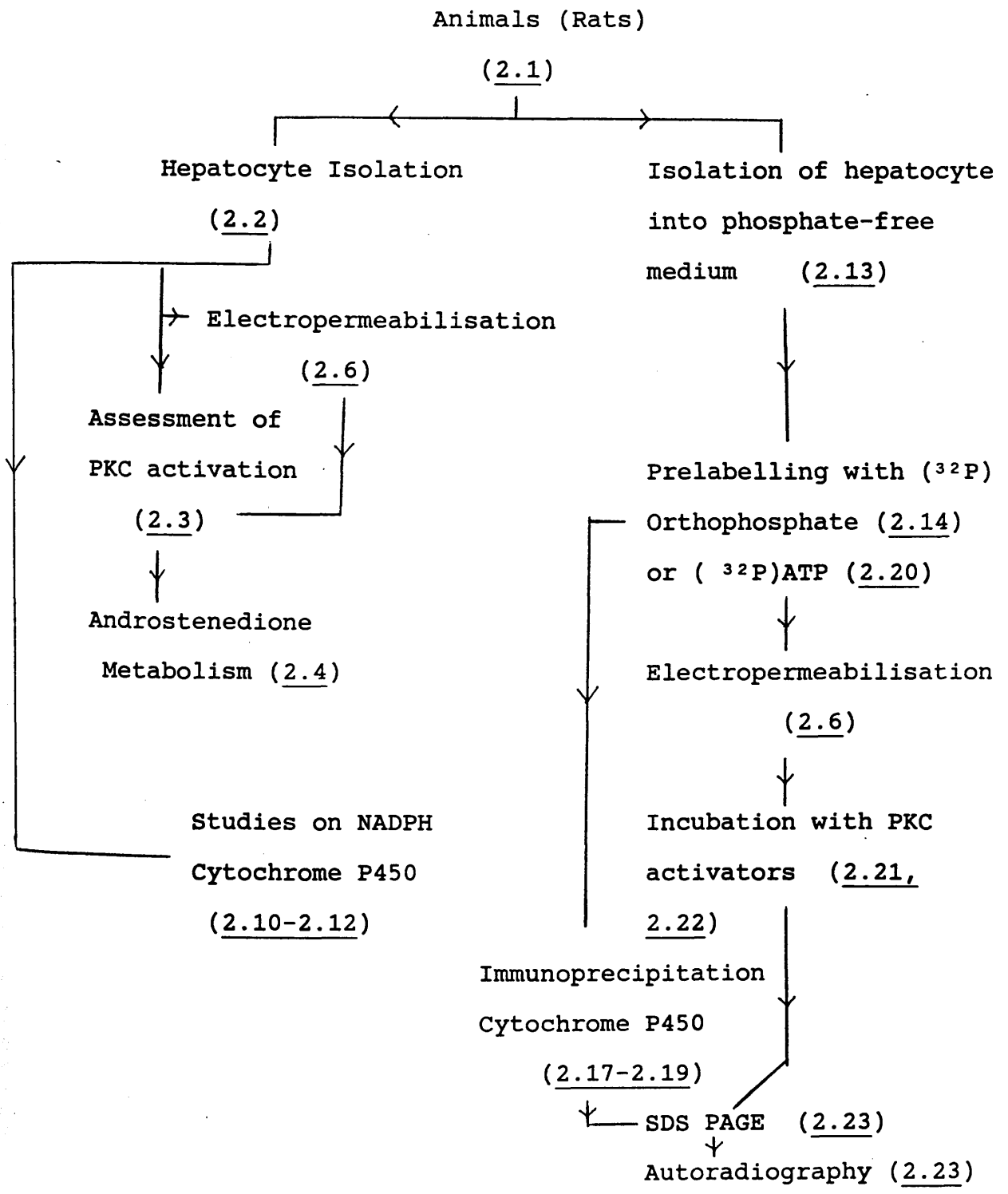
Cytochrome P450 antibodies specific for cytochrome P450 isozymes were a kind gift from Dr C.R. Wolf, ICRF, Edinburgh, Scotland, U.K.

All other chemicals and reagents were of the highest analytical grade commercially avaliable.

2.26 STATISTICS

Results were expressed as percentage of relevant control ± standard deviation. Statistical analysis was performed using Student's t-test. Statistical significance was set at $p<0.01$ in all cases.

SUMMARY OF METHODS



Flow diagram of experiments. The numbers in brackets refer to the relevant sections in the text.

RESULTS

RESULTS.

3.1 Metabolism of 4-androstene-3,17-dione in hepatocytes from freshly isolated male and female rat hepatocytes.

The first part of our study was to investigate the actual method of 4-androstene-3,17-dione metabolism and to determine what could be seen from the results of the assay and how these are interpreted. The methods section 2.4 (figure 9) shows the characteristic band pattern of 4-androstene-3,17-dione metabolism and each band is interpreted as follows;

Band 1	Start point for chromotography run and not analyzed further.
Band 2	Represents 7α-hydroxylase .
Band 3	Represents 6β-hydroxylase .
Band 4	Represents 16α-hydroxylase.
Band 5	Represents 17α(β)-hydroxysteroid dehydrogenase.
Band 6	Represents 3α(β)-hydroxysteroid dehydrogenase
Band 7	Represents unmetabolised androstene-dione substrate.
Band 8	Represents 5α-reductase.

The values for activity for the enzymes 3α(β)-hydroxysteroid dehydrogenase and 5α-reductase are amalgamated to give one overall value for the activity of 5α-reductase (see section 2.4 for explanation). The start band and the band for unmetabolised androstenedione substrate are not analyzed further and hence, the assay for androstenedione is concerned with determining the activities

of 5 different enzymes. Tabular data expresses the enzyme activities as pmole product per minute per 10^6 cells, whereas graphical data is transferred to mean percentage of relevant control to give a better illustration of changes in activity which may be occuring.

Table 3 shows the activities of each of the five enzymes metabolising 4-androstene-3,17-dione in freshly isolated hepatocytes from male and female rats. It can be seen that the well documented sexually differentiated pattern of enzyme profiles are expressed in the isolated rat hepatocyte. Thus;

7α-hydroxylase	Female ≥ Male
6β-hydroxylase	Female < Male
16α-hydroxylase	Female << Male
17-hydroxysteroid dehydrodgenase	Female ≤ Male
5α-reductase	Female >> Male

In the results in table 3, female 6β-hydroxylase activity is 54.7% of male 6β-hydroxylase activity and female 5α-reductase activity is 156.9% of male 5α-reductase activity. In our experiments in general,in contradiction to the above enzyme profile of activity, 7α-hydroxylase activity was greater in males than females and 17-hydroxysteroid dehydrogenase activity was also greater in males than females. Table 3 shows that these enzymes in females have activities of 85.6% and 81.4% respectively of corresponding male activities.

	7αOH	6βOH	16αOH	17OHSD	5αRed.
Male	89.3±10.5	64.3±7.9	102.3±7.2	85.5±6.4	413±24
Female	76.4±9.8	35.2±4.3	49.6±5.3	69.4±7.9	639±63

Table 3: The activities of 7α-hydroxylase (7αOH), 6β-hydroxylase (6βOH), 16α-hydroxylase (16αOH), 17-hydroxysteroid dehydrogenase (17OHSD) and 5α-reductase (5αRed.) in freshly isolated rat hepatocytes from males and females. Results expressed as pmole metabolite formed per minute per 10^6 cells ± S.D. for 5 cell samples.

3.2 Effects of phorbol esters upon the metabolism of 4-androstene-3,17-dione in isolated male rat hepatocytes.

3.2(a) Effects of 4β-Phorbol-12-myristate-13-acetate in male rat hepatocytes.

To account for biological variation and cellular differences from preparation to preparation, the effects of the different compounds investigated upon the activities of the enzymes metabolising 4-androstene-3,17-dione are expressed as percentage of the relevant control activity. By expressing results in this way, we compensate for the fact that control activities can vary during the time course of the experiment.

The effects of the biologically active protein kinase C -activating phorbol ester 4β-phorbol-12-myristate-13-acetate (PMA), at two different concentrations are shown in Figures 12 and 13.

Figure 12A & 12B shows the effect of PMA at a concentration of 50nM. It can be seen that all enzymes, apart from 5α-reductase, were showing reductions in activity after 10 minutes incubation with PMA. The reductions were to 70 - 90% of control and the reduction in activity for 17-hydroxysteroid dehydrodgenase was statistically significant (71% of control, $p<0.01$). By 30 minutes, enzyme activity was further reduced, apart from 16α-hydroxylase which had drifted back towards control activity. The reductions in enzyme activity seen at 60 minutes were maximal for the following enzymes: 7α-hydroxylase reduced to 54% of control, 6β-hydroxylase reduced to 56% of control and

5α-reductase reduced to 60% of control. These maximum reductions in enzyme activity were all statistically significant ($p<0.01$). The activities of all enzymes, except 16α-hydroxylase, had returned to control levels by 120 minutes. The maximum reduction in activity for 16α-hydroxylase was seen at 120 minutes (54% of control, $p<0.01$).

The effect of 1nM PMA is shown in figure 13A & 13B. The effects of 1nM PMA upon enzyme activity parallels quite closely that seen with 50nM PMA (figure 12A & 12B), indicating that the effects of PMA had reached their maximum at 1nM. All enzyme activities, apart from the 16α-hydroxylase noted above, showed maximum reductions after 60 minutes incubation with PMA. The reductions were: 7α-hydroxylase and 5α-reductase reduced to 55% of control, 6β-hydroxylase reduced to 56% of control and 17-hydroxysteroid dehydrogenase reduced to 69% of control ($p<0.01$ in all cases). The maximum reduction in activity for 16α-hydroxylase was at 20 minutes, with a reduction to 79% of control, but this was not statistically significant. All enzyme activities, except 6β-hydroxylase, had returned to control levels by 80 minutes. As both concentrations of PMA produced similar effects upon the enzymes metabolising 4-androstene-3,17-dione, 50nM PMA was chosen as the concentration of phorbol ester for subsequent experiments.

Table 4 shows the activities of all the enzymes in the presence of 50nM PMA with time. Table 5 shows the effect of 1nM PMA upon enzyme activity.

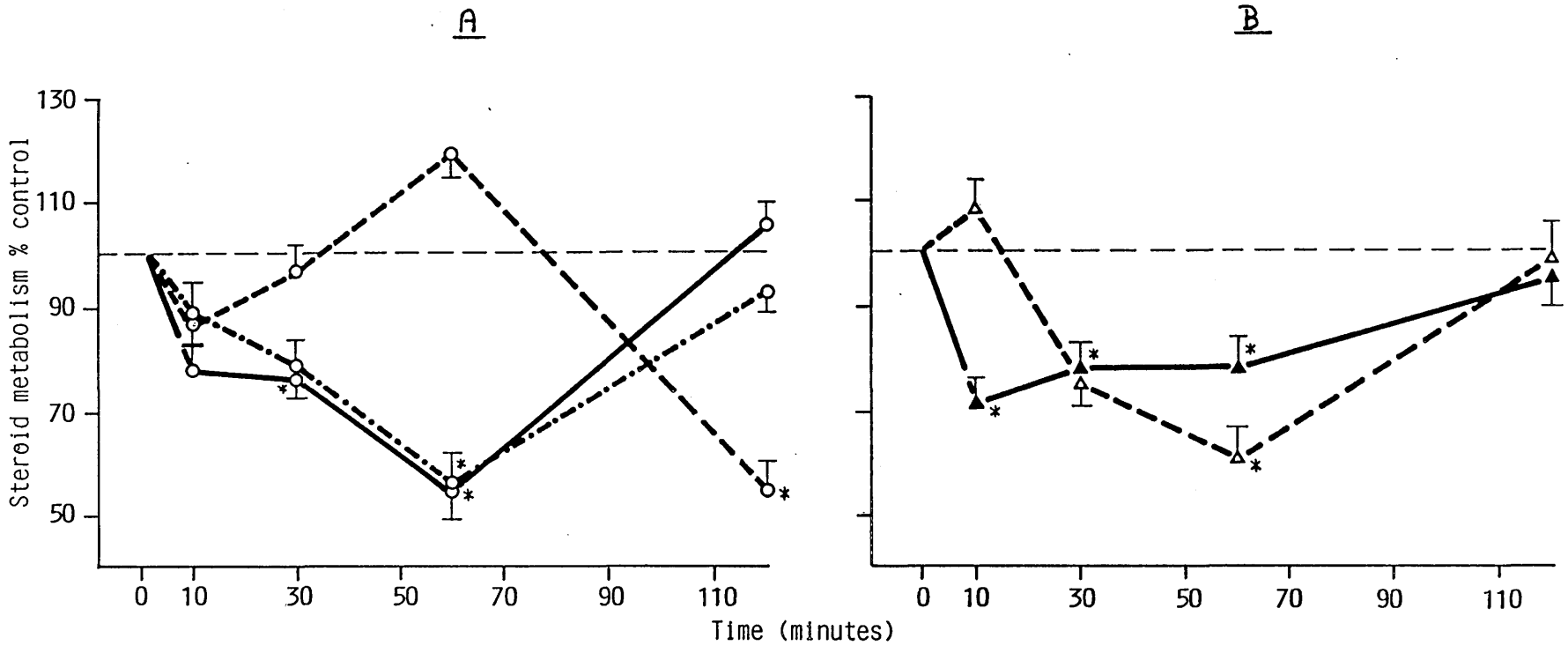


FIG. 12

Changes in 7α (7αOH) (——), 6β (6βOH) (-·-·-·) and 16α (16αOH) (– – – –) activities with time in hepatocytes isolated from male rats following incubation with PMA (50nM). Results expressed as percentage control ± S.D. of nine cell samples (*p<0.01). Control activities expressed as pmol metabolite formed per min. per 10^6 cell:-

	7αOH	6βOH	16αOH
10 min	89.1 ± 1.9	64.2 ± 7.4	53.4 ± 8.2
30 min	76.4 ± 2.1	68.7 ± 8.3	50.6 ± 1.5
60 min	79.3 ± 3.8	62.3 ± 2.5	59.3 ± 1.4
120 min	40.1 ± 1.6	39.4 ± 1.4	57.2 ± 3.9

Changes in 17OHSD (——), 5α (5αRED) (– – – –) activities with time in hepatocytes isolated from male rats following incubation with PMA (50nM). Results expressed as percentage control ± S.D. of nine cell samples (*p<0.01). Control activities expressed as pmol metabolite formed per min. per 10^6 cell:-

	17OH S.D.	5αRED
10 min	63.5 ± 2.4	72.3 ± 8
30 min	41.8 ± 4.2	65.4 ± 2.3
60 min	56.3 ± 8.1	65.3 ± 9.8
120 min	42.9 ± 1.9	46.8 ± 1.9

TABLE 4A

	7αOH	6βOH	16αOH	17OHSD	5αRed.
10 min	89.1±1.9	64.2±7.4	53.4±8.2	63.5±2.4	72.4±8.1
30 min	76.4±2.1	68.8±8.3	50.6±1.5	41.8±4.2	65.4±2.3
60 min	79.3±3.8	62.3±2.5	59.3±1.4	56.3±8.1	65.3±9.8
120 min	40.1±1.6	39.4±1.4	57.2±3.9	42.9±1.9	46.8±1.9

TABLE 4B

	7αOH	6βOH	16αOH	17OHSD	5αRed.
10 min	69.4±3.4	57.2±7.1	46.5±2.1	*45.2±4.1	78.4±8.1
30 min	*58.1±2.4	54.4±2.9	48.3±7.2	*32.6±6.1	49.2±6.1
60 min	*43.4±4.1	*34.5±4.1	70.4±3.9	*43.4±8.1	*39.3±6.5
120 min	42.6±4.2	36.2±6.1	*31.2±6.1	40.3±8.2	45.6±7.1

Table 4: Changes in the activities of 7α-hydroxylase (7αOH), 6β-hydroxylase (6βOH), 16α-hydroxylase (16αOH), 17-hydroxysteroid dehydrogenase (17OHSD) and 5α-reductase (5αRed.) with time in isolated male rat hepatocytes from control (Table 4A) and PMA-treated (50nM)(Table 4B) samples. Results expressed as pmole metabolite formed per minute per 10^6 cells ± S.D. of 9 cell samples. (* $p<0.01$)

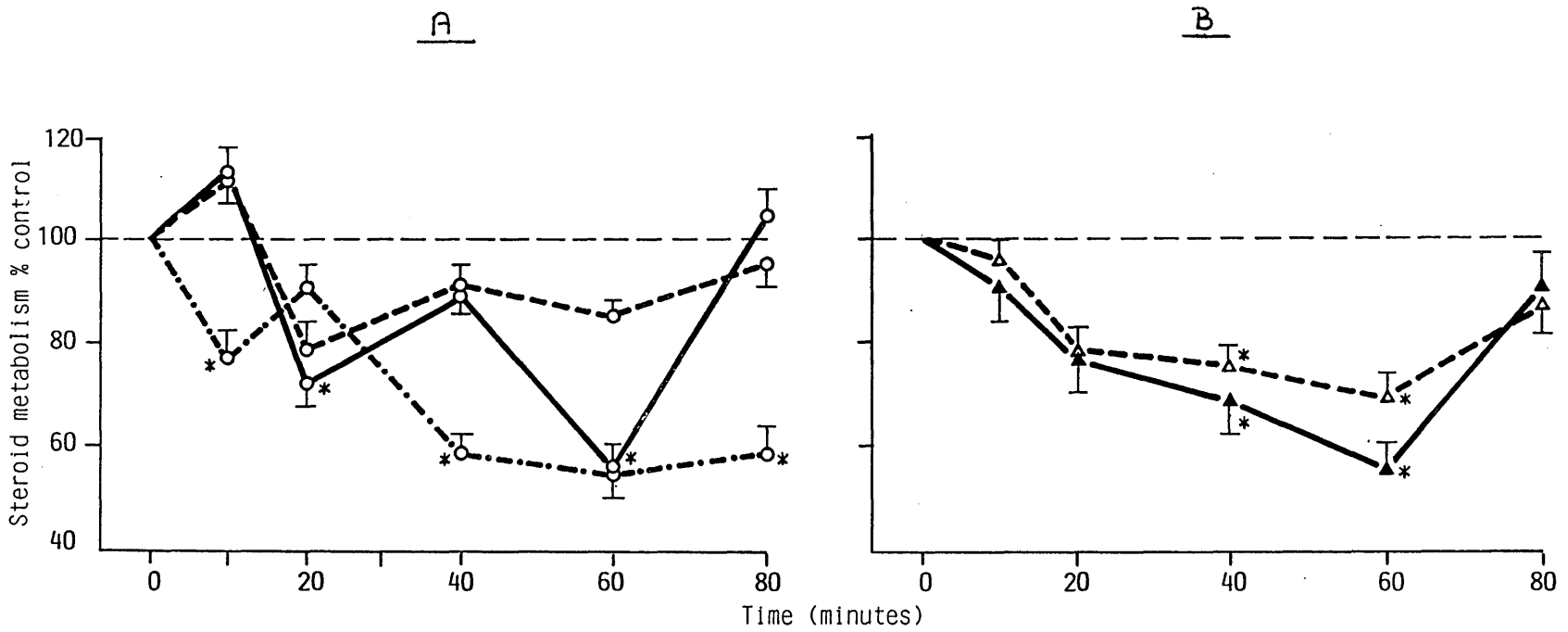


FIG.13

Changes in 7α (7αOH) (——), 6β (6βOH) (-·-·-·) and 16α (16αOH) (- - - -) activities with time in hepatocytes isolated from male rats following incubation with 1nM PMA. Results expressed as percentage control ± S.D. of six cell samples (*p<0.01). Control activities expressed as pmol metabolite formed per min. per 10^6 cell:-

	7αOH	6βOH	16αOH
10 min	62.4 ± 8.1	133 ± 5.6	66.3 ± 6.1
20 min	75.3 ± 5.1	116 ± 3.2	84.5 ± 2.2
40 min	66.2 ± 6.4	117 ± 6.1	70.2 ± 8.9
60 min	84.3 ± 5.3	110 ± 2.0	74.3 ± 2.1
80 min	56.2 ± 8.1	106 ± 5.1	63.8 ± 8.1

Changes in 17OHSD (——), 5α (5αRED) (- - - -) activities with time following incubation with PMA (1nM) in hepatocytes isolated from male rats. Results expressed as % control ± S.D. of 6 cell samples (*p<0.01). Control activities expressed as pmole metabolite formed per minute per 10^6 cell:-

	17OH S.D.	5αRED
10 min	102.0 ± 9.1	423 ± 4.4
20 min	96.8 ± 8.1	377 ± 6
40 min	97.3 ± 5.1	300 ± 2.8
60 min	107.6 ± 9	351 ± 4
80 min	90.0 ± 3.1	336 ± 10

TABLE 5A

	7αOH	6βOH	16αOH	17OHSD	5αRed.
10 min	62.4±8.1	133±6	66.3±6.1	102±9	423±4
20 min	75.3±5.1	116±3	84.5±2.2	97±8	377±6
40 min	66.2±6.4	117±6	70.2±8.9	97±5	300±3
60 min	84.3±5.3	110±2	74.3±2.1	107±9	351±4
80 min	56.2±8.1	106±5	63.8±8.1	90±3	336±10

TABLE 5B

	7αOH	6βOH	16αOH	17OHSD	5αRed.
10 min	70.3±8.1	*103±6	73.2±6.1	92.4±4.1	406±5
20 min	*54.4±4.3	106±2	66.4±4.1	74.5±2.0	295±8
40 min	59.4±3.9	* 69±5	64.3±5.1	*67.3±5.1	*225±8
60 min	*47.2±8.4	60±5	63.2±5.1	*59.4±8.1	*243±2
80 min	55.4±5.2	* 63±6	60.4±4.7	81.6±2.1	297±7

Table 5: Changes in the activities of 7α-hydroxylase (7αOH), 6β-hydroxylase (6βOH), 16α-hydroxylase (16αOH), 17-hydroxysteroid dehydrogenase (17OHSD) and 5α-reductase (5αRed.) with time in hepatocytes isolated from male rats from control (Table 5A) and PMA-treated (1nM)(Table 5B) samples. Results expressed as pmole metabolite formed per minute per 10^6 cells ± S.D. of 6 cell samples. (* $p<0.01$)

3.2(b) Effects of 4α-Phorbol in male rat hepatocytes. The effect of the phorbol ester, 4α-phorbol (100nM), which does not activate protein kinase C, is shown in figure 14A & 14B.

In comparison to the protein kinase C activating phorbol ester, PMA (Figures 12 & 13), the inactive 4α-phorbol shows some increases and decreases in enzyme activity as time proceeds. Although some of the reductions appear quite marked, such as 16α-hydroxylase (reduced to 80% of control at 60 minutes) none of the reductions in activity were statistically significant. There are also some marked increases in activity, notably for 16α-hydroxylase (126% of control at 10 minutes), 5α-reductase (136% of control at 10 minutes), 17-hydroxysteroid dehydrogenase (134% of control at 30 minutes) and 7α-hydroxylase (138% of control at 120 minutes). These increases were statistically significant ($p<0.01$), compared to control activities. Apart from these four significant increases in activity, 4α-phorbol caused only minor fluctuations in enzyme activity at all time points.

The effect of 4α-phorbol (100nM) upon each of the enzymes activities with time are shown in table 6.

3.2(c) Effects of 4α-Phorbol-12,13-didecanoate in male rat hepatocytes.

The effects of another phorbol ester, 4α-phorbol-12,13-didecanoate (4αPDD) (100nM), which is also inactive toward protein kinase C, are shown in figure 15A & 15B.

As seen with 4α-phorbol, (Figures 14A & 14B), 4αPDD

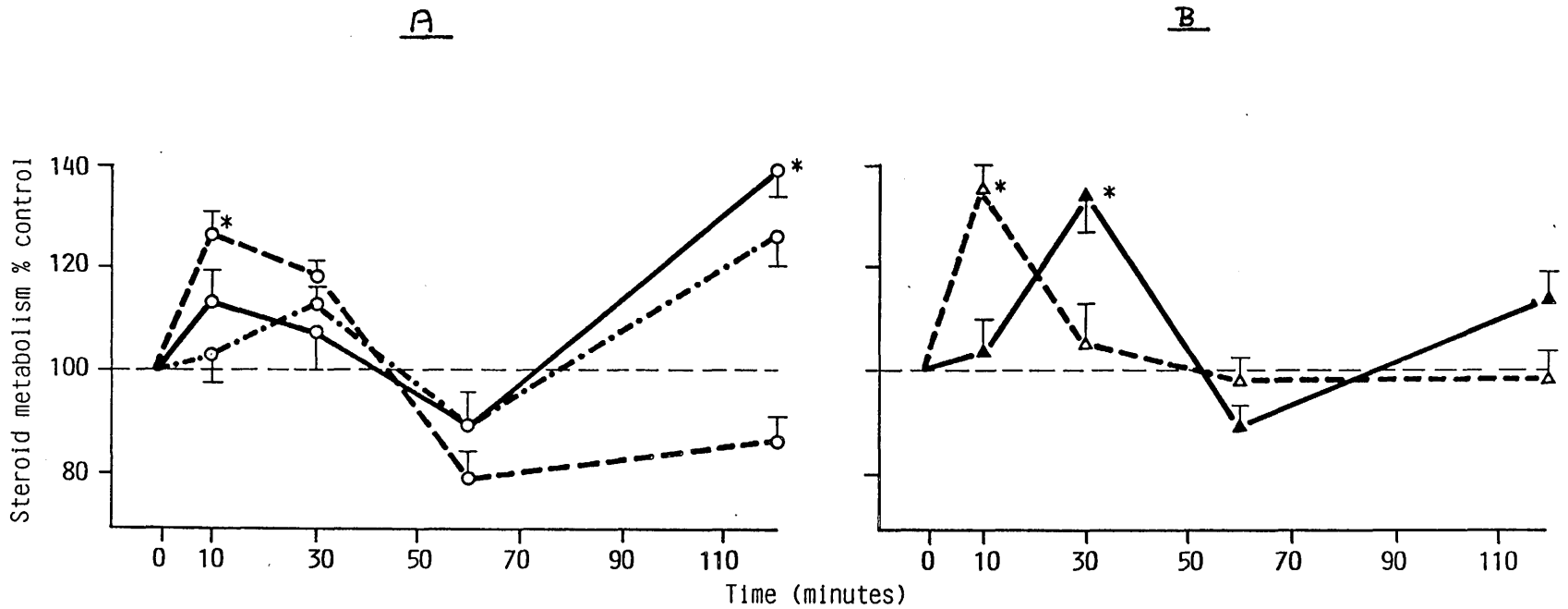


FIG. 14

Changes in 7α (7αOH) (——), 6β (6βOH) (-·-·-·) and 16α (16αOH) (– – – –) activities with time in hepatocytes isolated from male rats following incubation with 4αP (100nM). Results expressed as percentage control ± S.D. of nine cell samples (*p<0.01). Control activities expressed as pmol metabolite formed per min. per 10^6 cell:-

	7αOH	6βOH	16αOH
10 min	89.1 ± 1.9	64.2 ± 7.4	53.4 ± 8.2
30 min	76.4 ± 2.1	68.7 ± 8.3	50.6 ± 1.5
60 min	79.3 ± 3.8	62.3 ± 2.5	59.3 ± 1.4
120 min	40.1 ± 1.6	39.4 ± 1.4	57.2 ± 3.9

Changes in 17OHSD (——), 5α (5αRED) (– – – –) activities with time in hepatocytes isolated from male hepatocytes following incubation with 4αP (100nM). Results expressed as percentage control ± S.D. of nine cell samples (*p<0.01). Control activities expressed as pmole metabolite formed per minute per 10^6 cell:-

	17OH S.D.	5αRED
10 min	63.5 ± 2.4	72.3 ± 1.8
30 min	41.8 ± 4.2	65.4 ± 2.3
60 min	56.3 ± 8.1	65.3 ± 1.4
120 min	42.9 ± 1.9	46.8 ± 1.9

TABLE 6A

	7αOH	6βOH	16αOH	17OHSD	5αRed.
10 min	89.1±1.9	64.2±7.4	53.4±8.2	63.5±2.4	72.4±8.1
30 min	76.4±2.1	68.8±8.3	50.6±1.5	41.8±4.2	65.4±2.3
60 min	79.3±3.8	62.3±2.5	59.3±1.4	56.3±8.1	65.3±8.9
120 min	40.1±1.6	39.4±1.4	57.2±3.9	42.9±1.9	46.8±2.9

TABLE 6B

	7αOH	6βOH	16αOH	17OHSD	5αRed.
10 min	101.0±4.0	66.2±6.1	*67.5±3.1	65.4±2.3	*98.2±4.1
30 min	*81.3±6.1	77.3±4.1	59.2±5.1	*56.1±4.1	68.3±3.4
60 min	70.5±3.4	55.4±8.2	47.3±4.1	49.3±6.2	64.6±5.1
120 min	*64.2±2.1	49.3±2.1	49.5±2.8	48.5±9.1	49.3±4.3

Table 6: Changes in the activities of 7α-hydroxylase (7αOH), 6β-hydroxylase (6βOH), 16α-hydroxylase (16αOH), 17-hydroxysteroid dehydrogenase (17OHSD) and 5α-reductase (5αRed.) with time in isolated male rat hepatocytes from control (Table 6A) or 4α-phorbol-treated (100nM)(Table 6B) samples. Results expressed as pmole metabolite formed per minute per 10^6 cells ± S.D. of 9 cell samples. (*$p<0.01$)

causes minor increases and decreases in enzyme activity for all the enzymes metabolising 4-androstene-3,17-dione. None of these fluctuations in activity were statistically significant when compared to their relevant control activities at each time point, suggesting little or no effect of 4αPDD on the activities of the enzymes metabolising 4-androstene-3,17-dione. Table 7 shows the effect of 4α-phorbol-12,13-didecanoate (100nM) upon each of the enzyme activities with time.

3.3 Effects of phorbol esters upon the metabolism of 4-androstene-3,17-dione in isolated female rat hepatocytes.

3.3(a) Effect of 4β-Phorbol-12-myristate-13-acetate (PMA) in female rat hepatocytes.

The effects of PMA (50nM) upon the activities of all enzymes metabolising 4-androstene-3,17-dione in isolated female rat hepatocytes are shown in figure 16A & 16B. The pattern of activity in the female correlates quite closely to that seen for the male rat (Figure 12). All enzymes showed a reduction in activity at 10 minutes ranging between 80-90% of control. Apart from 7α-hydroxylase , all the enzymes continued to show a gradual decline in activity and the maximum degree of reduction was seen at 60 minutes. The reductions at this time were: 6β-hydroxylase (78% of control), 16α-hydroxylase (51% of control), 17-hydroxysteroid dehydrogenase (74% of control) and 5α-reductase (45% of control). These reductions were statistically significant ($p<0.01$). 7α-hydroxylase showed

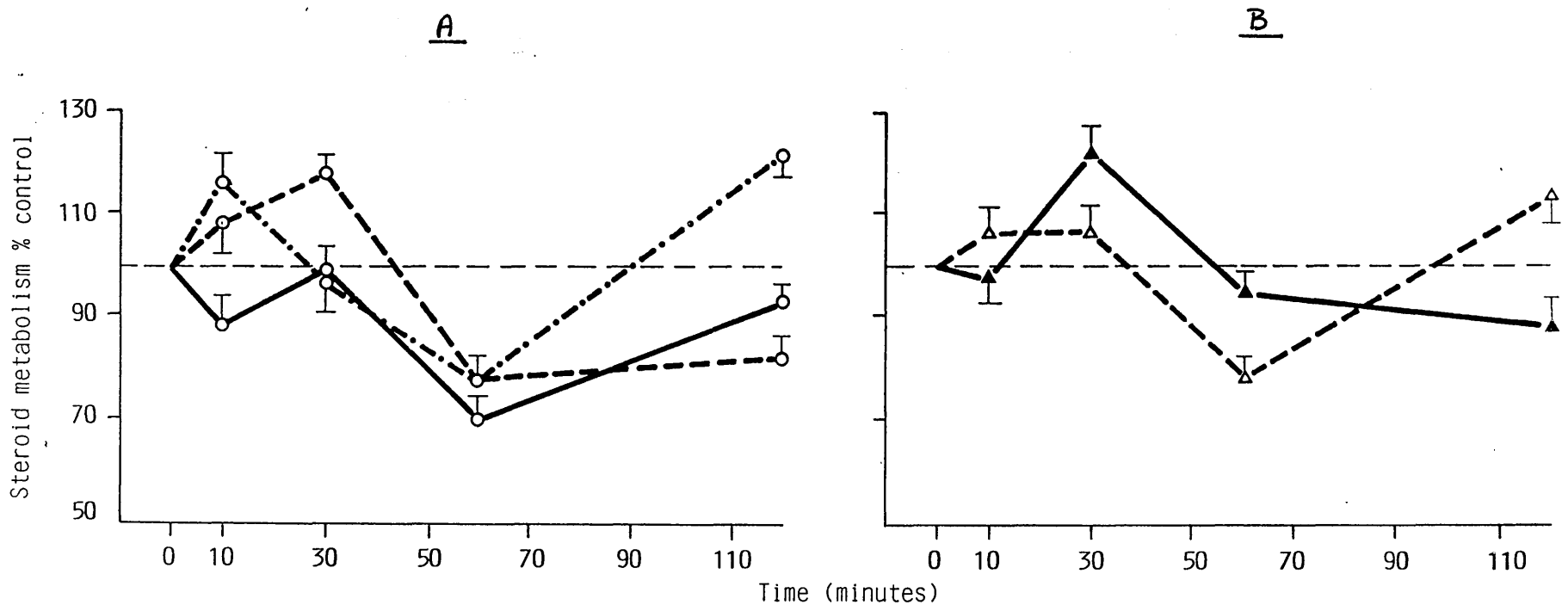


FIG.15 Changes in 7α (7αOH) (———), 6β (6βOH) (-·-·-) and 16α (16αOH) (– – –) activities with time in hepatocytes isolated from male rats following incubation with 4αPDD (100nM). Results expressed as percentage control ± S.D. of nine cell samples (*p<0.01). Control activities expressed as pmol metabolite formed per min. per 10^6 cell:-

	7αOH	6βOH	16αOH
10 min	89.1 ± 1.9	64.2 ± 7.4	53.4 ± 8.2
30 min	76.4 ± 2.1	68.7 ± 8.3	50.6 ± 1.5
60 min	79.3 ± 3.8	62.3 ± 2.5	59.3 ± 1.4
120 min	40.1 ± 1.6	39.4 ± 1.4	57.2 ± 3.9

Changes in 17OHSD (———), 5α (5αRED) (– – –) activities with time in hepatocytes isolated from male hepatocytes following incubation with 4αPDD (100nM). Results expressed as percentage control ± S.D. of nine cell samples (*P<0.01). Control activities expressed as pmole metabolite formed per minute per 10^6 cell:-

	17OH S.D.	5αRED
10 min	63.5 ± 2.4	72.3 ± 1.8
30 min	41.8 ± 4.2	65.4 ± 2.3
60 min	56.3 ± 8.1	65.3 ± 1.4
120 min	42.9 ± 1.9	46.8 ± 1.9

TABLE 7A

	7αOH	6βOH	16αOH	17OHSD	5αRed.
10 min	89.1±1.9	64.2±7.4	53.4±8.2	63.5±2.4	72.4±8.1
30 min	76.4±2.1	68.8±8.3	50.6±1.5	41.8±4.2	65.4±2.3
60 min	79.3±3.8	62.3±2.5	59.3±1.4	56.3±8.1	65.3±9.8
120 min	40.1±1.6	39.4±1.4	57.2±3.9	42.9±1.9	46.8±1.9

TABLE 7B

	7αOH	6βOH	16αOH	17OHSD	5αRed.
10 min	78.3±2.4	74.3±2.4	57.6±4.3	62.3±5.4	76.3±2.4
30 min	75.9±3.1	65.3±7.1	59.3±2.5	50.4±3.4	69.8±3.2
60 min	55.6±5.2	48.3±3.4	46.5±7.3	53.6±4.2	51.5±5.2
120 min	37.1±6.2	47.2±5.1	46.2±5.1	37.8±2.9	52.6±5.4

Table 7: Changes in the activities of 7α-hydroxylase (7αOH), 6β-hydroxylase (6βOH), 16α-hydroxylase (16αOH), 17-hydroxysteroid dehydrogenase (17OHSD) and 5α-reductase (5αRed.) with time in isolated male rat hepatocytes from control (Table 7A) and 4α-phorbol-12,13-didecanoate-treated (100nM)(Table 7B) samples. Results expressed as pmole metabolite formed per minute per 10^6 cells ± S.D. of 9 cell samples.

its maximum reduction in activity at 120 minutes (56% of control; $p<0.01$). Apart from 7α-hydroxylase, all activities had returned to control levels by 120 minutes.

The effects of PMA (50nM) upon each of the enzyme activities with time are shown in table 8.

3.3(b) Effects of 4α-Phorbol in female rat hepatocytes.

The effects of 4α-phorbol (100nM) upon the activities of all the enzymes metabolising 4-androstene-3,17-dione are shown in Figure 17A & 17B.

Again, as in the male rat (Figure 14A & 14B), 4α-phorbol caused only minor fluctuations in enzyme activity for all enzymes concerned. The one discrepancy appears to be for 6β-hydroxylase, where a non-significant increase in activity of 145% of control was seen at 120 minutes.

The effect of 4α-phorbol(100nM) upon the activities of all the enzymes with time is shown in Table 9.

3.3(c) Effects of 4α-Phorbol-12,13-didecanoate (4αPDD) in female rat hepatocytes.

The effects of 4αPDD (100nM) upon the activities of all the enzymes metabolising 4-androstene-3,17-dione are shown in Figure 18A & 18B.

As in the male rat (Figure 15A & 15B), 4αPDD exerted only slight alterations in enzyme activities. None of these alterations in activity were statistically significant compared to the respective control activity. The effects of 4αPDD (100nM) with time upon the activities of all enzymes is shown in table 10.

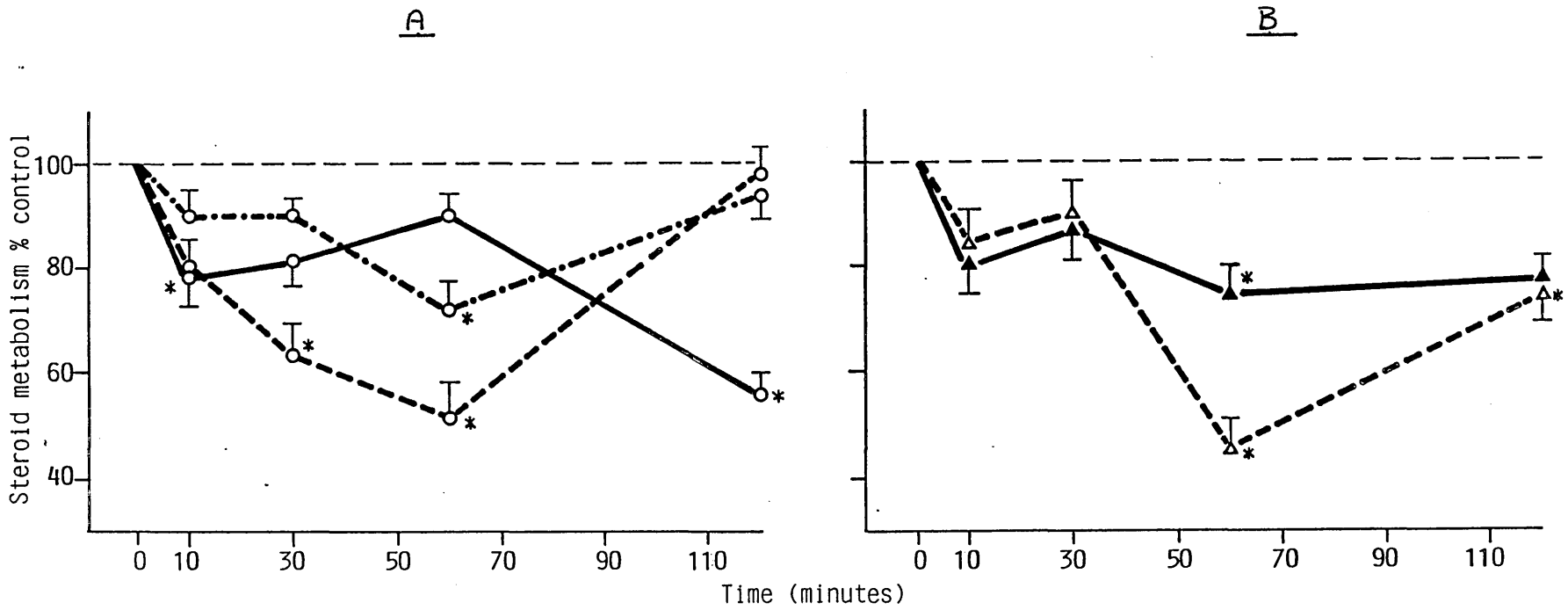


FIG. 16 Changes in 7α (7αOH) (———), 6β (6βOH) (-·-·-) and 16α (16αOH) (– – – –) activities with time in hepatocytes isolated from female rats following incubation with PMA (50nM). Results expressed as percentage control ± S.D. of nine cell samples (*p<0.01). Control activities expressed as pmol metabolite formed per min. per 10^6 cell:-

	7αOH	6βOH	16αOH
10 min	60.3 ± 4.3	59.2 ± 6.4	64.75 ± 3.9
30 min	58.4 ± 7.6	60.1 ± 6.3	76.50 ± 4.9
60 min	52.1 ± 2.9	58.4 ± 6.2	59.80 ± 5.3
120 min	54.5 ± 8.1	35.7 ± 7.1	65.30 ± 6.1

Changes in 17OHSD (———), 5α (5αRED) (– – – –) activities with time in hepatocytes isolated from female rats, following incubation with PMA (50nM). Results expressed as percentage control ± S.D. of nine cell samples (*P<0.01). Control activities expressed as pmole metabolite formed per minute per 10^6 cell:-

	17OH S.D.	5αRED
10 min	69.4 ± 2.3	225 ± 10.1
30 min	55.6 ± 8.1	173 ± 9.8
60 min	69.3 ± 9.4	276 ± 6.9
120 min	64.8 ± 3.8	236 ± 9.8

TABLE 8A

	7αOH	6βOH	16αOH	17OHSD	5αRed.
10 min	60.3±4.3	59.2±6.4	64.8±3.9	69.4±2.3	225±9
30 min	58.4±7.6	60.1±6.3	76.5±4.9	55.6±8.1	173±9
60 min	52.1±2.9	58.4±6.2	59.8±5.3	69.3±9.4	276±7
120 min	54.5±8.1	35.7±7.1	65.3±6.1	64.8±3.8	236±9

TABLE 8B

	7αOH	6βOH	16αOH	17OHSD	5αRed.
10 min	*46.3±5.1	53.2±6.1	51.8±2.4	55.2±2.4	189±8
30 min	47.4±3.1	54.6±8.1	*48.2±6.3	48.3±2.1	156±9
60 min	47.6±9.4	*42.4±6.1	*30.5±5.2	*51.6±8.4	*124±6
120 min	*30.5±4.2	33.2±8.4	64.2±5.1	49.3±6.1	*176±5

Table 8: Changes in the activities of 7α-hydroxylase (7αOH), 6β-hydroxylase (6βOH), 16α-hydroxylase (16αOH), 17-hydroxysteroid dehydrogenase (17OHSD) and 5α-reductase (5αRed.) with time in isolated female rat hepatocytes from control (Table 8A) and PMA-treated (50nM)(Table 8B) samples. Results expressed as pmole metabolite formed per minute per 10^6 cells ± S.D. of 9 cell samples. (* $p<0.01$)

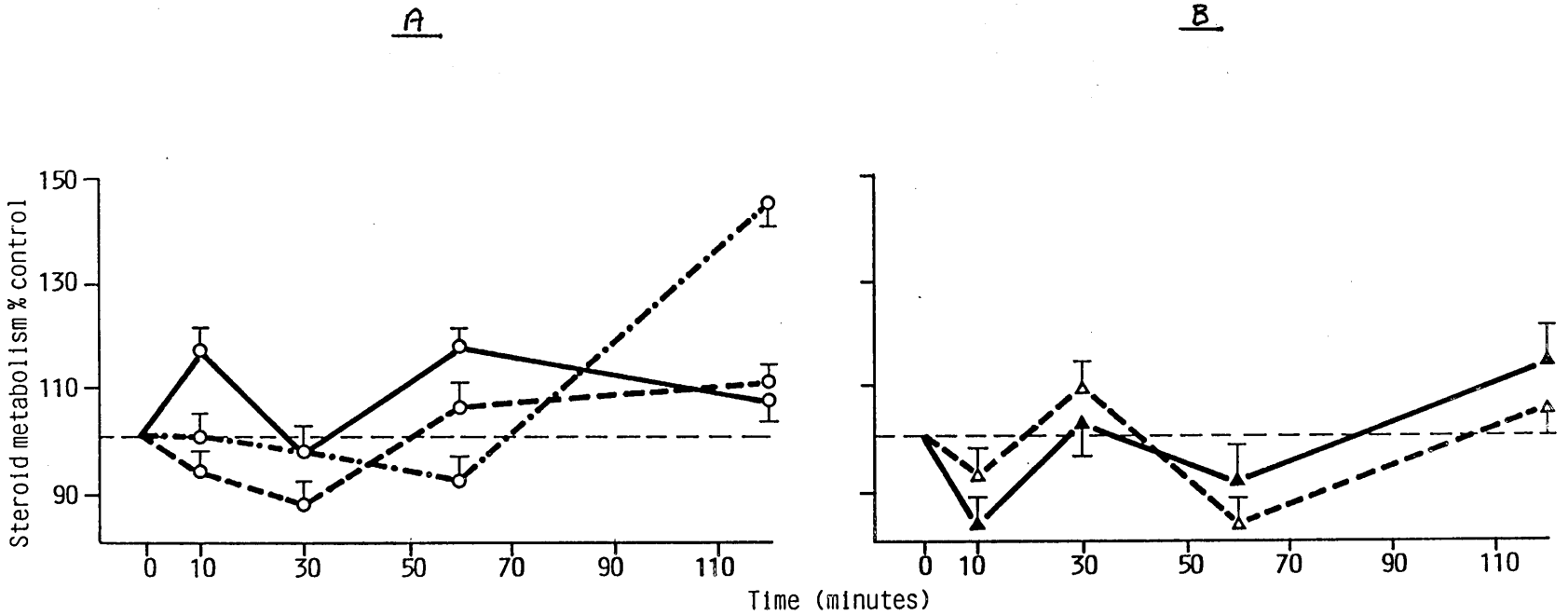


FIG. 17 Changes in 7α (7αOH) (——), 6β (6βOH) (-·-·-) and 16α (16αOH) (----) activities with time in hepatocytes isolated from female rats following incubation with 4αP (100nM). Results expressed as percentage control ± S.D. of nine cell samples (*p<0.01). Control activities expressed as pmol metabolite formed per min. per 10^6 cell:-

	7αOH	6βOH	16αOH
10 min	60.3 ± 4.8	59.2 ± 6.4	64.80 ± 3.9
30 min	58.4 ± 10.4	60.7 ± 6.8	76.50 ± 4.9
60 min	52.3 ± 2.3	58.3 ± 6.9	59.80 ± 5.3
120 min	54.6 ± 8.3	35.4 ± 10.1	65.30 ± 6.1

Changes in 17OHSD (——), 5α (5αRED) (----) activities with time in hepatocytes isolated from female rats, following incubation with4αP (100nM). Results expressed as percentage control ± S.D. of nine cell samples (*P<0.01). Control activities expressed as pmole metabolite formed per minute per 10^6 cell:-

	17OH S.D.	5αRED
10 min	69.4 ± 2.3	225 ± 10.1
30 min	55.6 ± 8.1	173 ± 9.8
60 min	69.3 ± 9.4	276 ± 6.9
120 min	64.8 ± 3.8	236 ± 9.8

TABLE 9A

	7αOH	6βOH	16αOH	17OHSD	5αRed.
10 min	60.3±4.3	59.2±6.4	64.8±3.9	69.4±2.3	225±9
30 min	58.4±7.6	60.1±6.3	76.5±4.9	55.6±8.1	173±9
60 min	52.1±2.9	58.4±6.2	59.8±5.3	69.3±9.4	276±7
120 min	54.5±8.1	35.7±7.1	65.3±6.1	64.8±3.8	236±9

TABLE 9B

	7αOH	6βOH	16αOH	17OHSD	5αRed.
10 min	70.1±7.4	59.6±2.4	60.9±4.1	56.2±6.1	207±8
30 min	56.3±2.1	59.8±5.4	66.6±5.3	56.3±2.4	188±8
60 min	61.9±8.4	53.2±9.4	63.4±2.8	63.5±7.1	213±9
120 min	58.3±8.4	51.6±2.4	71.9±3.6	73.2±2.9	245±6

Table 9: Changes in the activities of 7α-hydroxylase (7αOH), 6β-hydroxylase (6βOH), 16α-hydroxylase (16αOH), 17-hydroxysteroid dehydrogenase (17OHSD) and 5α-reductase (5αRed.) with time in isolated female rat hepatocytes from control (Table 9A) and 4α-phorbol-treated (100nM)(Table 9B) samples. Results expressed as pmole metabolite formed per minute per 10^6 cells ± S.D. of 9 cell samples.

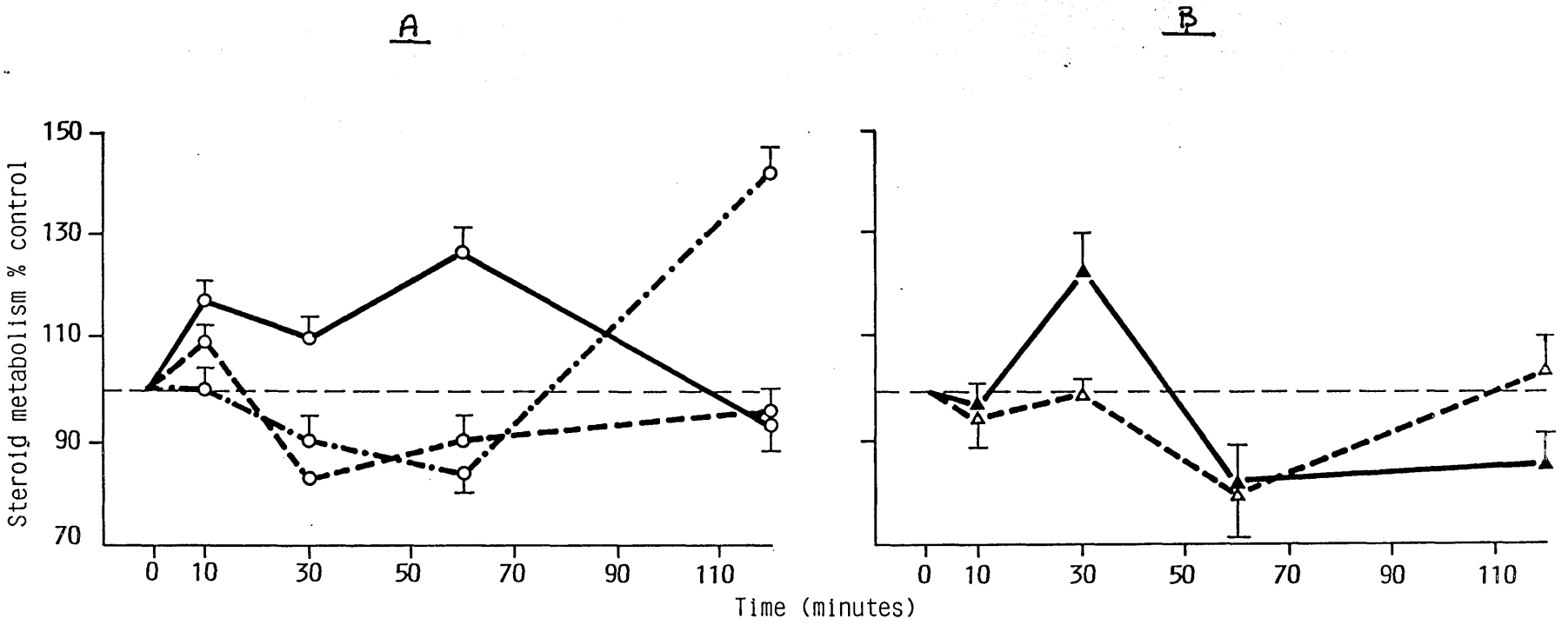


FIG. 18

Changes in 7α (7αOH) (——), 6β (6βOH) (-·-·-·) and 16α (16αOH) (----) activities with time in hepatocytes isolated from female rats following incubation with 4αPDD (100nM). Results expressed as percentage control ± S.D. of nine cell samples (*p<0.01). Control activities expressed as pmol metabolite formed per min. per 10^6 cell:-

	7αOH	6βOH	16αOH
10 min	60.3 ± 4.3	59.2 ± 6.4	64.8 ± 3.9
30 min	58.4 ± 7.6	60.1 ± 6.3	76.5 ± 4.9
60 min	52.1 ± 2.9	58.3 ± 6.2	59.8 ± 5.3
120 min	54.5 ± 8.1	35.7 ± 7.1	65.3 ± 6.1

Changes in 17OHSD (——), 5α (5αRED) (----) activities with time in hepatocytes isolated from female rats, following incubation with 4αPDD (100nM). Results expressed as percentage control ± S.D. of nine cell samples (*p<0.01). Control activities expressed as pmole metabolite formed per minute per 10^6 cell:-

	17OH S.D.	5αRED
10 min	69.4 ± 2.3	225 ± 10.1
30 min	55.6 ± 8.1	173 ± 9.8
60 min	69.3 ± 9.4	276 ± 6.9
120 min	64.8 ± 3.8	236 ± 9.8

TABLE 10A

	7αOH	6βOH	16αOH	17OHSD	5αRed.
10 min	60.3±4.3	59.2±6.4	64.8±3.9	69.4±2.3	225±9
30 min	58.4±7.6	60.1±6.3	76.5±4.9	55.6±8.1	173±9
60 min	52.1±2.9	58.4±6.2	59.8±5.3	69.3±9.4	276±7
120 min	54.5±8.1	35.7±7.1	65.3±6.1	64.8±3.8	236±9

TABLE 10B

	7αOH	6βOH	16αOH	17OHSD	5αRed.
10 min	70.3±5.1	59.6±5.1	70.6±4.1	67.4±5.4	214±11
30 min	64.3±2.9	54.3±8.4	63.5±2.9	67.8±2.3	171±7
60 min	66.8±5.1	49.2±9.1	53.8±5.4	56.8±5.2	222±4
120 min	56.3±4.2	52.6±3.2	62.7±2.1	55.4±4.8	244±5

Table 10: Changes in the activities of 7α-hydroxylase (7αOH), 6β-hydroxylase (6βOH), 16α-hydroxylase (16αOH), 17-hydroxysteroid dehydrogenase (17OHSD) and 5α-reductase (5αRed.) with time in isolated female rat hepatocytes from control (Table 10A) and 4α-phorbol-12,13-didecanoate-treated (100nM)(Table 10B) samples. Results expressed as pmole metabolite formed per minute per 10^6 cells ± S.D. of 9 cell samples.

3.4 Effect of 1,2-Dioctanoylglycerol upon the metabolism of 4-androstene-3,17-dione in isolated male rat hepatocytes.

1,2-Dioctanoylglycerol is a synthetic, cell-permeable analogue of the endogenous protein kinase C activator, diacylglycerol. The effect of 1,2-dioctanoylglycerol (DOG) (5μM), upon the metabolism of 4-androstene-3,17-dione is shown in Figure 19A & 19B. DOG, in comparison to the active phorbol ester PMA (Figures 12 & 13), produced a similar pattern in the reduction in enzyme activity to PMA but there are some apparent differences to the phorbol ester effect. Firstly, DOG exerts a much more rapid onset of effect upon enzyme activity than PMA, the maximum effect upon activity is seen at 20 minutes for all enzymes except 7α-hydroxylase. The maximum effect of PMA in comparison was seen at 60 minutes. The second main difference to PMA is that although a higher concentration of DOG was used than PMA, the magnitude of reduction in enzyme activity with DOG is not as marked as the reduction with phorbol ester. The reductions with DOG are to 78 - 85% of control, whereas maximum reductions with PMA were to 50 - 60% of control. These maximum reductions in activity with DOG, although quite small, were still statistically significant ($p<0.05$). The action of DOG had ceased by 60 minutes, by which time activities were back to, or approaching, control levels. 5α-reductase was the only enzyme still showing a reduction in activity at 60 minutes (85%) of control, but this was not statistically significant.

Table 11 shows the effect on reduction of enzyme

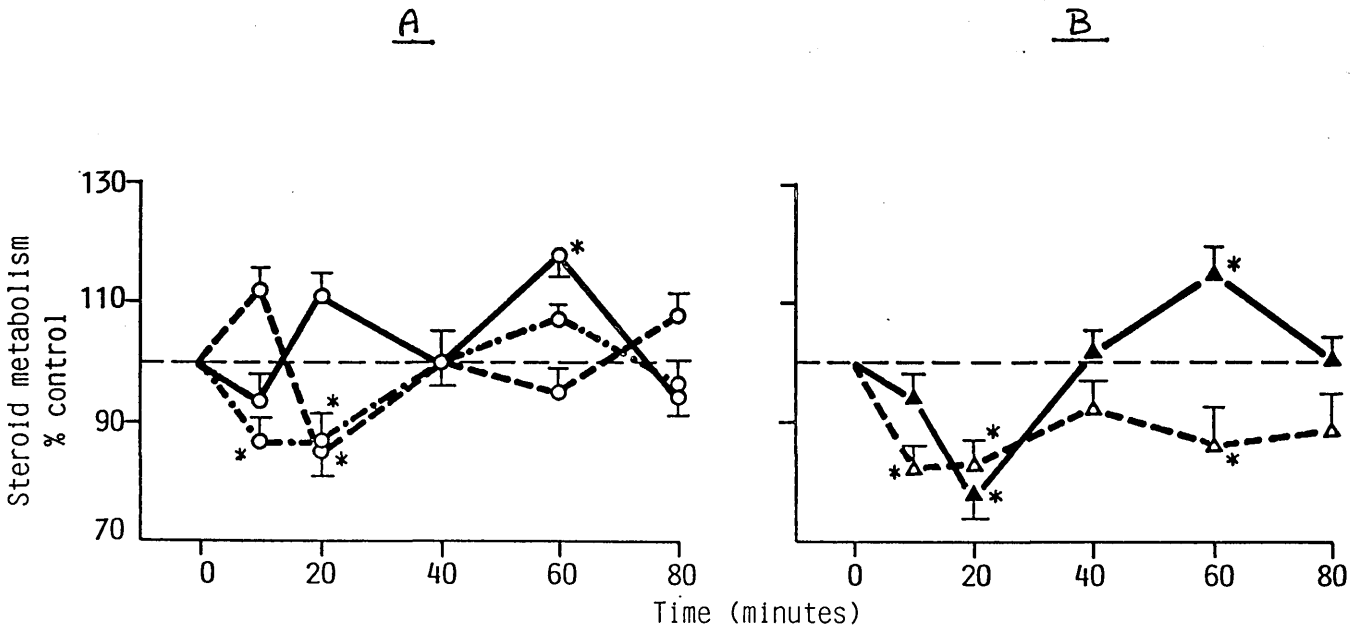


FIG. 19

Changes in 7α (7αOH) (———), 6β (6βOH) (–·–·–·) and 16α (16αOH) (– – – –) activities with time in hepatocytes isolated from male rats following incubation with 1,2-dioctanoylglycerol (5μM). Results expressed as percentage control ± S.D. of six cell samples (*p<0.01). Control activities expressed as pmol metabolite formed per min. per 10^6 cell:-

	7αOH	6βOH	16αOH
10 min	55.3 ± 7.4	76.8 ± 5.2	52.4 ± 7.4
20 min	56.8 ± 9.3	83.1 ± 10	59.6 ± 6.4
40 min	56.4 ± 7.1	74.6 ± 9.3	53.2 ± 7.6
60 min	56.8 ± 6.1	74.2 ± 7.3	55.3 ± 9.4
80 min	50.2 ± 7.1	84.1 ± 9.2	52.1 ± 10

Changes in 17OHSD (———), 5α (5αRED) (– – – –) activities with time in hepatocytes isolated from male rats, following incubation with 1,2-dioctanoylglycerol (5μM). Results expressed as percentage control ± S.D. of six cell samples (*p<0.01). Control activities expressed as pmole metabolite formed per minute per 10^6 cell:-

	17OH S.D.	5αRED
10 min	94.2 ± 6.1	304 ± 10
20 min	92.4 ± 7.2	317 ± 8.3
40 min	90.3 ± 10	275 ± 5.4
60 min	88.3 ± 5.4	280 ± 9.4
80 min	88.5 ± 6.2	278 ± 3.9

TABLE 11A

	7αOH	6βOH	16αOH	17OHSD	5αRed.
10 min	55.3±7.4	76.8±5.2	52.4±7.4	94.7±6.1	304±10
20 min	56.8±9.3	83.1±8.7	59.6±6.4	92.4±7.2	317±8
40 min	56.4±7.1	74.6±9.3	53.2±7.6	90.3±9.7	275±5
60 min	56.8±6.1	74.2±7.3	55.3±9.4	88.3±5.4	280±9
80 min	50.2±7.1	84.1±9.2	52.1±9.5	88.5±6.2	278±4

TABLE 11B

	7αOH	6βOH	16αOH	17OHSD	5αRed.
10 min	51.4±7.2	65.3±9.1	58.2±4.1	87.4±5.1	*247±8
20 min	62.3±8.1	*71.4±5.1	*50.3±6.1	*71.6±2.1	*261±8
40 min	56.2±6.4	74.2±6.3	53.9±7.1	91.3±6.4	249±7
60 min	66.3±1.1	79.8±9.4	52.6±8.4	*100 ± 7	*239±2
80 min	47.4±7.1	81.6±5.1	56.3±9.1	88.2±6.1	245±3

Table 11: Changes in the activities of 7α-hydroxylase (7αOH), 6β-hydroxylase (6βOH), 16α-hydroxylase (16αOH), 17-hydroxysteroid dehydrogenase (17OHSD) and 5α-reductase (5αred.) with time in isolated male rat hepatocytes from control (Table 11A) and 1,2-dioctanoylglycerol-treated (5μM)(Table 11B) samples. Results expressed as pmole metabolite formed per minute per 10^6 cells ± S.D. of 6 cell samples. (* $p<0.01$)

activity by 1,2-dioctanoylglycerol (5μM) with time.

3.5 Effect of Vasopressin upon the metabolism of 4-Androstene-3,17-dione in isolated male rat hepatocytes.

The effects of the hormone, vasopressin, upon the metabolism of 4-androstene-3,17-dione are shown in figures 20 and 21.

Figure 20A and 20B shows the effect of a physiological concentration of Vasopressin (100nM) (Gárciá-Sáinz et al, 1987; Cabot et al, 1988). Figure 21A and 21B shows the effect of a supraphysiological concentration (100μM).

3.5(a) Effect of 100nM vasopressin. The effect of 100nM vasopressin is shown in Figure 20A & 20B, and it shows a high degree of similarity to the effects of PMA (Figure 12 & 13). The magnitude of reduction in enzyme activity with vasopressin correlates well with that seen with PMA and the maximum reductions in enzyme activity were; 7α-hydroxylase reduced to 49% of control, 6β-hydroxylase reduced to 57% of control, 16α-hydroxylase reduced to 60% of control, 17-hydroxysteroid dehydrogenase reduced to 68% of control and 5α-reductase reduced to 69% of control. These maximum reductions in activity were all statistically significant ($p<0.01$). Apart from 16α-hydroxylase, these maximum reduction in activity were all seen after 40 minutes incubation with vasopressin, which again correlates well with the time when the maximum effect of PMA was seen (60 minutes). Vasopressin differs from PMA in that there appears to be a biphasic mode of action upon enzyme activity. After 10 minutes incubation, for all enzymes,

there is a statistically significant reduction in the levels of enzyme activity ($p<0.01$) and the magnitude of this reduction is to between 55 - 72% of control activities. Between 10 and 20 minutes there appears to be a return of enzyme activities towards the control activities, and after 20 minutes there is a gradual decline in enzyme activity until 40 minutes, when the maximum reductions in activity are seen. The enzyme 16α-hydroxylase is the only exception to this pattern. 16α-hydroxylase still shows a biphasic pattern of reduction in activity, but the maximum reduction in activity is seen at 20 minutes and not at 40 minutes. After 60 minutes incubation, all enzyme activities had returned toward control activities. The reduction in activity with vasopressin at 10 minutes correlates more closely to the time of onset of action of 1,2-dioctanoylglycerol (Figure 19A & 19B), although the magnitude of reduction is more akin to that seen with PMA (Figures 12 & 13).

The effects of vasopressin (100nM) on all enzymes with time are shown in table 12.

3.5(b) Effect of 100μM Vasopressin. The effects of vasopressin (100μM) upon the metabolism of 4-androstene-3,17-dione is shown in Figure 21A and 21B.

The pattern of reduction of enzyme activity with 100μM vasopressin is similar to that seen with 100nM vasopressin (Figure 20A & 20B) and once again a biphasic pattern of reduction in activity is seen. The magnitude of reduction is also quite similar (between 50 - 70% of control) and

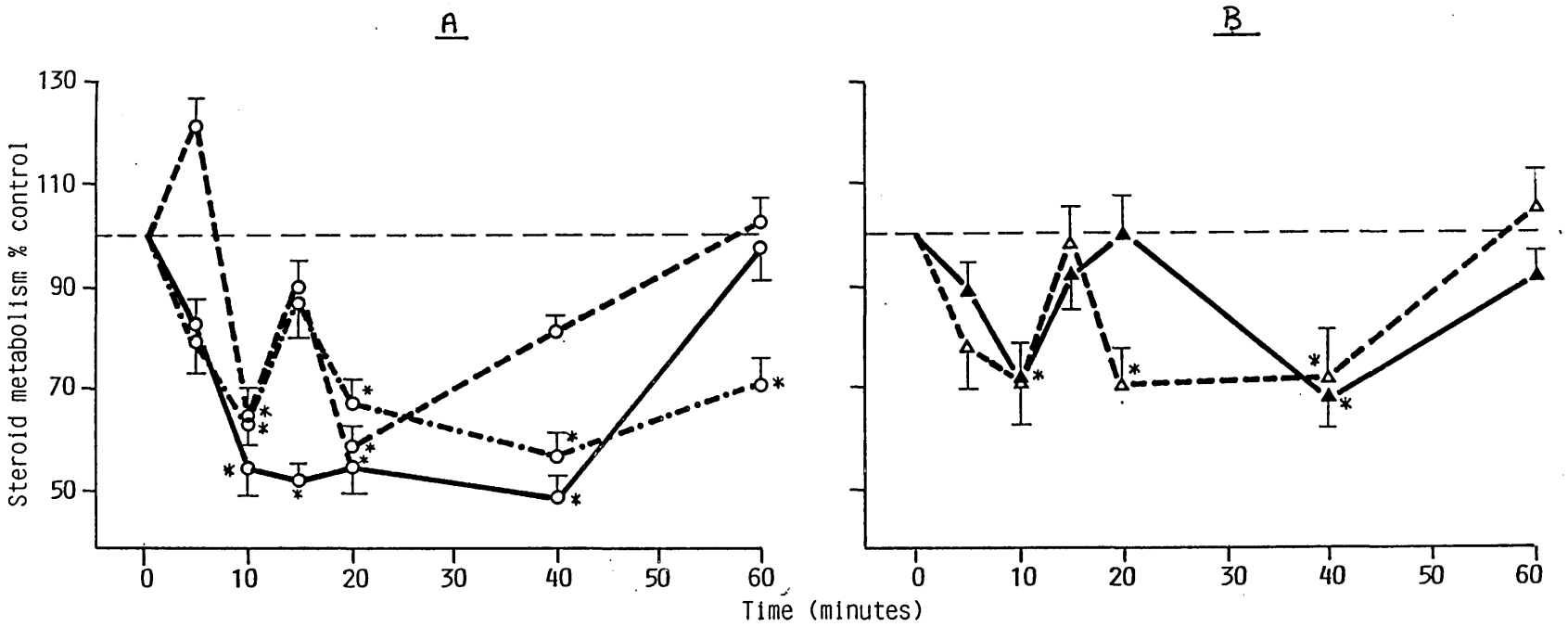


FIG-20

Changes in 7α (7αOH) (———), 6β (6βOH) (-·-·-) and 16α (16αOH) (– – –) activities with time in hepatocytes isolated from male rats following incubation with vasopressin (100nM). Results expressed as percentage control ± S.D. of six cell samples (*p<0.01). Control activities expressed as pmol metabolite formed per min. per 10^6 cell:-

	7αOH	6βOH	16αOH
5 min	52.1 ± 10.1	60.8 ± 6.1	46.3 ± 6.4
10 min	77.4 ± 9.3	86.3 ± 8.1	69.8 ± 6.3
15 min	71.2 ± 10	62.3 ± 7.8	52.6 ± 5.4
20 min	64.3 ± 8.1	75.6 ± 9.4	87.6 ± 4.1
40 min	86.7 ± 7.8	72.3 ± 7.9	63.9 ± 6.1
60 min	45.2 ± 6.1	55.4 ± 6.1	48.5 ± 3.1

Changes in 17OHSD (———), 5α (5αRED) (– – – –) activities with time in hepatocytes isolated from male rats, following incubation with Vasopressin (100nM). Results expressed as percentage control ± S.D. of six cell samples (*p<0.01). Control activities expressed as pmole metabolite formed per minute per 10^6 cell:-

	17OH S.D.	5αRED
5 min	66.3 ± 8.4	414 ± 11
10 min	86.4 ± 7.2	350 ± 7.6
15 min	64.6 ± 10.2	294 ± 8.3
20 min	69.1 ± 6.3	325 ± 6.0
40 min	75.5 ± 9.1	349 ± 7.1
60 min	65.2 ± 8.2	275 ± 4.6

TABLE 12A

	7αOH	6βOH	16αOH	17OHSD	5αRed.
5 min	52.1±8.6	60.8±6.1	46.3±6.4	66.3±8.4	414±11
10 min	77.4±9.3	86.3±8.1	69.8±6.3	86.4±7.4	350±8
15 min	71.2±7.9	62.3±7.8	52.6±5.4	64.6±10.2	294±8
20 min	64.3±8.1	75.6±9.4	87.6±4.1	69.1±6.3	325±6
40 min	86.7±7.8	72.3±7.9	63.9±6.1	75.5±9.1	349±7
60 min	45.2±6.1	55.4±6.1	48.5±3.1	65.2±8.2	275±5

TABLE 12B

	7αOH	6βOH	16αOH	17OHSD	5αRed.
5 min	42.3±3.1	48.3±5.1	56.3±7.1	59.2±5.1	323±5
10 min	*42.4±6.1	*54.6±9.1	*44.2±9.1	*62.3±6.1	250±7
15 min	*37.2±5.4	54.3±9.1	47.3±4.2	59.8±9.3	289±6
20 min	*35.4±9.2	*51.2±5.1	*51.6±5.2	69.4±6.1	*230±4
40 min	*42.3±2.1	*41.2±2.1	*51.4±7.2	*51.3±8.9	*252±5
60 min	44.6±8.4	*39.4±6.2	49.3±7.1	60.4±7.9	289±6

Table 12: Changes in the activities of 7α-hydroxylase (7αOH), 6β-hydroxylase (6βOH), 16α-hydroxylase (16αOH), 17-hydroxysteroid dehydrogenase (17OHSD) and 5α-reductase (5αRed.) with time in isolated male rat hepatocytes from control (Table 12A) and vasopressin-treated (100nM)(Table 12B) samples. Results expressed as pmole metabolite formed per minute per 10^6 cells ± S.D. of 6 cell samples. (* $p<0.01$)

maximum reductions were again statistically significant ($p<0.01$). The only major difference between the two concentrations of vasopressin is that 100µM vasopressin exerts its maximum reductions in activity at 10 minutes, instead of at 40 minutes as was seen with 100nM vasopressin. Reductions at both 10 and 40 minutes were still observed for both concentrations of vasopressin. Again with 100µM vasopressin, all enzyme activities had returned to control levels by 60 minutes.

The effect of 100µM vasopressin with time upon enzyme activity is shown in table 13.

3.6 Effect of A23187 upon the metabolism of 4-androstene-3,17-dione in isolated male rat hepatocytes.

The actions of the calcium ionophore A23187 (100nM) alone and in combination with PMA (50nM) upon the metabolism of 4-androstene-3,17-dione are shown in Figure 22.

Intact cells were incubated with A23187 and PMA for 60 minutes before being assayed with 4-androstene-3,17-dione; 60 minutes incubation was the time period chosen as it is at this time that the maximum effects of PMA had been seen previously (Section 3.2(a), Figures 12 & 13). From Figure 22 it can be seen that A23187 (100nM) alone had very little effect upon the activities of the enzymes. Small nonsignificant increases in activity were seen for all enzymes, apart from 5α-reductase which showed a slight nonsignificant decrease in activity (reduced to 78% of control). None of the increases were more than 120% of control.

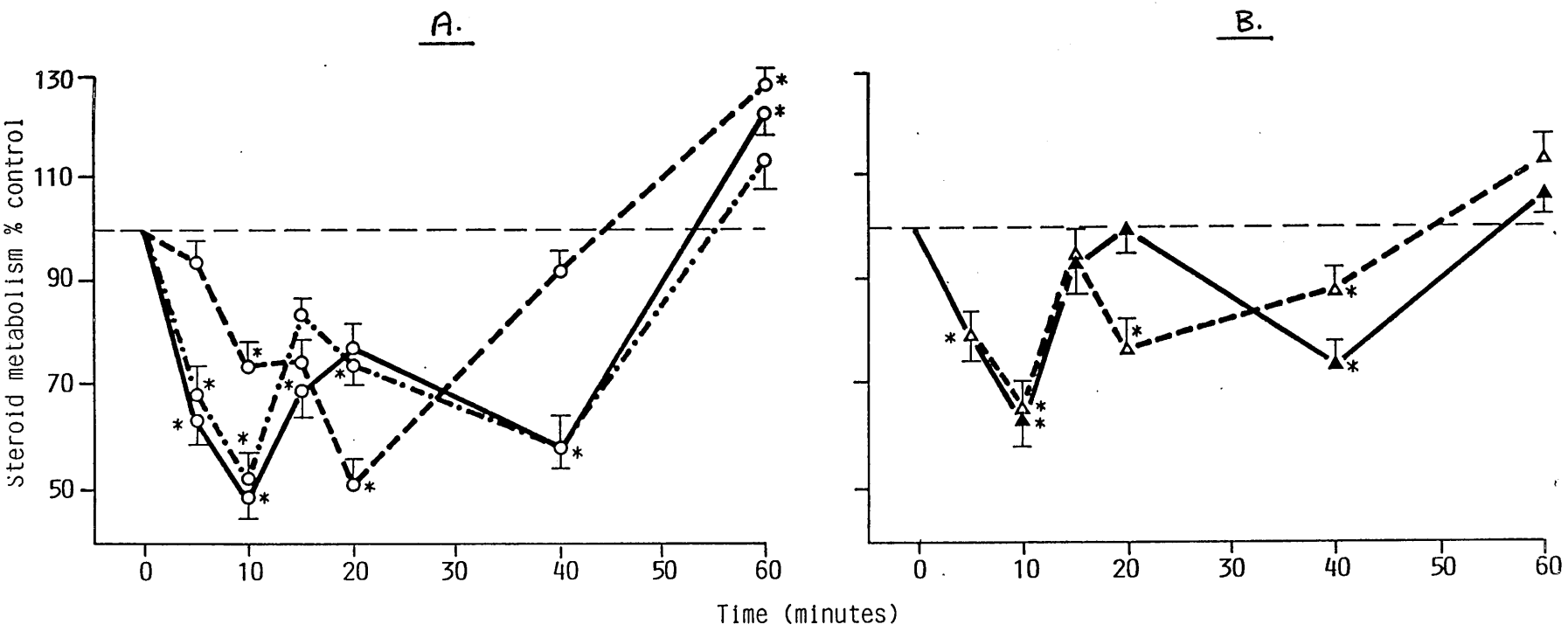


FIG. 21

Changes in 7α (7αOH) (———), 6β (6βOH) (-·-·-) and 16α (16αOH) (– – – –) activities with time in hepatocytes isolated from male rats following incubation with vasopressin (100μM). Results expressed as percentage control ± S.D. of six cell samples (*p<0.01). Control activities expressed as pmol metabolite formed per min. per 10^6 cell:-

	7αOH	6βOH	16αOH
5 min	52.1 ± 10.1	60.8 ± 6.1	46.3 ± 6.4
10 min	77.4 ± 9.3	86.3 ± 8.1	69.8 ± 6.3
15 min	71.2 ± 10	62.3 ± 7.8	52.6 ± 5.4
20 min	64.3 ± 8.1	75.6 ± 9.4	87.6 ± 4.1
40 min	86.7 ± 7.8	72.3 ± 7.9	63.9 ± 6.1
60 min	45.2 ± 6.1	55.4 ± 6.1	48.5 ± 3.1

Changes in 17OHSD (———), 5α (5αRED) (– – – –) activities with time in hepatocytes isolated from male rats, following incubation with Vasopressin (100μM). Results expressed as percentage control ± S.D. of six cell samples (*p<0.01). Control activities expressed as pmole metabolite formed per minute per 10^6 cell:-

	17OH S.D.	5αRED
5 min	66.3 ± 8.4	414 ± 11
10 min	86.4 ± 7.2	350 ± 7.6
15 min	64.6 ± 10.2	294 ± 8.3
20 min	69.1 ± 6.3	325 ± 6.0
40 min	75.5 ± 9.1	349 ± 7.1
60 min	65.2 ± 8.2	275 ± 4.6

TABLE 13A

	7αOH	6βOH	16αOH	17OHSD	5αRed.
5 min	52.1±8.9	60.8±6.1	46.3±6.4	66.3±8.4	414±11
10 min	77.4±9.3	86.3±8.1	69.8±6.3	86.4±7.4	350±8
15 min	71.2±7.7	62.3±7.8	52.6±5.4	64.6±10.2	294±8
20 min	64.3±8.1	75.6±9.4	87.6±4.1	69.1±6.3	325±6
40 min	86.7±7.8	72.3±7.9	63.9±6.1	75.5±9.1	349±7
60 min	45.2±6.1	55.4±6.1	48.5±3.1	65.2±8.2	275±5

TABLE 13B

	7αOH	6βOH	16αOH	17OHSD	5αRed.
5 min	*33.6±5.1	*41.2±6.2	43.2±9.1	*52.4±6.1	*286±4
10 min	*37.2±2.1	*44.3±8.2	*51.4±8.2	*53.9±2.1	*227±3
15 min	*49.4±7.1	52.1±8.1	39.6±2.4	59.6±8.4	276±5
20 min	49.3±6.9	*55.3±3.1	*44.1±7.4	68.4±6.1	247±6
40 min	*50.2±5.1	*42.6±8.1	62.3±4.1	*55.2±5.5	*303±5
60 min	55.4±5.1	65.4±3.1	64.5±3.1	69.8±9.1	308±4

Table 13: Changes in the activities of 7α-hydroxylase (7αOH), 6β-hydroxylase (6βOH), 16α-hydroxylase (16αOH), 17-hydroxysteroid dehydrogenase (17OHSD) and 5α-reductase (5αRed.) with time in isolated male rat hepatocytes from control (Table 13A) and vasopressin-treated (100μM)(Table 13B) samples. Results expressed as pmole metabolite formed per minute per 10^6 cells ± S.D. of 6 cell samples. (* $p<0.01$)

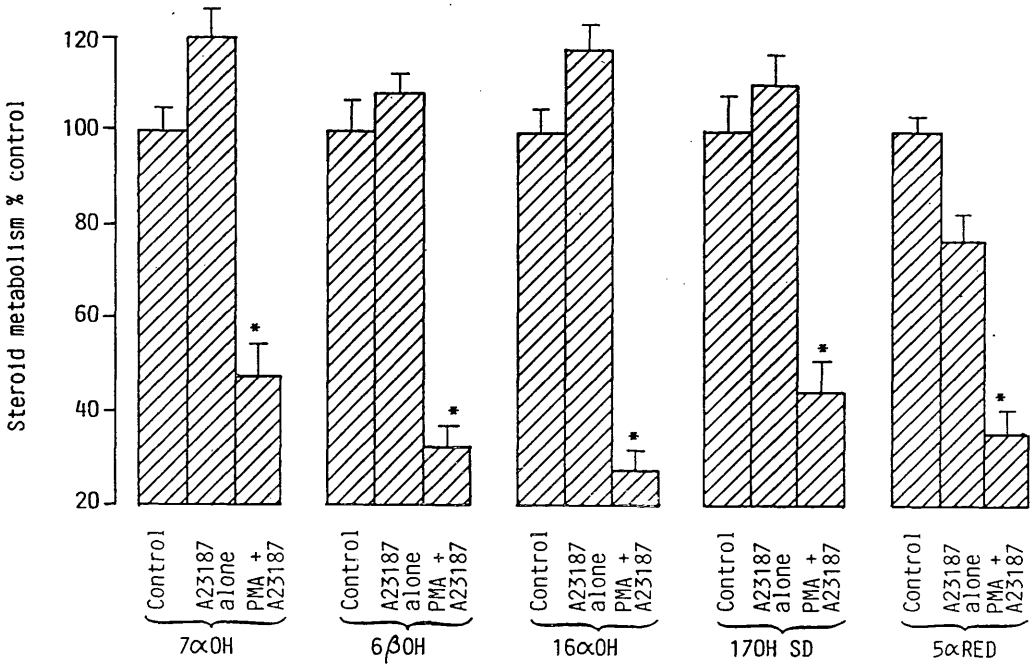


FIG. 22.

Changes in activity of the enzymes metabolising Androstene dione after 60 mins. incubation with either A23187 alone (100nM) or PMA + A23187 in combination (50nM/100nM). Results expressed as percentage control ± S.D. for six cell samples. Control activities expressed as pmole metabolite formed per minute per 10^6 cells:-

	7αOH	6βOH	16αOH	17OH S.D.	5αRED
60 min.	73.1 ± 5.1	130 ± 6.2	179 ± 5.2	240 ± 8.3	590 ± 4.2

	7αOH	6βOH	16αOH	17OHSD	5αRed.
Control	73.1±5.1	130±6	179±5	240±8	590±4
A23187	90.4±8.2	141±7	212±3	259±5	453±7
PMA+A23187	*35.1±7.1	*43.2±9.1	*50.4±4	*109±7	*213±3

Table 14: Changes in the activities of 7α-hydroxylase (7αOH), 6β-hydroxylase (6βOH), 16α-hydroxylase (16αOH), 17-hydroxysteroid dehydrogenase (17OHSD) and 5α-reductase (5αRed.) in isolated male rat hepatocytes after 60 minutes preincubation from control, A23187 alone (100nM) and PMA + A23187 (50nM + 100nM respectively) samples. Results expressed as pmole metabolite per minute per 10^6 cells ± S.D. of 6 cell samples. (* $p<0.01$)

When A23187 (100nM) and PMA (50nM) were used in combination, the activities of all the enzymes was found to be markedly reduced. These reductions were of greater magnitude than for PMA acting alone (section 3.2(a), Figures 12 & 13) and the reductions were; 7α-hydroxylase and 17-hydroxysteroid dehydrogenase both reduced to 46% of control, 6β-hydroxylase and 5α-reductase both reduced to 35% of control and 16α-hydroxylase reduced to 28% of control. All of the reductions were statistically significant ($p<0.01$). In section 3.2(a), the greatest reduction seen for PMA (50nM) was to 54% of control. The effects of A23187 (100nM) and A23187 + PMA (100nM & 50nM) upon enzyme activity are shown in Table 14.

3.7 Effect of Angiotensin II upon metabolism of 4-androstene-3,17-dione in isolated male rat hepatocytes.

The effect of the calcium-dependent hormone, angiotensin II (100nM), upon the metabolism of 4-androstene-3,17-dione is shown in figure 23A & 23B. The activities of the enzymes metabolising 4-androstene-3,17-dione were found to all show an increase in activity when incubated with angiotensin II. The maximum increases in activity were shown at different times for different enzymes. 7α-hydroxylase showed its maximum increase in activity at 20 minutes (143% of control), 6β-hydroxylase was maximal at 60 minutes (149% of control), 16α-hydroxylase was maximal at 10 minutes (161% of control), 17-hydroxysteroid dehydrogenase was maximal at 40 minutes (159% of control) and 5α-reductase was maximal at 40 minutes (126% of control). All of these maximum increases

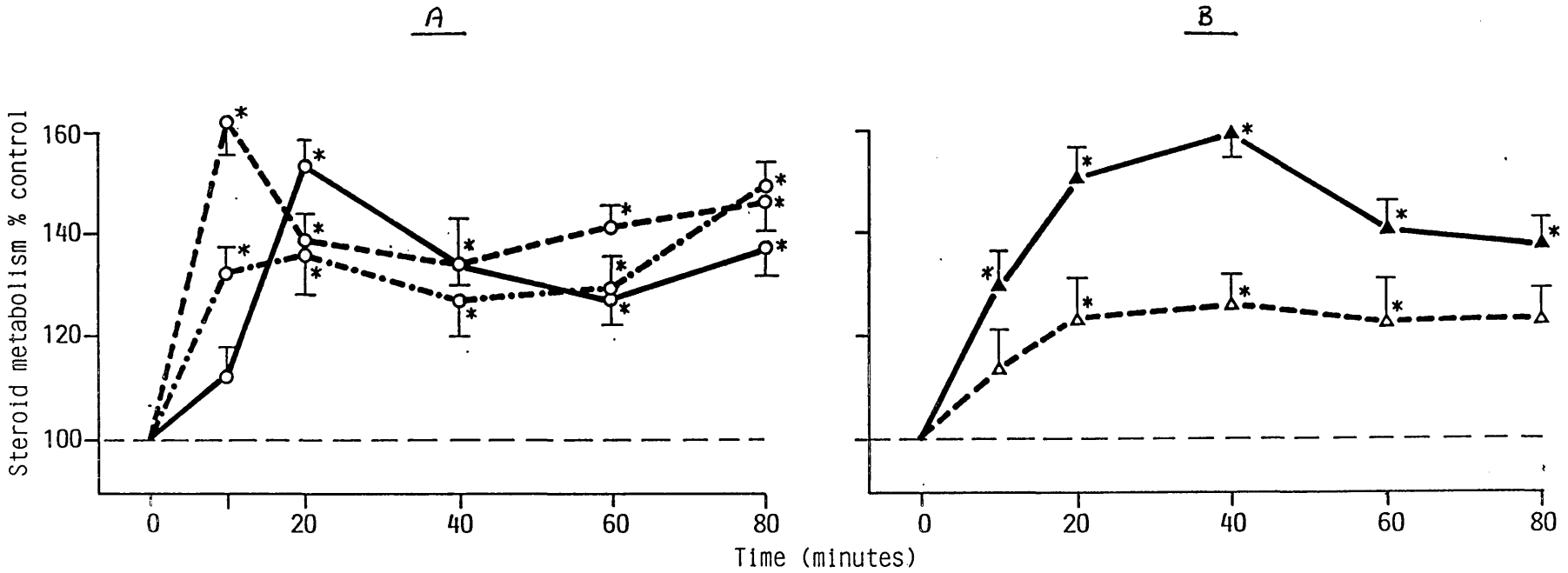


FIG. 23

Changes in 7α (7αOH) (———), 6β (6βOH) (-·-·-·) and 16α (16αOH) (- - - -) activities with time in hepatocytes isolated from male rats following incubation with Angiotensin II (100nM). Results expressed as percentage control ± S.D. of six cell samples (*p<0.01). Control activities expressed as pmol metabolite formed per min. per 10^6 cell:-

	7αOH	6βOH	16αOH
10 min	128 ± 7.4	132 ± 4.2	111 ± 9.6
20min	112 ± 6.1	131 ± 8.1	101 ± 9.1
40 min	110 ± 4.1	132 ± 10	112 ± 6.1
60 min	108 ± 9.1	129 ± 8.2	110 ± 2.1
80 min	111 ± 8.2	99 ± 4.1	98 ± 8.1

Changes in 17OHSD (———), 5α (5αRED) (- - - -) activities with time in hepatocytes isolated from male rats following incubation with Angiotensin II (100nM). Results expressed as percentage control ± S.D. of six cell samples (*P<0.01). Control activities expressed as pmole metabolite formed per minute per 10^6 cell:-

	17OH S.D.	5αRED
10 min	142 ± 6.2	460 ± 9.2
20 min	132 ± 2.1	418 ± 8.1
40 min	105 ± 7.3	411 ± 9.5
60 min	130 ± 2.4	408 ± 9.5
80 min	115 ± 9.1	392 ± 6.9

TABLE 15A

	7αOH	6βOH	16αOH	17OHSD	5αRed.
10 min	128±7	132±4	111±9	142±6	460±9
20 min	112±6	131±8	101±9	132±2	418±8
40 min	110±4	132±10	112±6	105±7	411±9
60 min	108±9	129±8	110±2	130±2	408±9
80 min	111±8	99±4	98±8	115±9	392±7

TABLE 15B

	7αOH	6βOH	16αOH	17OHSD	5αRed.
10 min	143±2	*174±6	*180±4	*185±3	519±6
20 min	*171±2	*178±4	*140±5	*199±2	*516±5
40 min	*147±3	*167±6	*150±3	*167±7	518±9
60 min	*138±2	*167±3	*155±4	*183±2	501±8
80 min	152±3	*148±9	*143±4	*159±2	442±5

Table 15: Changes in the activities of 7α-hydroxylase (7αOH), 6β-hydroxylase (6βOH), 16α-hydroxylase (16αOH), 17-hydroxysteroid dehydrogenase (17OHSD) and 5α-reductase (5αRed.) with time in isolated male rat hepatocytes from control (Table 15A) and angiotensin II-treated (100nM)(Table 15B) samples. Results expressed as pmole metabolite formed per minute per 10^6 cells ± S.D. of 6 cell samples. (* $p<0.01$)

were statistically significant ($p<0.01$). The activity of all the enzymes in the presence of angiotensin II (100nM) with time are shown in Table 15.

3.8 Assessment of Protein Kinase C activation by 4β-Phorbol-12-myristate-13-acetate.

The activation of the enzyme, protein kinase C, by the active phorbol ester 4β-phorbol-12-myristate-13-acetate (PMA) was assessed by the method of Pelech et al (1986). Figure 24 shows the result. It is clear that as the concentration of the phorbol ester increases from 0.1nM to 100nM, the activity of protein kinase C in each fraction is altered. Increasing the concentration of PMA causes the activity of protein kinase C in the soluble fraction to decrease, from 115 fmole ATP per minute per 10^6 cells at 0.1nM PMA to 40 fmole ATP per minute per 10^6 cells at 100nM PMA. The soluble fraction in this assay represents the cell cytosol of an intact cell, and so it appears that as the concentration of PMA is increased there is a gradual loss of protein kinase C from the cell cytosol. The particulate fraction, which is representative of the plasma membrane of an intact cell, showed the opposite effect to the soluble fraction, that is increasing the concentration of PMA caused an increase in protein kinase C activity from 81 fmole ATP per minute per 10^6 cells at 0.1nM PMA to 122 fmole ATP per minute per 10^6 cells at 100nM PMA. The increase in activity of protein kinase C in the particulate fraction occurred concomitantly with the decrease in activity seen in the soluble fraction. This suggests that the loss of protein

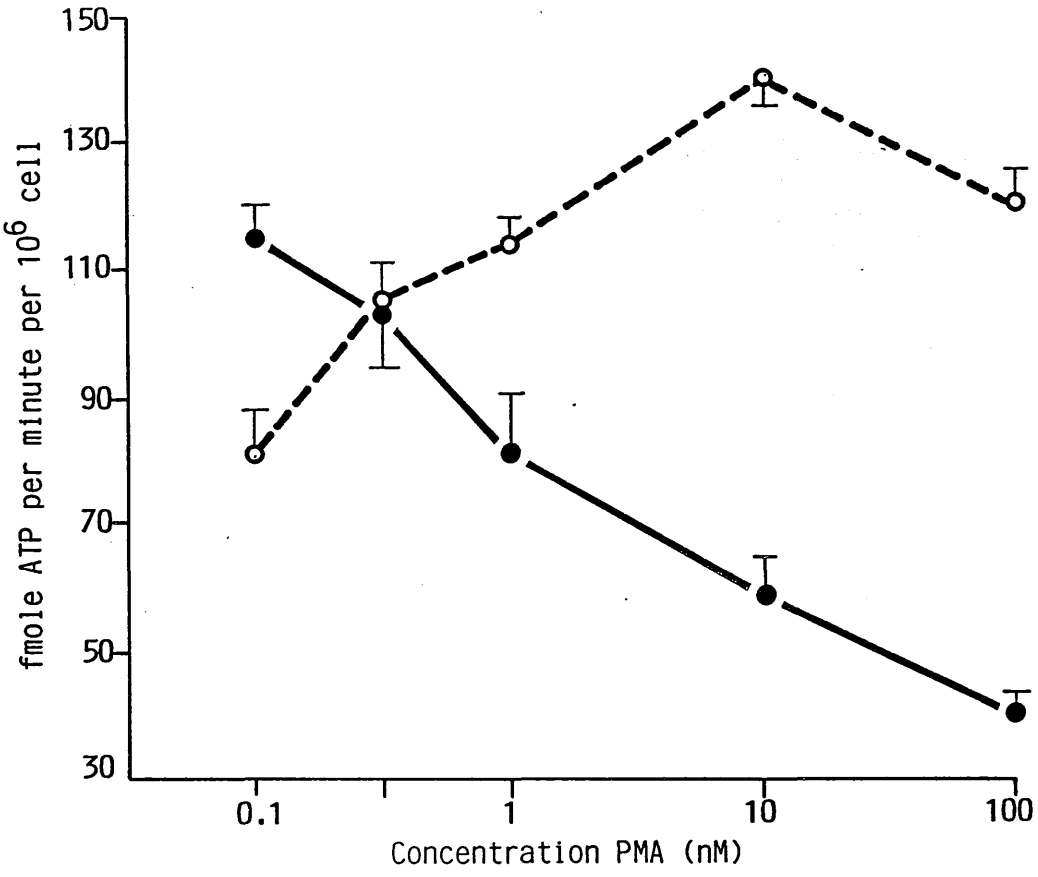


FIG. 24

Changes in Protein Kinase C activity in soluble (——) and particulate (– – – –) fractions with increasing concentrations of PMA. Control activities expressed as fmole ATP per minute per 10^6 cell ± S.D.

Soluble	65.7 ± 2.5
Particulate	129.4 ± 1.2

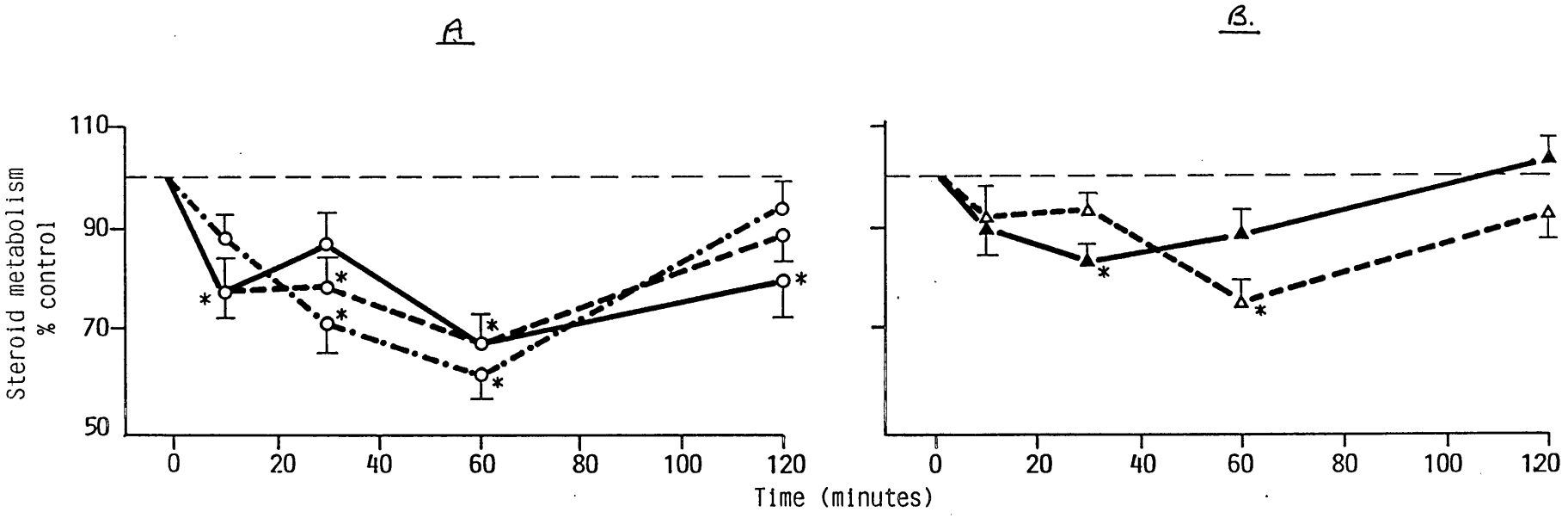
kinase C activity from the soluble fraction is reflected by the increase in activity of protein kinase C in the particulate fraction. This effect is characteristic of the fact that protein kinase C has undergone translocation, from cytosol to plasma membrane, and activation of protein kinase C has subsequently occured at the plasma membrane. The maximum level of protein kinase C activity in the particulate fraction was seen at 10nM PMA, which correlates well with the concentration of PMA used in our earlier studies (50nM).

3.9 Effects of 4β-Phorbol-12-myristate-13-acetate upon the NADPH/Cytochrome P450 enzyme system.

3.9(a) Effects of homogenisation and addition of excess cofactors on the actions of 4β-Phorbol-12-myristate-13-acetate in male rat hepatocytes.

The time-course of the effect of the active phorbol ester PMA (50nM) in homogenised hepatocytes which have been supplemented with an excess of the cofactor NADPH, before determination of 4-androstene-3,17-dione metabolism is shown in Figure 25A & 25B.

As was apparent in the intact hepatocyte (Figure 12 & 13), all of the enzymes metabolising 4-androstene-3,17-dione underwent an inhibition of activity in the presence of PMA, with the maximum reduction in activity being observed at 60 minutes (the same as the time of the maximum effect of PMA in section 3.2(a)). The reductions in activity were to 60 - 80% of control and were statistically significant



FIG·2S

Changes in 7α (7αOH) (———), 6β (6βOH) (-·-·-·) and 16α (16αOH) (– – – –) activities with time in hepatocytes isolated from male rats following incubation with PMA (50nM) in the presence of excess NADPH. Results expressed as percentage control ± S.D. of six cell samples (*p<0.01). Control activities expressed as pmol metabolite formed per min. per 10^6 cell:-

	7αOH	6βOH	16αOH
10 min	52 ± 3.1	60 ± 8.2	57 ± 3.1
30 min	42 ± 4.2	69 ± 9.1	58 ± 7.4
60 min	43 ± 6.1	59 ± 6.3	54 ± 6.2
120 min	39 ± 9.2	54 ± 9.3	43 ± 4.1

Changes in 17OHSD (———), 5α (5αRED) (– – – –) activities with time in hepatocytes isolated from male rats following incubation with PMA (50nM) in the presence of excess NADPH. Results expressed as percentage control ± S.D. of six cell samples (*p<0.01). Control activities expressed as pmole metabolite formed per minute per 10^6 cell:-

	17OH S.D.	5αRED
10 min	106 ± 4.2	307 ± 6.7
30 min	112 ± 3.1	258 ± 3.2
60 min	97 ± 5.1	260 ± 2.5
120 min	90 ± 3.1	265 ± 3.1

TABLE 16A

	7αOH	6βOH	16αOH	17OHSD	5αRed.
10 min	52±3	60±8	57±3	106±4	307±7
20 min	42±4	69±9	58±7	112±3	258±3
30 min	43±6	59±6	54±6	97±5	260±3
60 min	39±9	54±9	43±4	90±3	265±3

TABLE 16B

	7αOH	6βOH	16αOH	17OHSD	5αRed.
10 min	*40.2±5.1	53.2±7.1	*44.3±6.1	94.2±2.9	279±7
20 min	36.4±8.1	*49.4±9.4	*45.2±9.1	*93.4±4.5	239±3
40 min	*29.2±7.4	*36.4±7.1	*36.4±3.2	86.3±2.8	*196±3
60 min	*31.4±4.2	50.2±8.1	38.2±6.1	93.4±2.8	244±3

Table 16: Changes in the activities of 7α-hydroxylase (7αOH), 6β-hydroxylase (6βOH), 16α-hydroxylase (16αOH), 17-hydroxysteroid dehydrogenase (17OHSD) and 5α-reductase (5αRed.) with time in isolated male rat hepatocytes from control (Table 16A) and PMA-treated (50nM)(Table 16B) samples in the presence of excess cofactor, NADPH. Results expressed as pmole metabolite formed per minute per 10^6 cells ± S.D. of 6 cell samples. (* $p<0.01$)

($p<0.01$).

The effects of PMA acting in the presence of excess NADPH are shown in Table 16.

3.9(b) Effects on the rate of loss of NADPH caused by 4β-Phorbol-12-myristate-13-acetate in male rat hepatocytes.

The time-course of the rate of degradation of NADPH in homogenised hepatocytes from control (DMSO) or PMA-treated samples (50nM), is shown in Figure 26. As time proceeds, the rate of loss of NADPH in both control and PMA-treated samples remains at a fairly constant level (between 0.65 and 0.85 µmole NADPH per minute per 10^6 cells). There is little difference between the rate of loss of NADPH for control and PMA-treated samples, except at 40 and 50 minutes where there is a difference between control and PMA-treated samples - control samples metabolised 0.67 µmole NADPH per minute per 10^6 cells and PMA-treated samples 0.89 µmole NADPH per minute per 10^6 cells. The deviation between the two samples was not, however, statistically significant.

The effect of PMA (50nM) upon the rate of loss of NADPH is shown in Table 17.

3.9(c) Effect of 4β-Phorbol-12-myristate-13-acetate upon NADPH/Cytochrome c (P450) Reductase in male rat hepatocytes.

The effect of PMA (50nM) upon NADPH/cytochrome c (P450) reductase activity with time is shown in Figure 27.

In both control (DMSO) and PMA-treated samples, there is a gradual decline in cytochrome c (P450) reductase activity after 20 minutes, but there is no significant

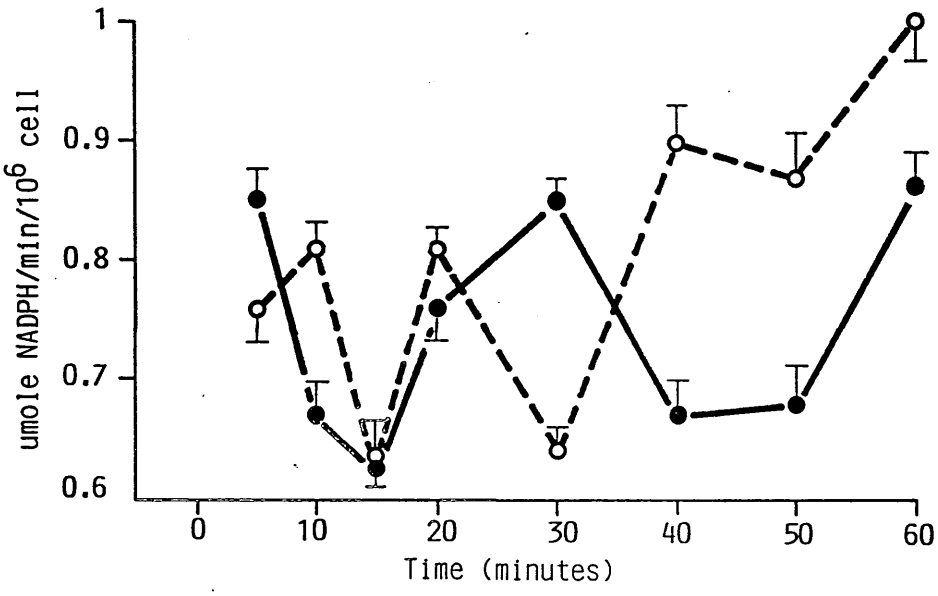


FIG.26

Rate of degradation of NADPH with time in hepatocytes isolated from male rats after incubation with DMSO [Control] (——) and PMA (50nM) (– – –). Results expressed as µmole NADPH/minute/10^6 cells. Control activities expressed as µmole NADPH/minutes/10^6 cell:-

	5 min	10 min	15 min	20 min	30 min	40 min	50 min	60 min
Control	0.85 ± 0.36	0.67 ± 0.39	0.62 ± 0.24	0.76 ± 0.43	0.85 ± 0.26	0.67 ± 0.2	0.67 ± 0.46	0.86 ± 0.38

Time(Min)	Control	PMA
5	850±4	761±3
10	669±4	808±5
15	624±2	631±5
20	764±4	814±4
30	853±3	640±4
40	665±2	898±2
50	669±5	865±4
60	855±4	1000±3

Table 17: Rate of loss of the cofactor NADPH from isolated male rat hepatocytes from control and PMA-treated (50nM) samples. Results expressed as nmole NADPH per minute per 10^6 cells ± S.D. of 9 cell samples.

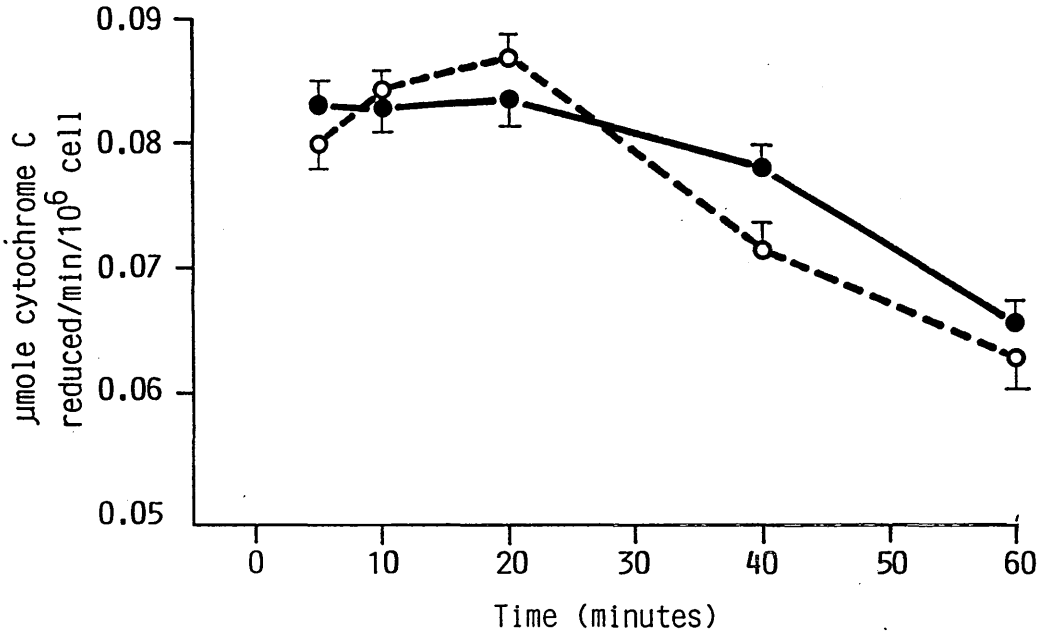


FIG 27

Reduction of cytochrome C (P450) reductase in the presence of DMSO (Control) (——) and PMA (50nM) (– – –) with time in hepatocytes isolated from male rats. Control activities expressed as µmole cytochrome C reduced/minute/10^6 cell:-

	5 min	10 min	20 min	40 min	60 min
Control	0.083 ± 0.025	0.083 ± 0.015	0.084 ± 0.015	0.078 ± 0.02	0.066 ± 0.014

Time(Min)	Control	PMA
5	83±2	80±2
10	83±2	84±2
20	84±1	87±2
40	78±2	72±2
60	66±4	63±4

Table 18: Effect of PMA (50nM) in isolated male rat hepatocytes upon NADPH cytochrome c (P450) reductase with time. Results expressed as nmole cytochrome c per minute per 10^6 cells ± S.D. of 9 cell samples.

difference between the two samples.

The effect of PMA (50nM) upon NADPH cytochrome c (P450) reductase with time is shown in Table 18.

3.10 Effect of Cycloheximide upon the actions of 4β-Phorbol-12-myristate-13-acetate in electropermeabilised cells.

The effects of PMA (50nM) upon the metabolism of 4-androstene-3,17-dione in the absence and presence of cycloheximide (10μM) are shown in Figure 28 (A - E). Cycloheximide, an inhibitor of mRNA translation at the ribosomal level (Peshka, 1971), was used to determine whether or not there was a role of protein synthesis in the action of PMA.

Blocking protein synthesis with cycloheximide was found to cause an inhibition of PMAs effect upon all the enzymes metabolising 4-androstene-3,17-dione. This effect occured throughout the time course, apart from 17-hydroxysteroid dehydrogenase, which still showed a statistically significant reduction in activity at 10 minutes in the presence of cycloheximide (74% of control, $p<0.01$). PMA acting alone reduced activity in the same manner as before (section 3.2) and maximum reductions in activity were at 40 minutes. Significant reductions in activity ($p<0.01$) were seen for all enzymes from 20 minutes onwards.

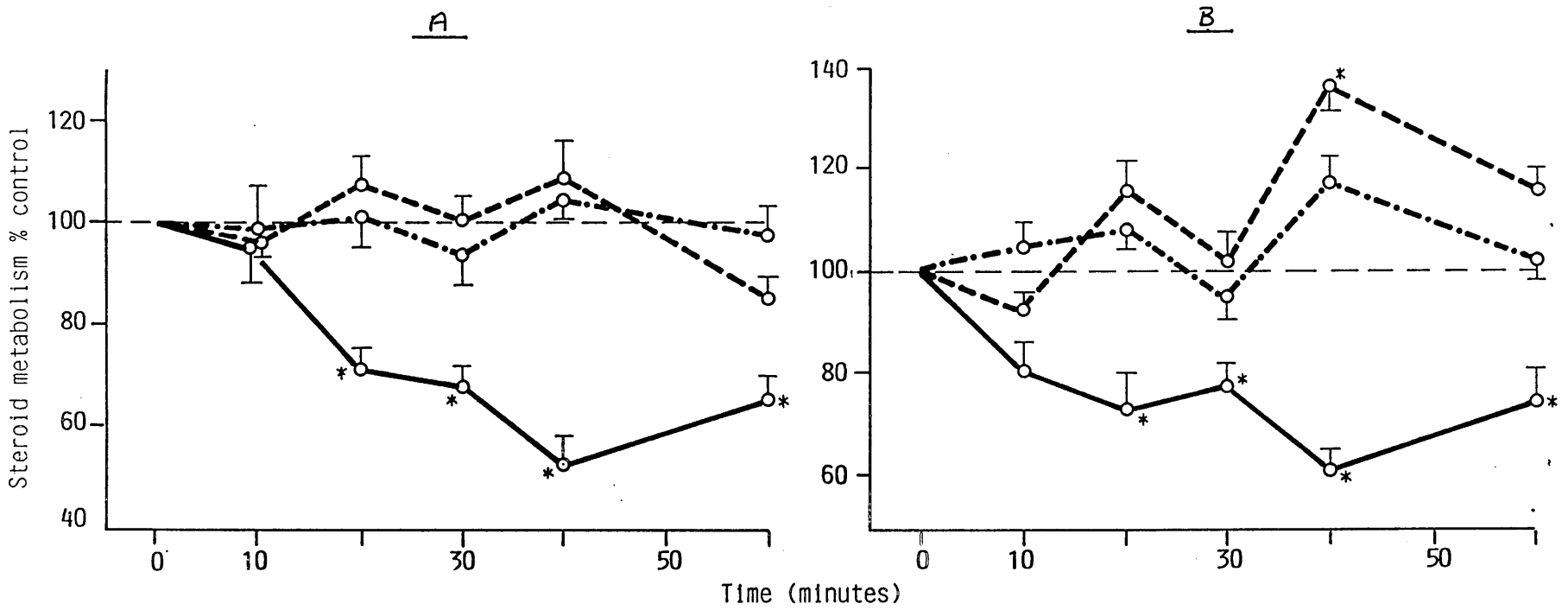


FIG. 28

Changes in the activity of 7αOH in hepatocytes isolated from male rats following incubation with PMA (50nM) (——), (Control + Cycloheximide [10μM]) (-·-·-) and (PMA (50nM)) + Cycloheximide [10μM]) (- - - -). Results expressed as percentage control ± S.D. of six cell samples (*p<0.01). Control activities expressed as pmol metabolite formed per min. per 10^6 cell:-

	10 min	20 min	30 min	40 min	60 min
Control	84 ± 5.3	101 ± 6.4	99 ± 2.1	119 ± 8.4	105 ± 4.2
Control (Cycloheximide)	83 ± 2.9	102 ± 5.3	92 ± 3.8	123 ± 5.1	102 ± 4.1
(PMA + Cycloheximide)	81 ± 3.1	108 ± 2.5	99 ± 6.7	128 ± 8.9	89 ± 5.4

Changes in the activity of 6β (6βOH) in hepatocytes isolated from male rats after incubation with PMA (50nM) (——), [Control + Cycloheximide (10μM)](-·-·-) and (PMA (50nM) + Cycloheximide (10μM)] (- - - -). Results expressed as percentage control ± S.D. of six cell samples (*p<0.01). Control activities expressed as pmol metabolite formed per minute per 10^6 cell:-

	10 min	20 min	30 min	40 min	60 min
Control	91 ± 10.1	89 ± 9.8	86 ± 4.8	114 ± 11.1	89 ± 4.3
Control (Cycloheximide)	96 ± 4.2	97 ± 4.3	82 ± 3.1	134 ± 6.1	92 ± 3.4
(PMA + Cycloheximide)	85 ± 3.1	103 ± 9.4	88 ± 6.2	156 ± 4.2	103 ± 4.6

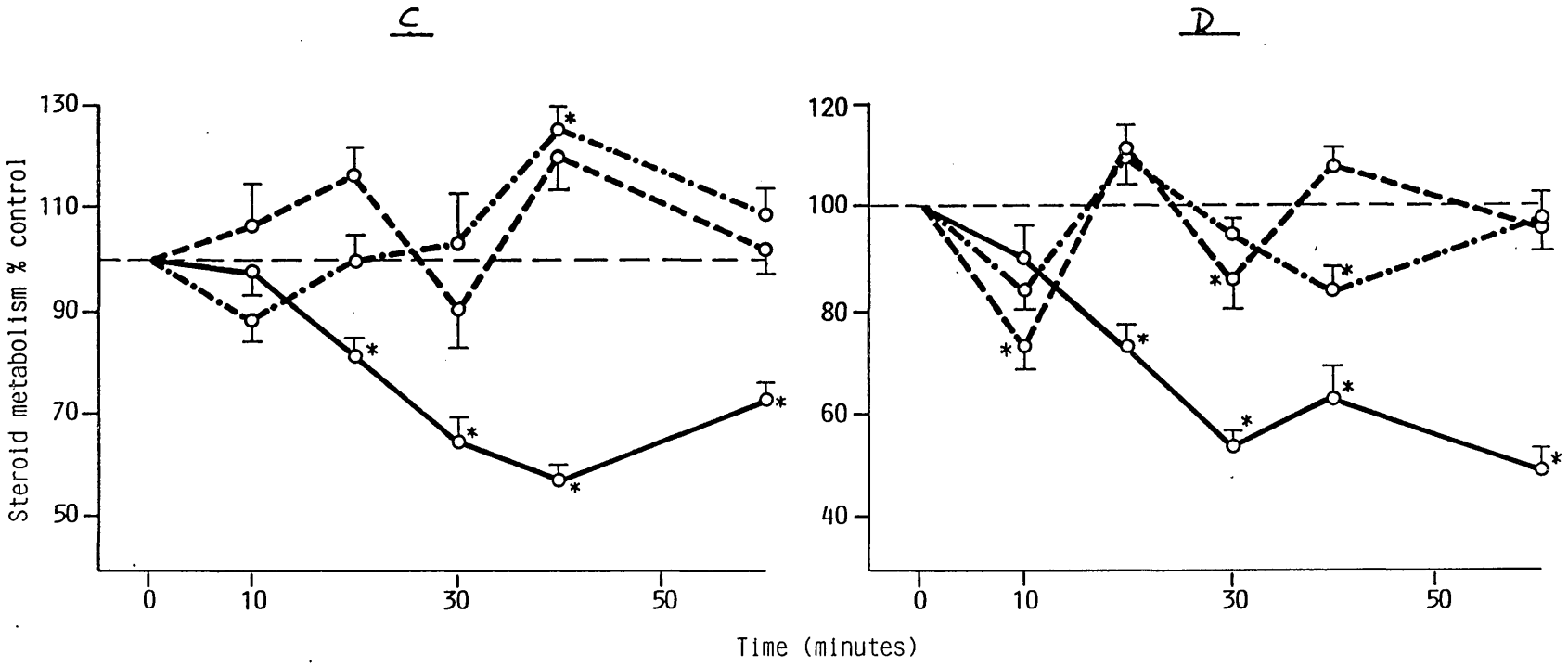


FIG. 28 Changes in the activity of 16α (16αOH) in hepatocytes isolated from male rats following incubation with PMA (50nM) (——), (Control + Cycloheximide [10μM]) (–·–·–) and (PMA (50nM)) + Cycloheximide [10μM]) (– – – –). Results expressed as percentage control ± S.D. of six cell samples (*$p<0.01$). Control activities expressed as pmol metabolite formed per min. per 10^6 cell:-

	10 min	20 min	30 min	40 min	60 min
Control	103±3.1	99±8.1	110±9.4	122±3.2	104±6.1
Control (Cycloheximide)	91±6.2	99±10	113±7.2	153±6.1	113±9
(PMA + Cycloheximide)	110±9.1	115±7.1	99±9.1	146±8.1	106±5.3

Changes in the activity of 17OH S.D. in hepatocytes isolated from male rats after incubation with PMA (50nM) (——), [Control + Cycloheximide (10μM)](–·–·–) and (PMA (50nM) + Cycloheximide (10μM)] (– – – –). Results expressed as percentage control ± S.D. of six cell samples (*$p<0.01$). Control activities expressed as pmol metabolite formed per minute per 10^6 cell:-

	10 min	20 min	30 min	40 min	60 min
Control	154±9.2	131±5.4	145±4.1	154±7.1	162±4.1
Control (Cycloheximide)	129±3.1	144±8.9	138±6.1	130±9.4	158±9.2
(PMA + Cycloheximide)	113±5.1	145±6.2	125±6.1	165±5.1	156±4.2

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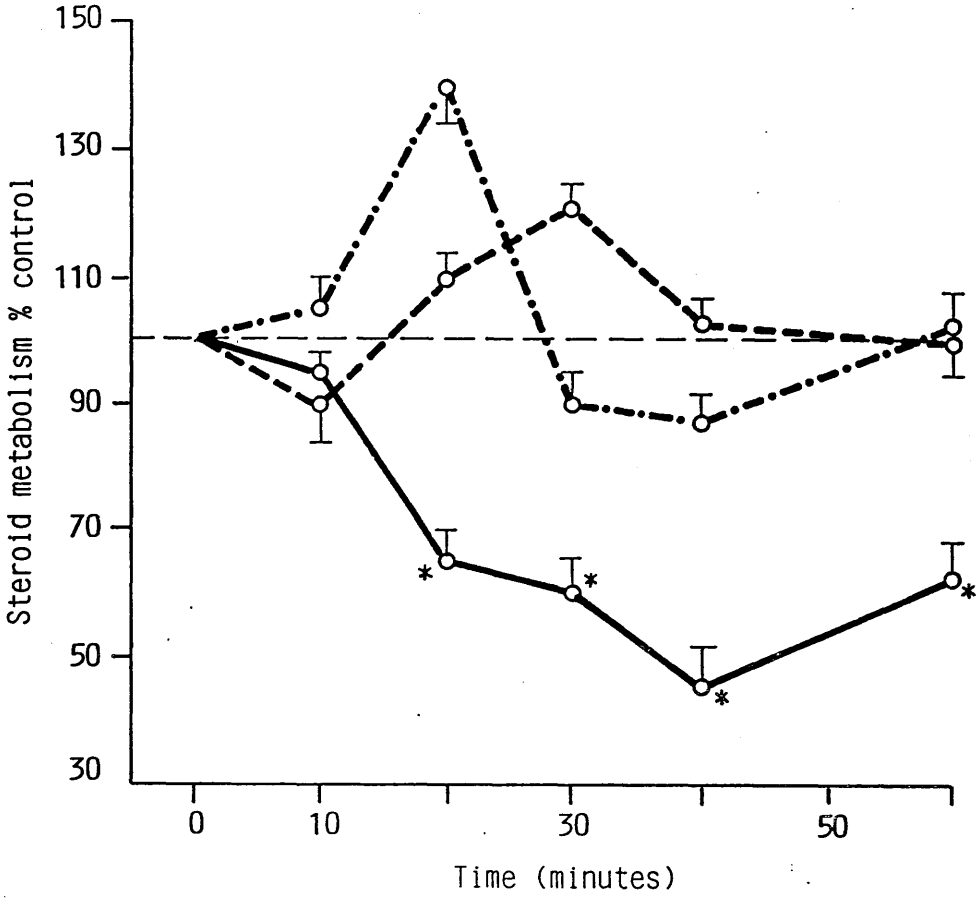


FIG. 28

Changes in the activity of 5α (5αRED) in hepatocytes isolated from male rats following incubation with PMA (50nM) (——), (Control + Cycloheximide [10μM]) (-·-·-·) and (PMA (50nM) + Cycloheximide [10μM]) (– – –). Results expressed as percentage control ± S.D. of six cell samples (*p<0.01). Control activities expressed as pmol metabolite formed per min. per 10^6 cell:-

	10 min	20 min	30 min	40 min	60 min
Control	774 ± 7.5	514 ± 5.2	612 ± 4.1	796 ± 9.8	613 ± 5.2
Control (Cycloheximide)	809 ± 8.6	721 ± 6.1	552 ± 5.8	691 ± 10.6	624 ± 4.2
(PMA + Cycloheximide)	697 ± 6.7	563 ± 5.4	743 ± 3.3	821 ± 7.2	612 ± 5.2

3.11 The effects of protein kinase inhibitors upon the actions of 4β-Phorbol-12-myristate-13-acetate in alteration of 4-androstene-3,17-dione metabolism in electropermeabilised hepatocytes.

3.11(a) Effect of K252a (general non-selective protein kinase inhibitor).

The effect of K252a (20nM) (inhibits all protein kinases) (Kase et al, 1986; Kase et al 1987), is shown in Figure 29 (A - E). This shows the effect of K252a upon the time course of PMA's (50nM) effect for each enzyme metabolising 4-androstene-3,17-dione.

For each enzyme, PMA acting alone reduces activity in its usual manner (as described in section 3.2(a), Figure 12). Maximum reductions were at 60 minutes and statistically significant ($p<0.01$). Cells with both PMA and K252a added show that PMA was still able to reduce enzyme activity in a similar way as to when PMA acted alone. Some enzymes still showed a maximum decrease in activity at 60 minutes, such as 6β-hydroxylase (84% control, $p<0.01$) and 16α-hydroxylase (69% of control, $p<0.01$).

3.11(b) Effect of KT5720 (inhibitor of cyclic AMP-dependent protein kinase).

The effect of preincubating hepatocytes with KT5720 (60nM) (specific protein kinase A inhibitor) upon the actions of PMA (50nM) on 4-androstene-3,17-dione metabolism is shown in Figure 30 (A - E).

PMA acting alone again produced its usual effect on

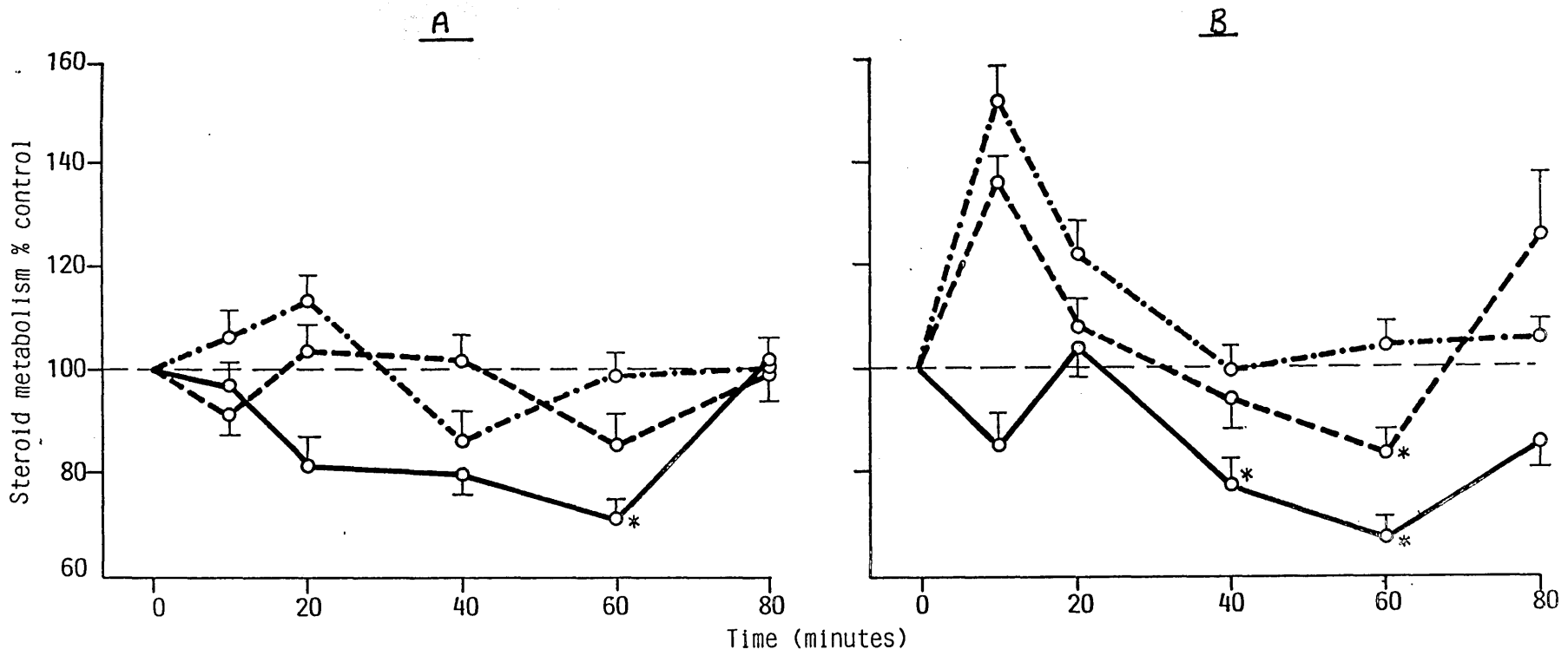


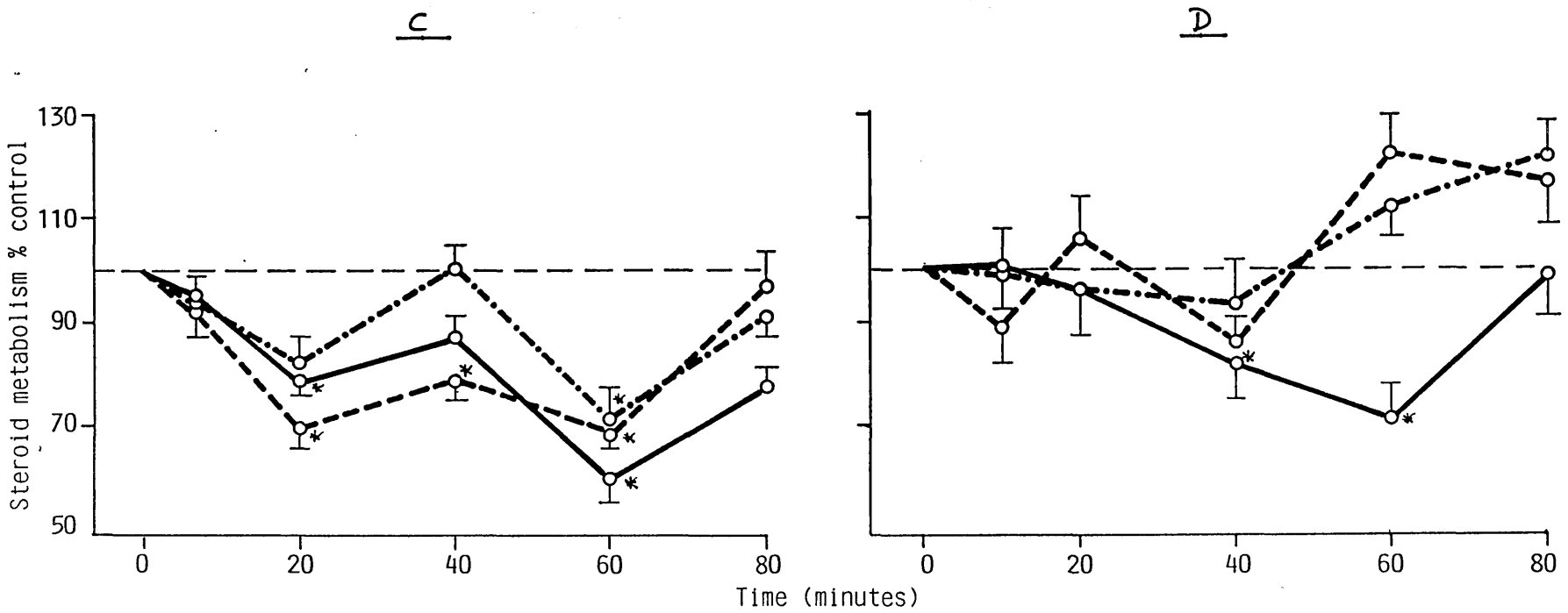
FIG. 29

Changes in the activity of 7α (7αOH) in hepatocytes isolated from male rats following incubation with PMA (50nM) (——), (Control + K252a [20nM]) (-·-·-·) and (PMA (50nM)) + K252a [20nM]) (- - -). Results expressed as percentage control ± S.D. of six cell samples (*p<0.01). Control activities expressed as pmol metabolite formed per min. per 10^6 cell:-

	10 min	20 min	40 min	60 min	80 min
Control	70 ± 3.1	70 ± 7.1	81 ± 8.1	80 ± 5.1	74 ± 9.2
Control (K252a)	74 ± 5.2	79 ± 6.2	70 ± 3.1	78 ± 7.4	74 ± 5.1
(PMA + K252a)	64 ± 6.1	73 ± 4.1	82 ± 5.1	68 ± 4.1	73 ± 5.4

Changes in the activity of 6β (6βOH) in hepatocytes isolated from male rats after incubation with PMA (50nM) (——), [Control + K252a (20nM)](-·-·-) and (PMA (50nM) + K252a (20nM)] (- - - -). Results expressed as percentage control ± S.D. of six cell samples (*p<0.01). Control activities expressed as pmol metabolite formed per minute per 10^6 cell:-

	10 min	20 min	40 min	60 min	80 min
Control	75 ± 5.1	73 ± 4.2	87 ± 3.1	83 ± 8.2	75 ± 3.2
Control (+ K252a)	113 ± 9.1	89 ± 4.1	86 ± 2.0	86 ± 7.1	79 ± 8.4
(PMA + K252a)	102 ± 4.2	79 ± 6.1	82 ± 6.1	69 ± 6.4	94 ± 10.5



FIG·29

Changes in the activity of 16αOH in hepatocytes isolated from male rats following incubation with PMA (50nM) (——), (Control + K252a [20nM]) (-·-·-) and (PMA (50nM)) + K252a [20nM]) (- - -). Results expressed as percentage control ± S.D. of six cell samples (*p<0.01). Control activities expressed as pmol metabolite formed per min. per 10^6 cell:-

	10 min	20 min	40 min	60 min	80 min
Control	66 ± 9.1	71 ± 5.3	70 ± 7.3	103 ± 2.3	79 ± 3.2
Control (K252a)	62 ± 7.3	58 ± 6.4	70 ± 5.6	73 ± 2.3	72 ± 9.4
(PMA + K252a)	61 ± 9.3	50 ± 6.1	55 ± 5.3	71 ± 3.2	77 ± 9.1

Changes in the activity of 17OH S.D. in hepatocytes isolated from male rats after incubation with PMA (50nM) (——), [Control + K252a (20nM)](-·-·-) and (PMA (50nM) + K252a (20nM)] (- - - -). Results expressed as percentage control ± S.D. of six cell samples (*p<0.01) Control activities expressed as pmol metabolite formed per minute per 10^6 cell:-

	10 min	20 min	40 min	60 min	80 min
Control	99 ± 12.1	94 ± 10.2	97 ± 7.3	84 ± 8.6	82 ± 7.3
Control (+ K252a)	97 ± 11.3	90 ± 7.4	93 ± 6.3	94 ± 7.3	100 ± 6.2
(PMA + K252a)	88 ± 8.2	100 ± 8.4	83 ± 3.1	92 ± 6.4	96 + 5.2

E

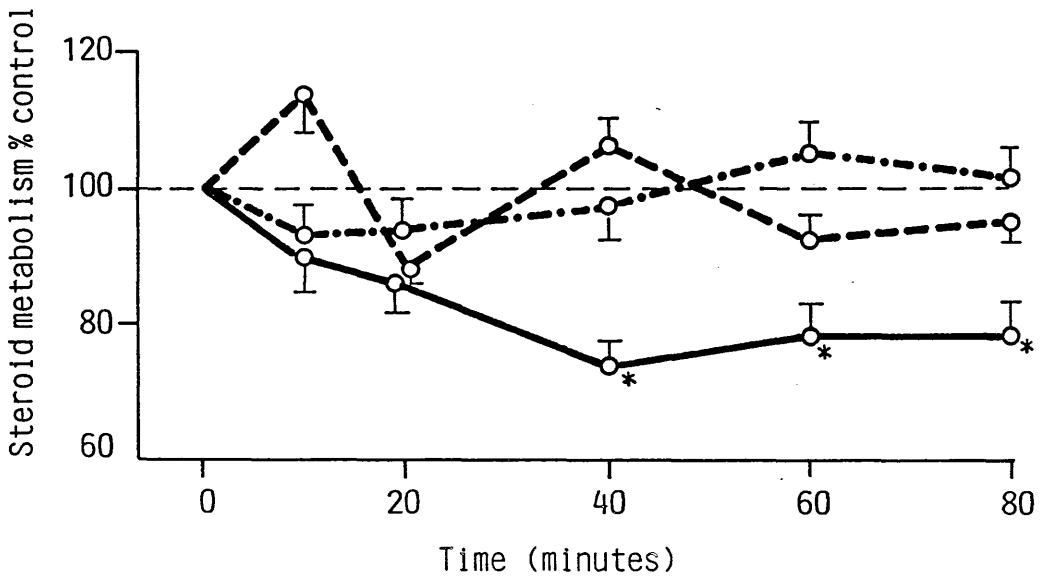


FIG. 29

Changes in the activity of 5α (5αRED) in hepatocytes isolated from male rats following incubation with PMA (50nM) (——), (Control + K252a [20nM]) (-·-·-) and (PMA (50nM) + K252a [20nM]) (– – –). Results expressed as percentage control ± S.D. of six cell samples (*$p<0.01$). Control activities expressed as pmol metabolite formed per min. per 10^6 cell:-

	10 min	20 min	40 min	60 min	80 min
Control	385 ± 9.3	436 ± 4.2	386 ± 7.1	375 ± 5.2	374 ± 3.4
Control + K252a	357 ± 8.6	409 ± 4.1	375 ± 5.2	394 ± 5.7	378 ± 2.6
(PMA + K252a)	437 ± 7.0	385 ± 4.1	408 ± 4.3	345 ± 8.3	355 ± 3.6

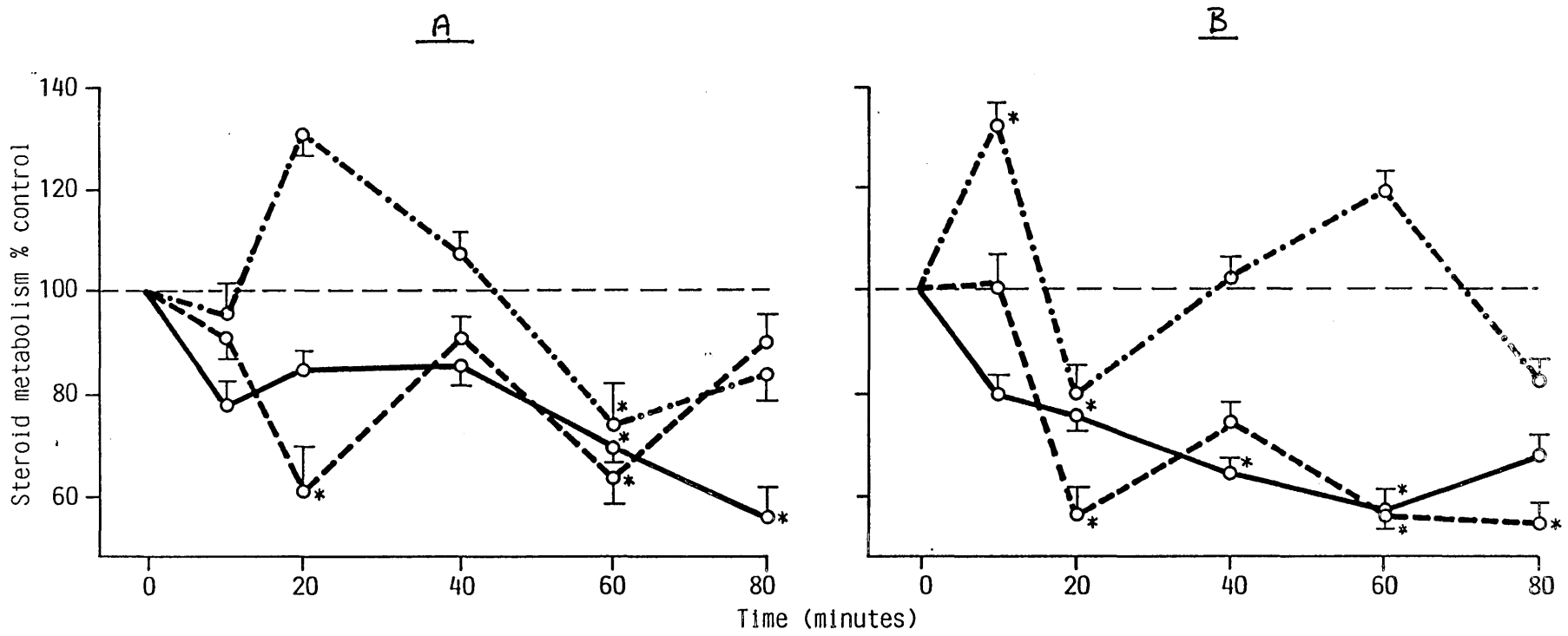


FIG.30

Changes in the activity of 7α (7αOH) in hepatocytes isolated from male rats following incubation with PMA (50nM) (——), (Control + KT5720 [60nM]) (-·-·-·) and (PMA (50nM)) + KT5720 [60nM]) (----). Results expressed as percentage control ± S.D. of six cell samples (*p<0.01). Control activities expressed as pmol metabolite formed per min. per 10^6 cell:-

	10 min	20 min	40 min	60 min	80 min
Control	126 ± 3.3	123 ± 3.1	95 ± 3.2	122 ± 7.1	125 ± 7.2
Control (+KT5720)	121 ± 2.1	161 ± 5.4	103 ± 3.1	90 ± 9.4	105 ± 4.2
(PMA + KT5720)	115 ± 2.6	75 ± 4.1	80 ± 2.1	78 ± 5.3	113 ± 3.1

Changes in the activity of 6β (6βOH) in hepatocytes isolated from male rats after incubation with PMA (50nM) (——), [Control + KT5720(60nM)](-·-·-) and (PMA (50nM) + KT5720 (60nM)] (-----). Results expressed as percentage control ± S.D. of six cell samples (*p<0.01). Control activities expressed as pmol metabolite formed per minute per 10^6 cell:-

	10 min	20 min	40 min	60 min	80 min
Control	158 ± 3.9	177 ± 8.4	169 ± 3.4	165 ± 3.9	199 ± 6.1
Control (+ KT5720)	212 ± 3.2	144 ± 7.3	176 ± 2.9	199 ± 5.1	166 ± 2.4
(PMA + KT5720)	161 ± 5.6	102 ± 6.3	128 ± 5.7	95 ± 2.4	112 ± 8.1

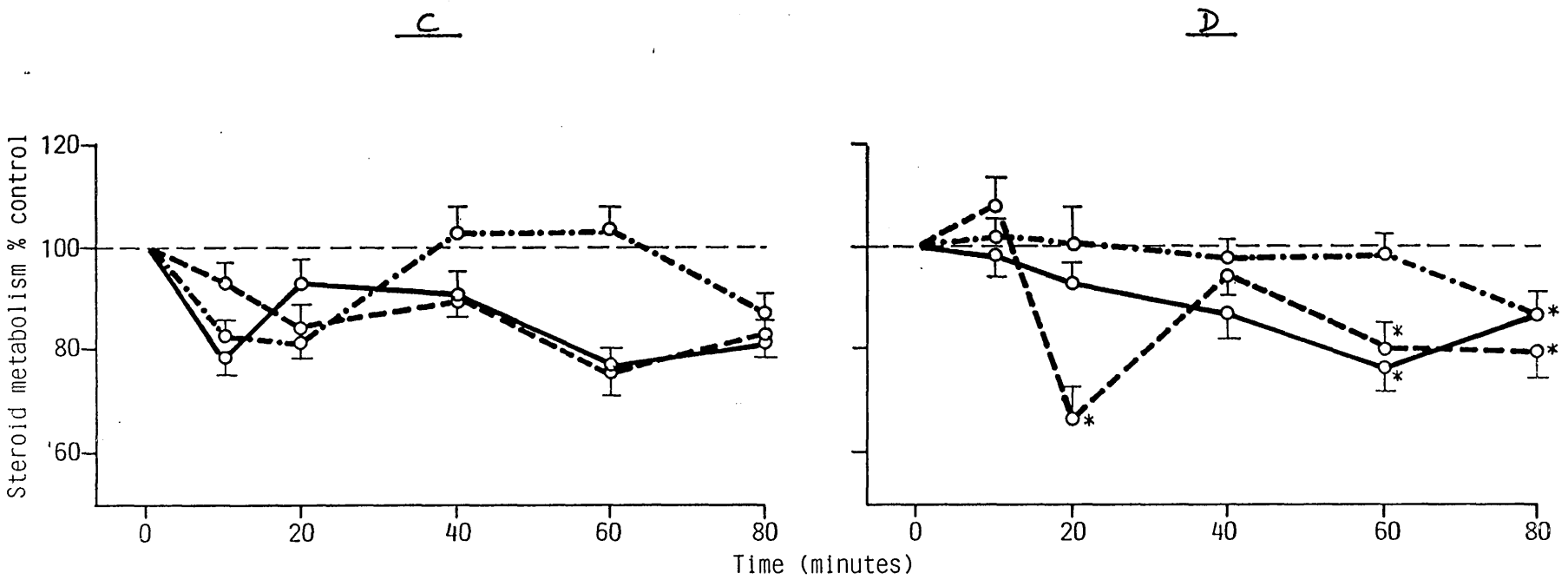


FIG.30 Changes in the activity of 16α (16αOH) in hepatocytes isolated from male rats following incubation with PMA (50nM) (——), (Control + KT5720 [60nM]) (-·-·-·) and (PMA (50nM)) + KT5720[60nM]) (- - - -). Results expressed as percentage control ± S.D. of six cell samples (*p<0.01). Control activities expressed as pmol metabolite formed per min. per 10^6 cell:-

	10 min	20 min	40 min	60 min	80 min
Control	184 ± 2.5	160 ± 3.4	143 ± 2.6	136 ± 2.1	161 ± 2.8
Control (+KT5720)	152 ± 2.7	130 ± 2.9	162 ± 3.5	142 ± 2.6	140 ± 2.8
(PMA + KT5720)	171 ± 5.0	134 ± 5.1	129 ± 3.1	104 ± 4.9	134 ± 3.1

Changes in the activity of 17OH S.D. in hepatocytes isolated from male rats after incubation with PMA (50nM) (——), [Control + KT5720 (60nM)](-·-·-) and (PMA (50nM) + KT5720 (60nM)] (- - - - -). Results expressed as percentage control ± S.D. of six cell samples (*P<0.01). Control activities expressed as pmol metabolite formed per minute per 10^6 cell:-

	10 min	20 min	40 min	60 min	80 min
Control	309 ± 2.4	314 ± 2.3	303 ± 2.6	302 ± 2.4	316 ± 3.8
Control (+ KT5720)	316 ± 2.3	314 ± 2.4	298 ± 2.5	300 ± 2.4	278 ± 2.3
(PMA + KT5720)	335 ± 5.2	207 ± 3.2	289 ± 3.1	243 ± 3.7	253 ± 2.9

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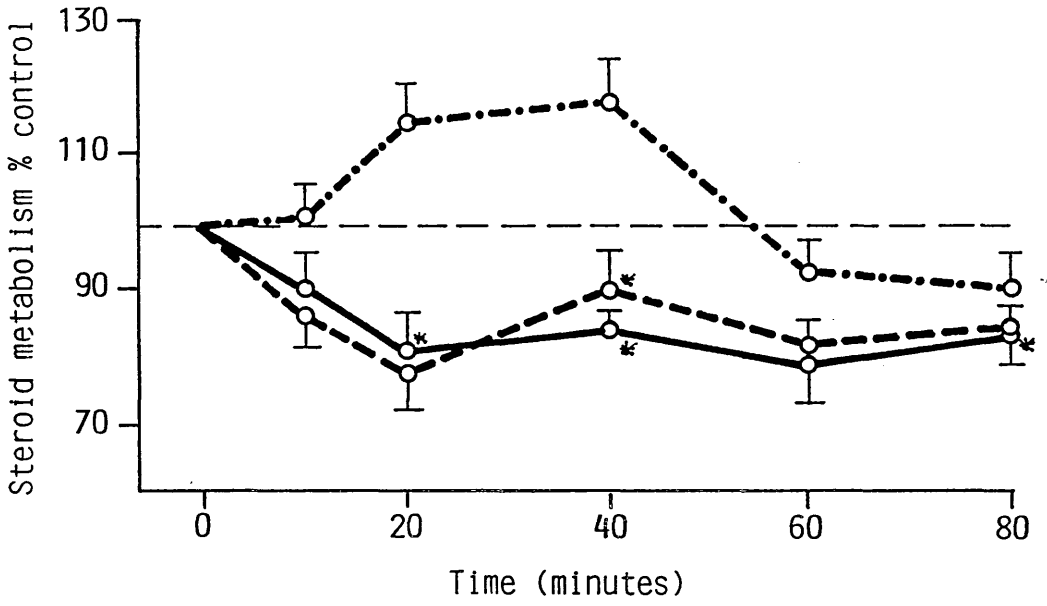

FIG.30 Changes in the activity of 5α (5αRED) in hepatocytes isolated from male rats following incubation with PMA (50nM) (——), (Control + KT5720 [60nM]) (-·-·-·) and (PMA (50nM) + KT5720 [60nM]) (----). Results expressed as percentage control ± S.D. of six cell samples (*$p<0.01$). Control activities expressed as pmol metabolite formed per min. per 10^6 cell:-

	10 min	20 min	40 min	60 min	80 min
Control	486 ± 5.8	499 ± 9.1	428 ± 5.0	478 ± 4.8	484 ± 9.1
Control + KT5720	493 ± 7.1	575 ± 8.4	503 ± 5.5	445 ± 3.3	436 ± 3.6
(PMA + KT5720)	417 ± 4.2	390 ± 6.9	387 ± 6.6	394 ± 5.8	462 ± 4.8

enzyme activity - maximum reductions at 60 minutes, $p<0.01$. Cells with PMA and KT5720 added also showed a reduction in activity for all enzymes at all time points studied. PMA in the presence of KT5720 was still able to cause significant reductions in enzyme activity for all the enzymes. Hence, PMA appears to be capable of reducing enzyme activity equally efficiently either alone or in the presence of KT5720.

3.11(c) Effect of K252b (specific inhibitor of protein kinase C).

The effect of preincubating hepatocytes with K252b (20nM) upon the actions of PMA (50nM) on metabolism of 4-androstene-3,17-dione are shown in Figure 31 (A - E).

Inhibiting protein kinase C with K252b revealed a more efficient blockade of PMA action than inhibition with K252a (Figure 29). K252b was found to block the effect of PMA for all enzymes studied and returned enzyme activities towards control levels at all time points investigated. PMA in the presence of K252b still showed several isolated reductions in activity, such as 7α-hydroxylase (86% of control at 60 minutes, $p<0.01$) and 17-hydroxysteroid dehydrogenase (80% of control at 20 minutes, $p<0.01$) but the overall effect was a blockade of PMA actions with K252b. PMA alone reduced activity in its usual way (as described in section 3.2(a)).

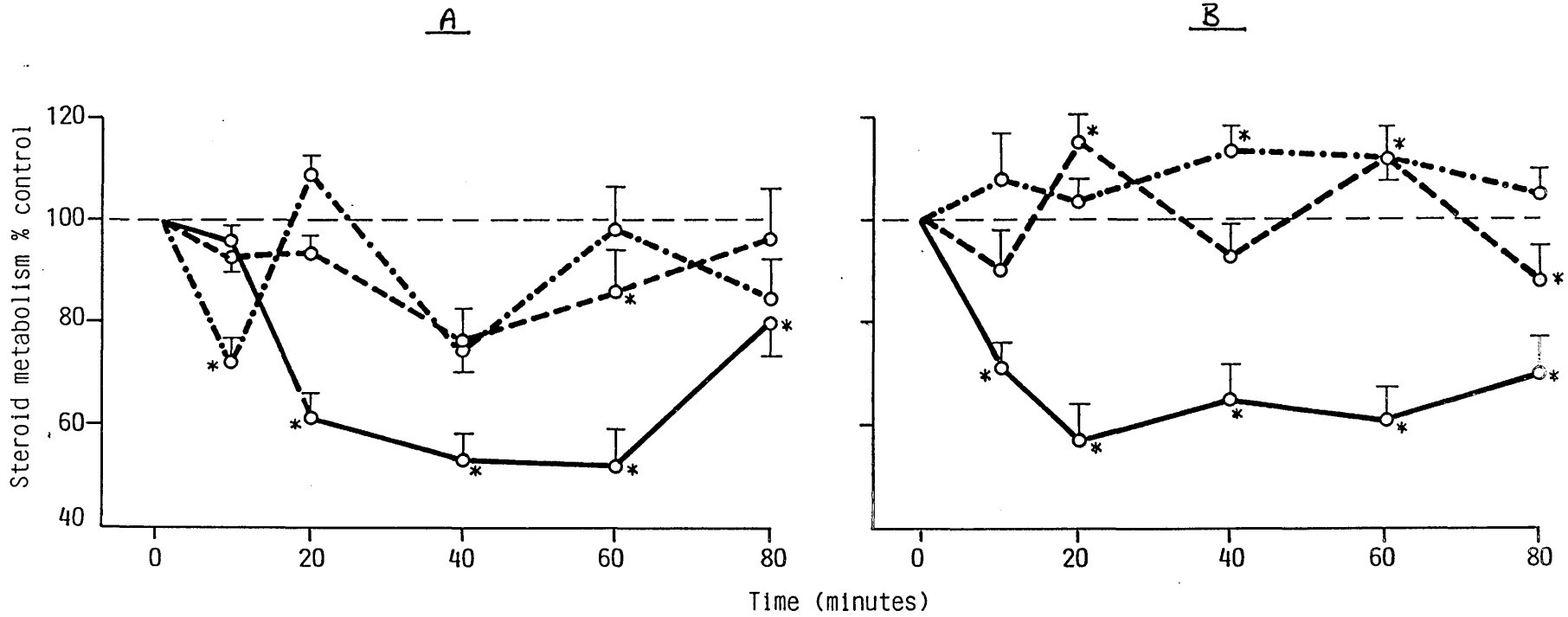

FIG.31 Changes in the activity of 7α (7αOH) in hepatocytes isolated from male rats following incubation with PMA (50nM) (——), (Control + K252b [20nM]) (-·-·-) and (PMA (50nM) + K252b [20nM]) (– – –). Results expressed as percentage control ± S.D. of six cell samples (*p<0.01). Control activities expressed as pmol metabolite formed per min. per 10^6 cell:-

	10 min	20 min	40 min	60 min	80 min
Control	130 ± 3.5	168 ± 2.6	184 ± 8.5	175 ± 9.6	137 ± 4.2
Control (+K252b)	94 ± 6.1	183 ± 4.3	136 ± 7.4	172 ± 9.8	117 ± 10.1
(PMA + K252b)	121 ± 8.1	158 ± 5.1	138 ± 6.2	150 ± 7.8	132 ± 9.4

Changes in the activity of 6β (6βOH) in hepatocytes isolated from male rats following incubation with PMA (50nM) (——), (Control + K252b [20nM]) (-·-·-) and (PMA (50nM) + K252b [20nM]) (– – –). Results expressed as percentage control ± S.D. of six cell samples (*p<0.01). Control activities expressed as pmol metabolite formed per min. per 10^6 cell:-

	10 min	20 min	40 min	60 min	80 min
Control	207 ± 7.6	187 ± 5.1	166 ± 9.1	155 ± 9.2	177 ± 4.1
Control (+K252b)	223 ± 7.6	192 ± 5.4	188 ± 7.6	173 ± 8.4	185 ± 5.3
(PMA + K252b)	187 ± 9.1	215 ± 5.2	155 ± 5.2	174 ± 8.3	155 ± 3.1

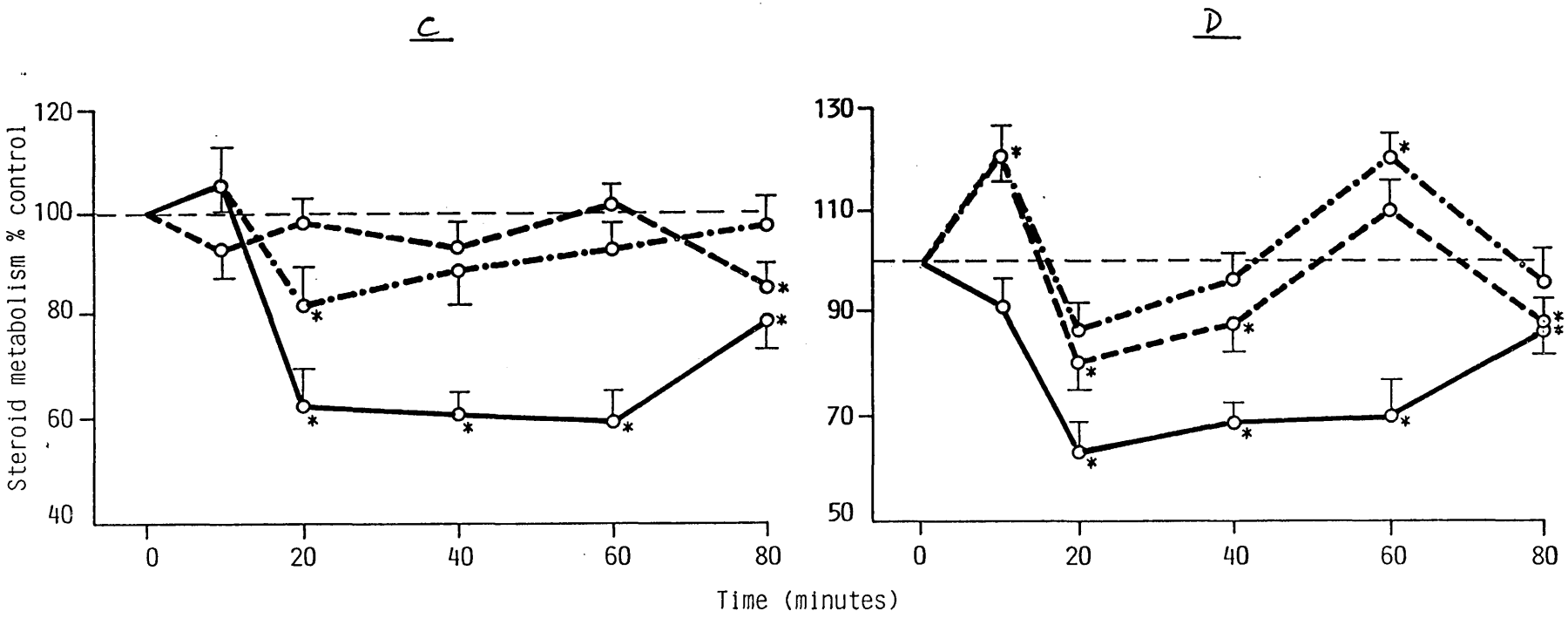

FIG. 31

Changes in the activity of 16α (16αOH) in hepatocytes isolated from male rats following incubation with PMA (50nM) (——), (Control + K252b [20nM]) (-·-·-) and (PMA (50nM) + K252b [20nM]) (– – –). Results expressed as percentage control ± S.D. of six cell samples (*p<0.01). Control activities expressed as pmol metabolite formed per min. per 10^6 cell:-

	10 min	20 min	40 min	60 min	80 min
Control	199 ± 8.2	214 ± 8.3	185 ± 9.1	181 ± 6.9	191 ± 5.6
Control (+K252b)	209 ± 8.4	176 ± 9.1	165 ± 9.1	161 ± 5.2	186 ± 5.0
(PMA + K252b)	186 ± 6.3	209 ± 8.4	172 ± 4.6	182 ± 5.1	163 ± 4.1

Changes in the activity of 17OH S.D. in hepatocytes isolated from male rats following incubation with PMA (50nM) (——), (Control + K252b [20nM]) (-·-·-) and (PMA (50nM) + K252b [20nM]) (– – –). Results expressed as percentage control ± S.D. of six cell samples (*p<0.01). Control activities expressed as pmol metabolite formed per min. per 10^6 cell:-

	10 min	20 min	40 min	60 min	80 min
Control	316 ± 3.2	396 ± 4.2	336 ± 6.1	305 ± 7.1	344 ± 3.5
Control (+K252b)	378 ± 2.9	340 ± 6.4	322 ± 5.3	364 ± 4.2	329 ± 6.1
(PMA + K252b)	379 ± 2.4	318 ± 4.4	295 ± 6.1	337 ± 3.6	301 ± 7.1

E

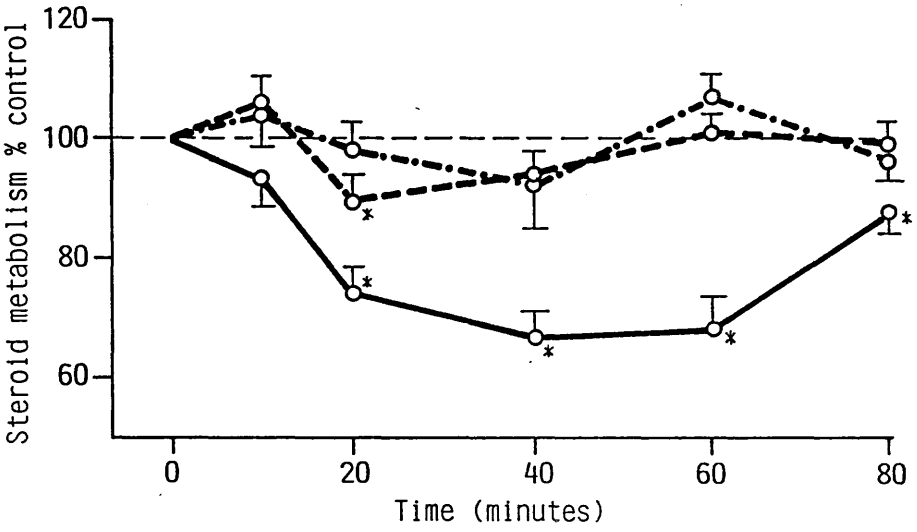

FIG. 31.

Changes in the activity of 5α (5αRED) in hepatocytes isolated from male rats following incubation with PMA (50nM) (——), (Control + K252b [20nM]) (-·-·-) and (PMA (50nM) + K252b [20nM]) (– – –). Results expressed as percentage control ± S.D. of six cell samples (*$p<0.01$). Control activities expressed as pmol metabolite formed per min. per 10^6 cell:-

	10 min	20 min	40min	60min	80 min
Control	516 ± 5.4	559 ± 5.2	512 ± 6.3	459 ± 3.0	465 ± 4.2
(Control + K252b)	535 ± 4.5	546 ± 7.2	473 ± 6.6	490 ± 3.2	448 ± 7.2
(PMA + K252b)	548 ± 6.3	498 ± 5.1	480 ± 4.1	462 ± 3.0	462 ± 3.2

3.12 Effect of 50nM 4β-Phorbol-12-myristate-13-acetate (PMA) upon the phosphorylation of protein in intact hepatocytes.

Figure 32 illustrates the SDS-PAGE of six different immunoprecipitated isozymes of cytochrome P450, precipitated by their specific antibodies . These isozymes were precipitated out from freshly isolated hepatocytes from male rats, which had not received any form of treatment, to assure us that the precipitation technique was working efficiently.

Figure 33 shows the effect of 50nM PMA upon the ^{32}P labelling of whole cells and the immunoprecipitated isozymes of cytochrome P450 UT-1 (Figure 33, Lane F), cytochrome P450 MC-1b (Lane G), cytochrome P450 PB2c (Lane H) and cytochrome P450 PB3a (Lane I). The immunoprecipitated samples were compared to whole cell samples from control (Lane B) and PMA-treated (Lane C) hepatocytes. The immunoprecipitated cytochrome P450 isozymes and PMA-treated whole cell samples were preincubated with PMA for 60 minutes before solubilisation (whole cells) or immunoprecipitation (isozymes).

It is apparent from Figure 33 that the whole cell samples show phosphate incorporation into proteins, which can be detected by autoradiography - Lane D is the autoradiogram of control whole cells (Lane B) and Lane E is the autoradiogram of PMA-treated whole cells (Lane C). However, the immunoprecipitated cytochrome P450 isozymes, which produce a reasonable amount of protein, as detected by SDS-PAGE, at approximate molecular weight 42,000 - 50,000, failed to show any incorporation of ^{32}P into any of the

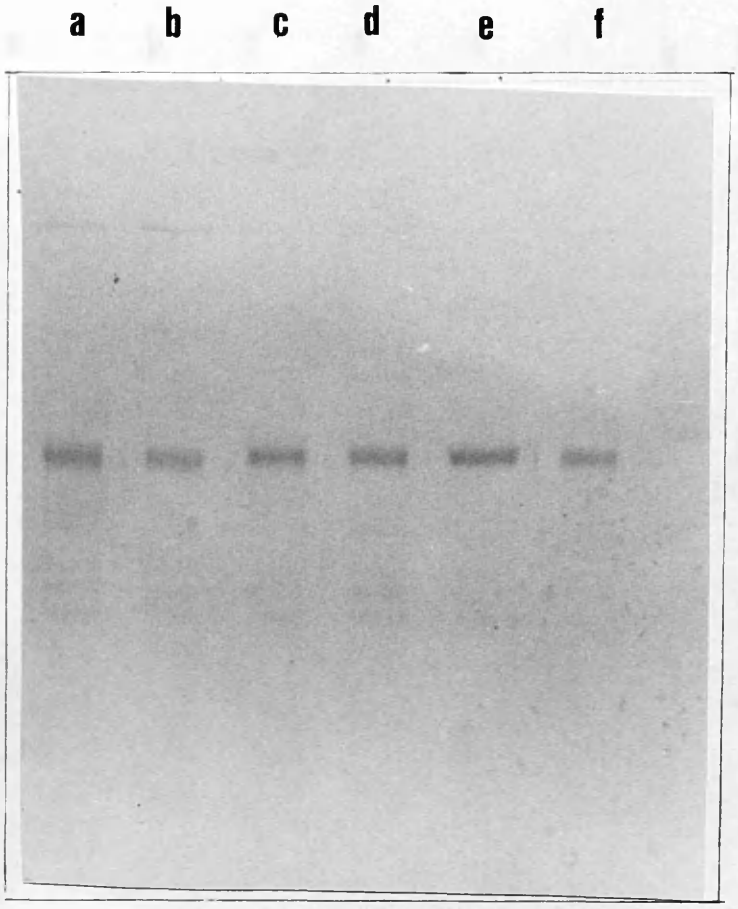

FIGURE 32. SDS-PAGE of a variety of immunoprecipitated cytochrome P450 isozymes from untreated male rat hepatocytes.

Lane A; Cytochrome P450IIC11 (P450 PB2c).

Lane B; Cytochrome P450IIA1 (P450 UT-1).

Lane C; Cytochrome P450IA1 (P450 MC-1b).

Lane D; Cytochrome P450IIB1 (P450 PB3a)

Lane E; Cytochrome P450IA2 (P450 MC-1a).

Lane F; Cytochrome P450IIC6 (P450 PB-1b).

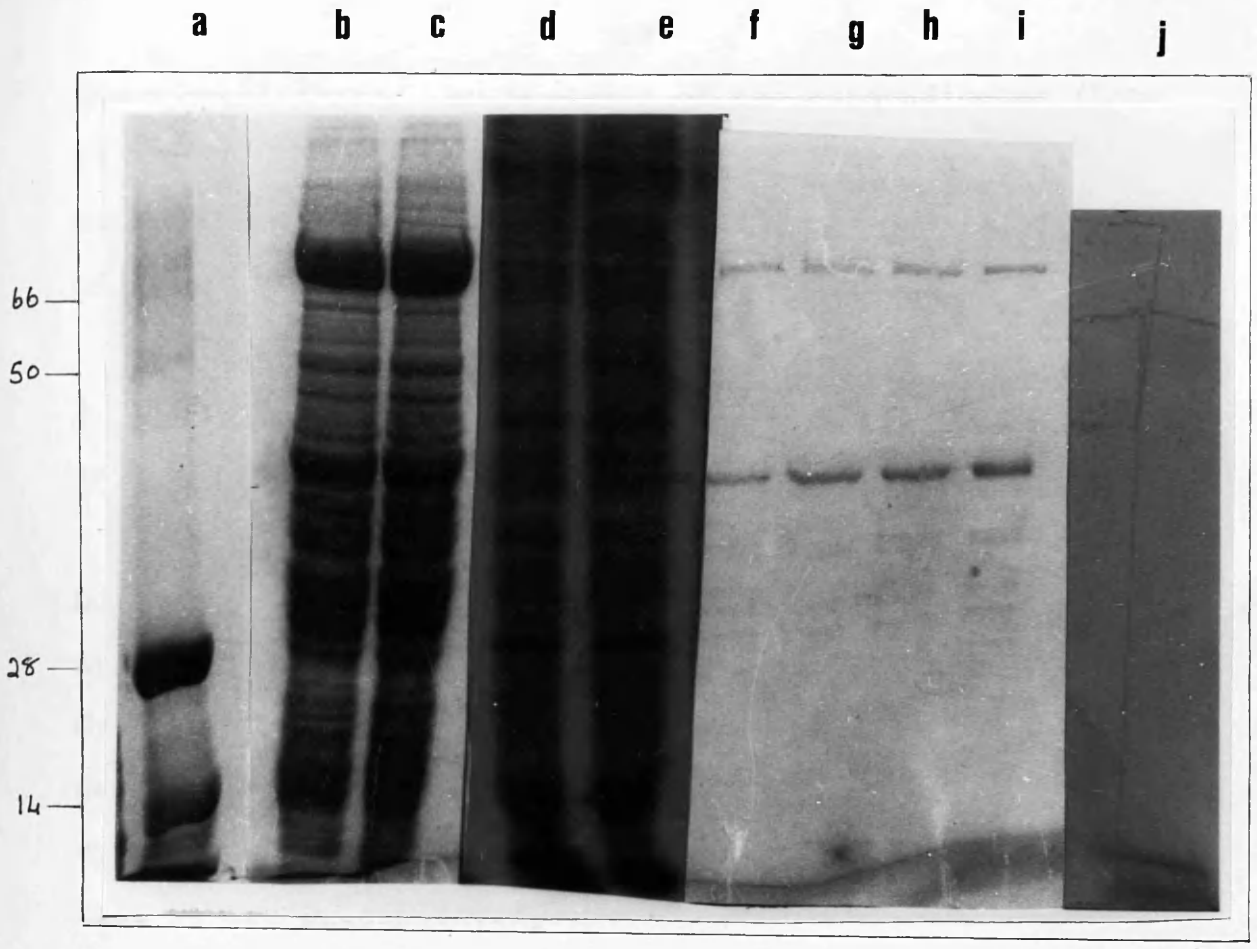

FIGURE 33. SDS PAGE and autoradiograms from intact male rat hepatocytes and immunoprecipitated cytochrome P450 isozymes from male rats, prelabelled with (^{32}P) and incubated for 60 minutes in the presence or absence (control) of 50nM 4β-Phorbol-12-myristate-13-acetate (PMA).

Lane A shows SDS PAGE of molecular weight markers. Lane B shows SDS PAGE of control whole cell hepatocytes. Lane C shows SDS PAGE of PMA-treated whole cell hepatocytes. Lane D shows the autoradiogram from control (Lane B) hepatocytes. Lane E shows autoradiogram of PMA-treated (Lane C) hepatocytes.

Lane F shows SDS PAGE of cytochrome P450IIA1 (P450 UT-1). Lane G shows SDS PAGE of cytochrome P450IA1 (P450 MC-1b) . Lane H shows SDS PAGE of cytochrome P450IIC11 (P450 PB2c). Lane I shows SDS PAGE of cytochrome P450 IIB1 (P450 PB3a). All immunoprecipitates treated with 50nM PMA for 60 minutes. Lane J shows the autoradiogram for a representative immunoprecipitated cytochrome P450 isozyme.

immunoprecipitates, by analysis of the autoradiogram (Lane J). Although only one autoradiogram is shown, the autoradiograms for all immunoprecipitated samples were identical, that is there is no incorporation of ^{32}P.

3.13 Assessment of Haem precipitation from whole cell samples and immunoprecipitated cytochrome P450 isozymes.

Table 19 shows the amount of haem precipitated from both whole cell and immunoprecipitated cytochrome P450 isozymes from control and PMA-treated (50nM) hepatocytes. Cytochrome P450 is a haem-containing flavoprotein and the detection of the presence of haem in the immunoprecipitated cytochrome P450 isozymes has been employed here to confirm that we are precipitating out haem, and hence cytochrome P450, in our samples.

It is apparent from table 19 that control whole cell samples contain the highest amount of haem (0.698 nmoles), whilst PMA-treated whole cell samples have significantly less (0.239 nmoles). The immunoprecipitated cytochrome P450 isozymes, which were preincubated with PMA (50nM) for 60 minutes before immunoprecipitation, showed very low levels of haem compared to control whole cell samples, although a reasonable amount of haem was detected for each immunoprecipitate (between 0.053 and 0.132 nmoles). The lower values in the immunoprecipitated samples reflect the fact that the immunoprecipitates contain only one form of cytochrome P450 each, whilst the whole cell sample contain the complete range of cytochrome P450 isozymes.

	nmole Haem per sample
Control (whole cell)	0.698±0.366
PMA (whole cell)	0.239±0.239
αPB UT-1 (+PMA)	0.132±0.108
αMC-1b (+PMA)	0.097±0.076
αPB 2c (+PMA)	0.053±0.081
αPB 3a (+PMA)	0.111±0.097

Table 19: Assessment of haem precipitation in whole cell preparations from control and PMA-treated (50nM) hepatocytes and in immunoprecipitated cytochrome P450 isozymes, treated with PMA (50nM) for 60 minutes. Results expressed as nmole haem produced per sample (mean) ± S.D. of 6 cell samples.

3.14 Direct analysis of ^{32}P incorporation into immunoprecipitated cytochrome P450 isozymes by liquid scintillation counting.

Table 20 shows the amount of ^{32}P present in whole cell and immunoprecipitated cytochrome P450 isozymes from control and PMA-treated (50nM for 60 minutes) samples.

Both control and PMA-treated whole cell samples show high counts for ^{32}P, although there is no significant difference in the amount of ^{32}P present in either sample, suggesting that PMA has not enhanced ^{32}P incorporation into the whole cell samples, compared to the control whole cell samples.

The immunoprecipitated cytochrome P450 isozymes show a markedly lower degree of ^{32}P present compared to the control whole cell samples, again reflecting the fact that only one isozyme of cytochrome P450 is present in each isozyme. The number of counts for each immunoprecipitate for control and PMA-treated samples shows no significant difference, again suggesting that PMA has not enhanced ^{32}P incorporation into the immunoprecipitated isozymes.

3.15 Effect of 50nM 4β-Phorbol-12-myristate-13-acetate (PMA) upon protein phosphorylation in electropermeabilised hepatocytes.

Figure 34 shows a typical gel from whole cell samples, prelabelled with (γ-^{32}P)ATP, and treated either with or without (control) PMA (50nM). Figure 34 shows an effect of PMA with time, 5 - 20 minutes.

	counts per minute per sample	% of control
Control (whole cell)	360613±7760	100
PMA (whole cell)	371156±10002	103
αPB UT-1 (control)	332±163	0.09
αMC-1b (control)	418±171	0.116
αPB 2c (control)	514±67	0.143
αPB 3a (control)	296±149	0.082
αPB UT-1 (+PMA)	571±352	0.158
αMC-1b (+PMA)	418±174	0.116
αPB 2c (+PMA)	513±353	0.142
αPB 3a (+PMA)	461±239	0.128

Table 20: Assessment of ^{32}P incorporation into whole cell and immunoprecipitated cytochrome P450 isozymes from control or PMA-treated (50nM for 60 minutes) hepatocytes. Results expressed as counts per minute per sample (mean) ± S.D. of 6 cell samples.

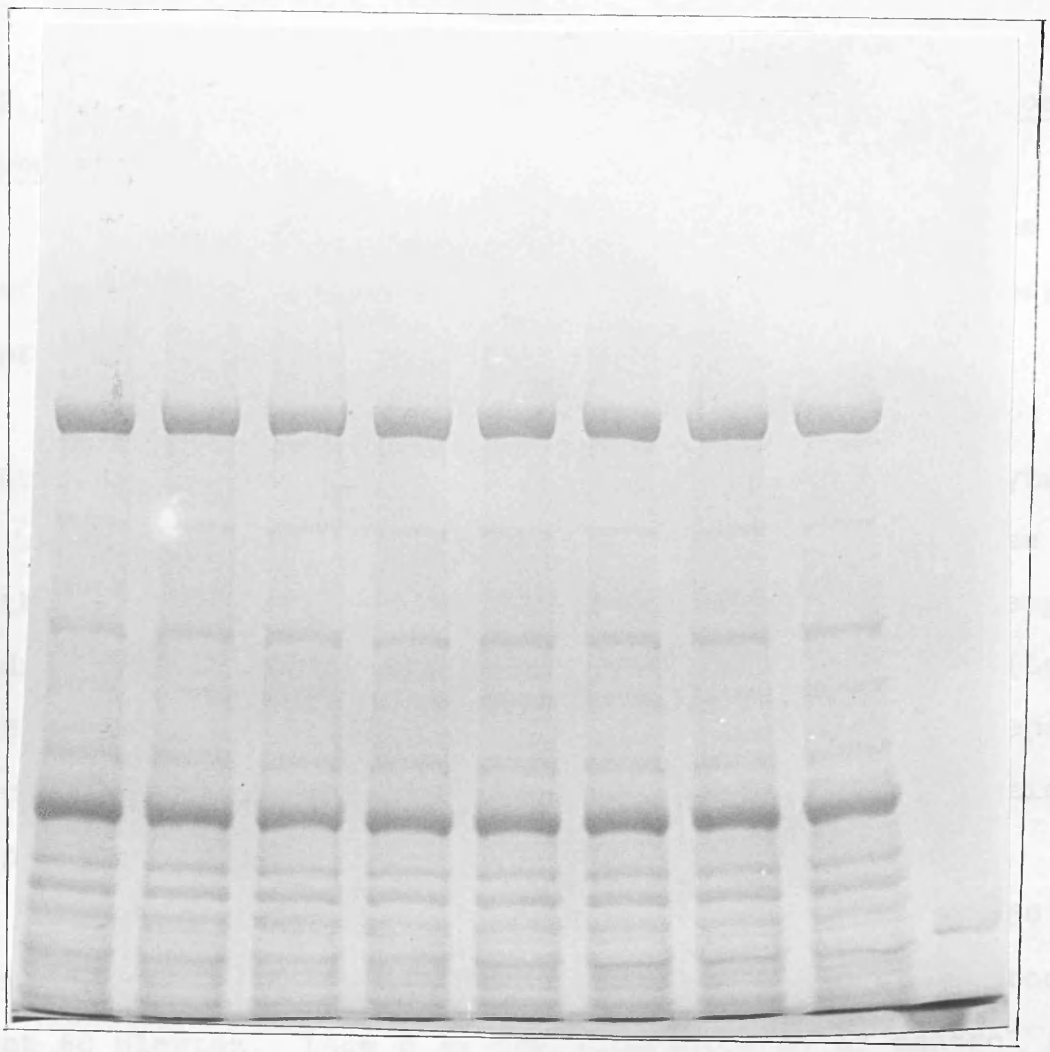

FIGURE 34. Representative SDS PAGE gel from intact male rat hepatocytes, prelabelled with (γ^{32}P)ATP, and incubated in the presence and absence (control) of 50nM 4β-Phorbol-12-myristate-13-acetate (PMA).

Lane A shows SDS PAGE of control hepatocytes at 5 minutes, Lane B shows SDS PAGE of PMA-treated hepatocytes at 5 minutes.

Lane C shows SDS PAGE of control hepatocytes at 10 minutes, Lane D shows SDS PAGE of PMA-treated hepatocytes at 10 minutes.

Lane E shows SDS PAGE of control hepatocytes at 15 minutes, Lane F shows SDS PAGE of PMA-treated hepatocytes at 15 minutes.

Lane G shows SDS PAGE of control hepatocytes at 20 minutes, Lane H shows SDS PAGE of PMA-treated hepatocytes at 20 minutes.

Lane I shows SDS PAGE of molecular weight markers.

3.15(a) Effect of PMA (50nM) with time upon protein phosphorylation.

Figure 35 shows SDS-PAGE and autoradiograms of the effect of PMA with time in electropermeabilised hepatocytes prelabelled with (γ-^{32}P)ATP.

Lane B shows SDS-PAGE of control hepatocytes at 5 minutes and Lane C shows SDS-PAGE of PMA-treated hepatocytes at 15 minutes. The SDS-PAGE shows no apparent differences in the amount of protein present in each lane. The corresponding autoradiograms, Lane D autoradiogram of control (Lane B) and Lane E autoradiogram of PMA-treated (Lane C) hepatocytes, show no visible ^{32}P incorporation into any particular protein for either sample.

Lane F shows SDS-PAGE of control hepatocytes at 60 minutes and Lane G shows SDS-PAGE of PMA-treated hepatocytes at 60 minutes. Lane H is the autoradiogram of control (Lane F) and Lane I is the autoradiogram of PMA-treated hepatocytes (Lane G). Again the autoradiograms show no significant ^{32}P incorporation into any particular proteins for either sample.

5 and 60 minutes were used in Figure 35 as example times to illustrate the result. The actual time course for the experiment was treatment with PMA for 60 minutes with samples taken at 5 minute intervals for analysis. All samples showed the same result, that is no visible incorporation of ^{32}P.

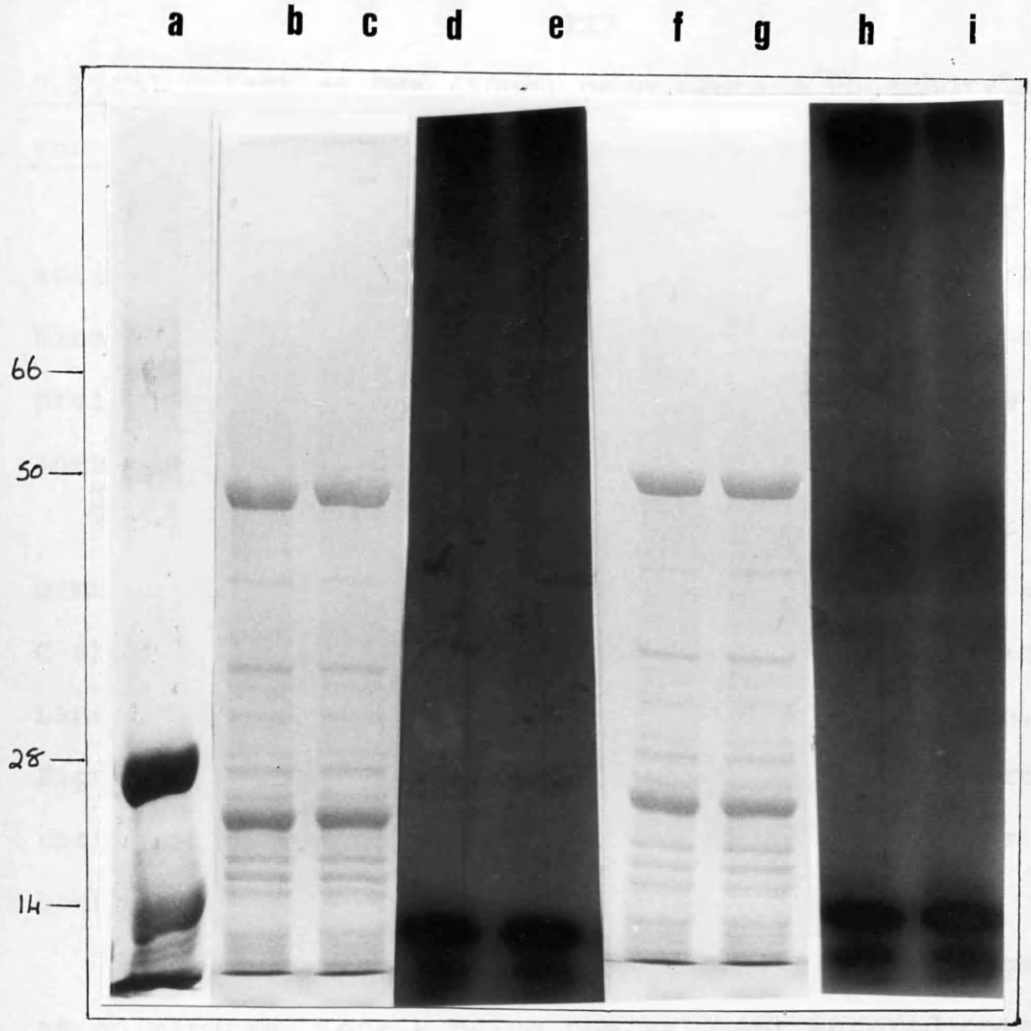

FIGURE 35. SDS PAGE and autoradiograms of electropermeabilised male rat hepatocytes, prelabelled with (γ^{32}P)ATP, and incubated for various times in the presence and absence (control) of 50nM 4β-Phorbol-12-myristate-13-acetate (PMA).

Lane A shows SDS PAGE of molecular weight markers. Lane B shows SDS PAGE of control hepatocytes at 5 minutes. Lane C shows SDS PAGE of PMA-treated hepatocytes at 5 minutes. Lane D shows autoradiogram of control hepatocytes at 5 minutes (Lane B). Lane E shows autoradiogram of PMA-treated hepatocytes at 5 minutes (Lane C).

Lane F shows SDS PAGE of control hepatocytes at 60 minutes. Lane G shows SDS PAGE of PMA-treated hepatocytes at 60 minutes. Lane H shows the autoradiogram of control hepatocytes at 60 minutes (Lane F). Lane I shows the autoradiogram of PMA-treated hepatocytes at 60 minutes (Lane G).

3.15(b) Effect of PMA (50nM) upon protein phosphorylation in the presence and absence of K252b.

Figure 36 shows SDS-PAGE and autoradiograms for PMA acting alone or in the presence of the specific protein kinase C inhibitor K252b (section 3.9(c)). Cells were preincubated for 30 minutes with K252b (20nM) before incubation with PMA (50nM) for 10 - 60 minutes.

Lane B shows SDS-PAGE of control hepatocytes at 60 minutes, Lane D is the corresponding autoradiogram, and Lane C shows SDS-PAGE of PMA-treated hepatocytes at 60 minutes, Lane E being the corresponding autoradiogram. Again, as in Figure 35, despite the presence of many different proteins there is no indication that incorporation of ^{32}P has occured.

Lane F is the SDS-PAGE of (control + K252b) hepatocytes at 60 minutes, Lane H being the relevant autoradiogram and Lane G is the SDS-PAGE of (PMA + K252b) at 60 minutes, Lane I being the relevant autoradiogram. The presence of K252b in the sample has had no effect on the amount of protein present in each sample, reflected by the SDS-PAGE, but once again there is no sign of any ^{32}P incorporation into any proteins in the presence of K252b.

Hence, PMA in the presence and absence of the inhibitor K252b failed to show any ^{32}P incorporation into any proteins with time. 60 minutes was chosen as the representative time to show the result, all other times showing the same effect.

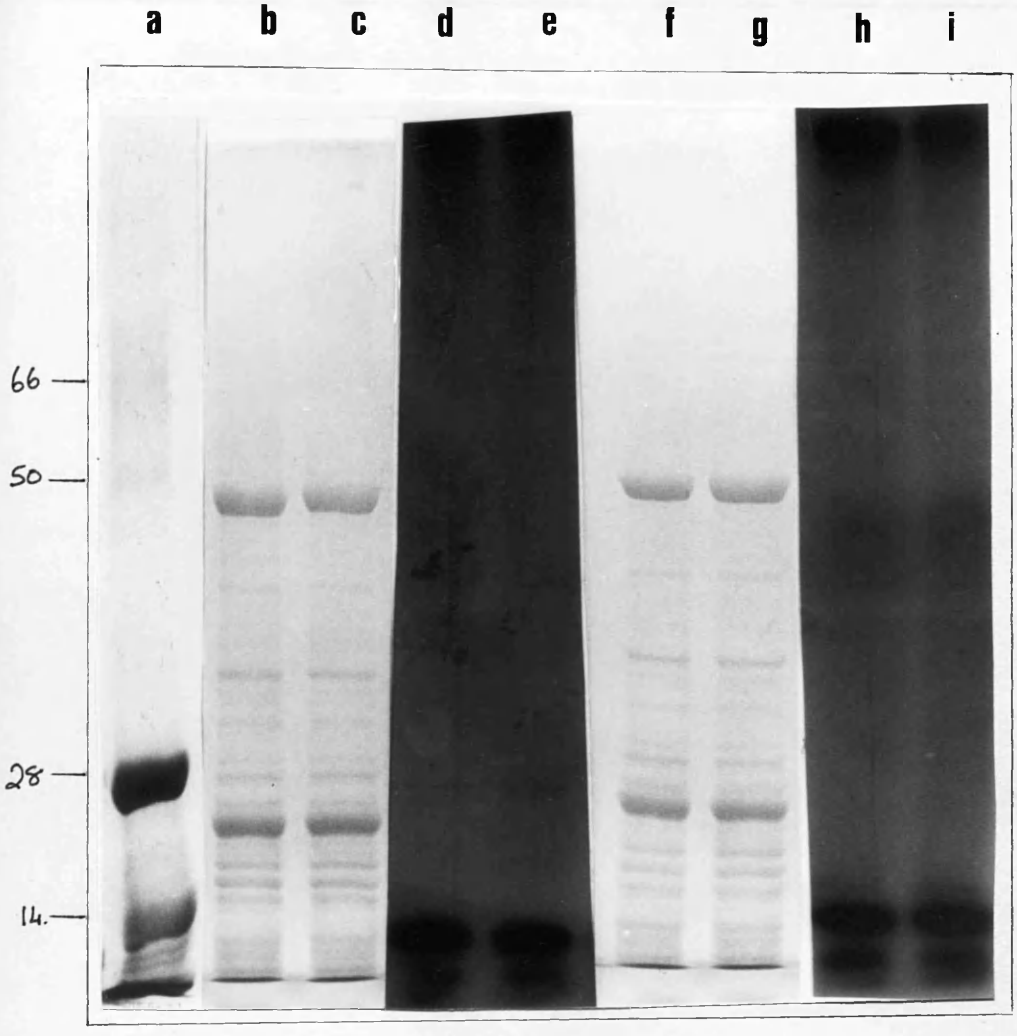

FIGURE 36. SDS PAGE and autoradiograms of electropermeabilised male rat hepatocytes, prelabelled with (γ^{32}P)ATP, and incubated for various times in the presence and absence (control) of 50nM 4β-Phorbol-12-myristate-13-acetate (PMA) and in the presence and absence (control) of K252b (20nM).

Lane A shows SDS PAGE of molecular weight markers. Lane B shows SDS PAGE of control hepatocytes at 60 minutes. Lane C shows SDS PAGE of PMA-treated hepatocytes at 60 minutes. Lane D shows autoradiogram of control hepatocytes at 60 minutes (Lane B). Lane E shows autoradiogram of PMA-treated hepatocytes at 60 minutes (Lane C).

Lane F shows SDS PAGE of (control hepatocytes + K252b) at 60 minutes. Lane G shows SDS PAGE of (PMA-treated hepatocytes + K252b) at 60 minutes. Lane H shows autoradiogram of (control hepatocytes + K252b) at 60 minutes (Lane F). Lane I shows autoradiogram of (PMA-treated hepatocytes + K252b) at 60 minutes (Lane G).

DISCUSSION

DISCUSSION

4.1 The use of 4-Androstene-3,17-dione as a substrate.

The use of xenobiotic substances has been the principal mode of study for discerning mechanisms of action and regulation of the hepatic monooxygenase system. It became apparent, however, that there are marked differences in the kinetics of the metabolism of drugs and steroids by this enzyme system and also some apparent differences in the control of drug-and steroid-metabolising enzyme activities. Kinetic studies have shown that the hepatic monooxygenase system generally has a higher apparent Km for xenobiotics than for steroid substrates (Skett, 1978). To attempt to overcome some of these differences, we utilized the endogenous steroid substrate, 4-androstene-3,17-dione, in our study.

4-Androstene-3,17-dione is a convenient and easy substrate to use, being readily available in both an unlabelled and (^{14}C)-labelled form. Metabolism of this steroid substrate has been well characterised by Gustafsson & Stenberg (1974) and the metabolites can be readily separated in the laboratory by one-dimensional thin layer chromatography. Radio-gas chromatography and gas chromatography-mass spectrometry provide more detailed separation and identification of the metabolites (Gustafsson & Stenberg, 1974), but these techniques were not used in this study.

The activities of the enzymes metabolising 4-androstene-3,17-dione are regulated either by an integral cytochrome P450 component or independently of cytochrome

P450, and so by using 4-androstene-3,17-dione as a substrate, we are able to compare cytochrome P450-dependent and -independent effects at the same time. The P450-containing enzymes are 7α-hydroxylase (Waxman et al, 1987), 6β-hydroxylase (Waxman et al, 1985) and 16α-hydroxylase (Waxman, 1984), whilst the P450-independent enzymes are 17α(β)-hydroxysteroid dehydrogenase and 5α-reductase (Golf & Graef, 1978).

Another advantage of 4-androstene-3,17-dione as a substrate is that the activities of the enzymes mediating its metabolism are subject to sexual differentiation (Gustafsson & Stenberg, 1974a) and so the role of sex differences in metabolism can also be readily investigated.

4-Androstene-3,17-dione therefore lends itself as a convenient substrate to assess cytochrome P450-dependent versus cytochrome P450-independent monooxygenases and also to assess changes in enzyme activity arising from feminisation or masculinisation of hepatic enzyme profiles.

4.2 The use of the isolated rat hepatocyte.

The early studies investigating hormonal regulation of the hepatic monooxygenase system relied upon the rather crude method of injecting hormones directly into the whole animal and then studying in vivo effects. An obvious problem with this method is that the animal will still be subject to extensive internal regulation from the endocrine system and so any effect which may be seen is probably not a true reflection of a single hormonal effect but is more

likely to be a complex hormonal effect modified by the rest of the functional endocrine system.

As techniques became more refined, in vitro systems came into use. These systems were typically either microsome preparations or reconstituted enzymes systems. Neither of these systems is a true representation of the physiological situation; microsomes are only artefacts of the preparation technique, and so their use as models of the physiological situation is limited. Although these in vitro systems overcame some of the problems of endocrine interactions, they are of no use in the study of extracellular effects or to compare the effects of intracellular pathways.

The next system to emerge as a model to investigate hepatic function was the isolated rat hepatocyte. Isolated rat hepatocytes are now the most widely employed experimental model to investigate hepatic function (Cockcroft & Taylor, 1987; Gabbay & Lardy, 1987).

Although hepatocytes represent a more physiological situation than microsomes or reconstituted systems, they still present a problem in that they do not conserve the physiological structure of the liver and also they can lack cell-cell contacts when in a homogenous suspension or when cultured.

The maintenance of cell contact is important because, in the intact liver, cooperation between the different cell types in the liver is vital to maintain hepatic function.

The parenchymal cells of the liver, which represent the isolated hepatocyte, are the principal stores of cytochrome

P450 isozymes. The content of cytochrome P450 isozymes can vary between different regions of the liver, eg there is a 30 fold higher concentration of cytochrome P450IIE1 in the centrilobular than in the periportal region of rat liver (Ingleman-Sundberg et al, 1988). The content of other types of enzymes in the liver can also vary, eg phase 2 conjugating enzymes are at a much higher concentration in the centrilobar region than the periportal region of the liver lobule (Ioannides & Park, 1987).

The loss of the structural arrangement of hepatic cells upon isolation must be borne in mind when extrapolating data from isolated hepatocytes to the physiological situation.

Another problem with using cultured hepatocytes is that the hepatic monooxygenase system suffers rapid loss of cytochrome P450 content during culture (Stewart et al, 1985). The constituency of the culture medium has been found to be vital in order to maintain cytochrome P450 levels and cytochrome P450 activities. Many cell lines, including hepatocytes, require the presence of serum to sustain their growth in culture, although fetal calf serum was found to cause a loss of androstenedione-16α-hydroxylase and androstenedione-5α-reductase activities (Stenberg et al, 1978).

Turner et al (1988) reported that amounts of cytochrome P450 isozymes, their inducibility by phenobarbitone and their activity toward the substrate DMBA (7,17-dimethyl benz(a)anthracene) depends upon the constituency of the medium. Studies into the supplementation of culture medium with different substances

found that levels of cytochrome P450 could be fairly well maintained for short periods of time upon the inclusion of different supplements. Some of the supplements used were DMSO (dimethyl sulfoxide) (Muakkassah-Kelly, 1987), collagen (Dich et al, 1988), dexamethasone (Michalopoulos & Pitot, 1975) and adrenocorticoids (Michalopoulos et al, 1976). Another study employed the co-culture of hepatocytes with epithelial cells (Agius, 1988). These supplemented culture media still cause problems in that some of the cytochrome P450 isozymes can become more susceptible to *in vitro* degradation than others (Turner et al, 1988) and this can result in a change in the overall profile of the hepatocyte monooxygenases. Dexamethasone and adrenocorticoids present additional problems in that they themselves can produce effects upon enzyme activities (van der Hoeven & Galivan, 1987; Karasik & Kahn, 1988).

Some of these difficulties with culture medium and conditions have been overcome in our laboratory. Hussin & Skett (1986) characterised a system for the primary culture of adult rat hepatocytes maintained in Ham's F10 medium supplemented with 0.1% bovine serum albumin and 100IU penicillin/streptomycin. The absence of hormones and serum in the medium allows the examination of hormonal regulation of hepatic monooxygenases at both extracellular and intracellular levels.

Our cultures were typically at a cell density of 10^6 cells per cm^2 and it has been reported that proliferation of hepatocytes in culture occurs at low cell densities but is inhibited by these high cell densities (Nakamura et al,

1983a). It has been postulated that a growth-inhibiting factor is produced by the plasma membrane of mature rat hepatocytes which inhibits growth and differentiation of the cells (Nakamura et al, 1983a). This postulated factor may prevent growth and promote differentiation of hepatocytes when cultured at the high cell densities used in this present study.

Over the time period used in this study, we consider the isolated rat hepatocyte, maintained in Ham's F10 and supplemented with 0.1% bovine serum albumin and penicillin/streptomycin, to be a valid, although not perfect model, for the study of hormonal and intracellular regulation of hepatic steroid metabolising enzymes.

4.3 The effects of activating protein kinase C directly or indirectly upon hepatic steroid metabolising enzymes.

The main problem in investigating physiological regulation of intracellular functions of protein kinase C (PKC) is that the endogenous activator of the kinase, diacylglycerol, is produced only transiently and is subject to rapid inactivation (Berridge,1985). We overcame this problem in two ways;

Firstly, the effects of the tumor-promoting phorbol ester, 4β-phorbol-12-myristate-13-acetate (PMA) upon hepatic steroid metabolism were assessed. PKC is the recognised intracellular receptor for PMA (Castagna et al, 1982; Nishizuka, 1984a) and so by using PMA we can investigate whether or not direct activation of PKC plays a role in mediating changes in hepatic steroid metabolism.

Secondly, the effects upon hepatic steroid metabolism of activating PKC by using agents known to activate this kinase was investigated. The agents employed were 1,2-dioctanoylglycerol (a cell-permeable analogue of the endogenous activator of PKC), the hormone vasopressin (acts by causing receptor-mediated hydrolysis of inositol phospholipids to activate PKC), A23187 alone and in combination with PMA (A23187 is a calcium ionophore and high intracellular concentrations of calcium can activate PKC) and finally angiotensin II (a calcium-elevating hormone, which exerts some of its effects via PKC).

4.3(a) The effect of PMA upon hepatic steroid metabolism.

Incubation of isolated rat hepatocytes with PMA caused a marked inhibitory effect upon the activities of the enzymes metabolising 4-androstene-3,17-dione. Both 50nM PMA (figure 12) and 1nM PMA (figure 13) exerted similar effects upon inhibition of enzyme activity, implying that 1nM PMA is capable of activating PKC maximally, and this correlates well with previous studies (Standaert et al, 1987; Shoji et al, 1987; Kiss et al, 1988c).

The effect of PMA appears to be transient, with maximum inhibitions of enzyme activity being seen at between 40 and 60 minutes, after which time the action of PMA wore off. The cessation of PMA's effect probably reflects the fact that long term activation of PKC by PMA causes down-regulation of the kinase (Terbush & Holz,1986; Darbon et al, 1987). The usual time period for PKC being down-regulated by PMA is between 3 and 6 hours (Cherqui et

al, 1987; Hepler et al, 1988),although down-regulation of PKC has been reported after 60 minutes (von Reucker et al,1988). Down-regulation of PKC by PMA is the most plausible reason for a return of enzyme activities to control levels, as most of PMAs effects are known to be mediated by this kinase. PMA caused quite marked inhibitions in enzyme activity, typically between 60 and 50 % of control activities. This implies that PKC plays quite an important role in controlling the activity of 4-androstene-3,17-dione metabolising enzymes.

The effect of PMA appears to be sexually-independent as it exerted similar effects in female rats (figure 16) to that seen in the male rats (figure 12). As all the enzyme activities were affected similarly, it suggests that the action of PMA upon the enzyme activities is not a feminising effect of PMA. Feminisation of the enzyme profile would have resulted in the suppression of 16α- and 6β-hydroxylase activities, little effect upon the activities of 7α-hydroxylase and 17α(β)-hydroxysteroid dehydrodgenase and a stimulation of 5α-reductase activity. The inability of PMA to feminise the male profile of steroid metabolising enzyme activity may indicate that activation of PKC has no role to play in controlling <u>in vivo</u> sex differences of hepatic monooxygenases.

4.3(b) Effect of 1,2-Dioctanoylglycerol upon hepatic steroid metabolism.

Incubation of intact rat hepatocytes with the synthetic diacylglycerol, 1,2-dioctanoylglycerol (DOG) was also found to produce an inhibition of the activities of the enzymes metabolising 4-androstene-3,17-dione (Figure 19). It appears, therefore, that DOG can produce a similar effect to that seen with PMA, presumably via activation of PKC.

Similarities between the effects of PMA and DOG have been previously reported, eg both compounds enhance catecholamine secretion and protein phosphorylation in intact and permeabilised adrenal chromaffin cells (Lee & Holz, 1986), both compounds block the anti-IgM-induced inositol phosphate production and the anti-IgM-stimulated increases in cytoplasmic calcium in WEH-231 cells (Gold & De Franco, 1987). The similarity in effect between phorbol esters and synthetic diacylglycerols reflects the fact that both compounds bind to a similar site on the PKC molecule, supposedly a 32kD protein fragment (the regulatory fragment of PKC) (Lee & Bell, 1986).

There are some subtle differences between the effects of PMA and DOG upon steroid metabolising enzymes in isolated rat hepatocytes.

Firstly, DOG appears to have a more rapid loss of activity than PMA; the maximum effect with DOG was seen at 20 minutes whilst the maximum effect of PMA was typically between 40 and 60 minutes. The rapid loss of activity with DOG is a reflection of the fact that although it is a synthetic analogue of the endogenous PKC activator, it is

nevertheless a target for degradation by diacylglycerol kinase and is converted to its corresponding phosphatidic acid. The loss of effect with DOG after 20 minutes suggests that DOG has been broken down by this time. Endogenous diacylglycerol is also rapidly degraded to phosphatic acid and so, in the physiological situation, there is only a limited time period within which PKC can become activated. Preventing breakdown of diacylglycerol or its synthetic analogues by the use of diacylglycerol kinase inhibitors would result in an elevated level of diacylglycerol and cause a more sustained activation of PKC. The use of diacylglycerol kinase inhibitors has been employed to study prolonged activation of PKC (Chataway & Barritt, 1988).

The other difference between PMA and DOG is that the degree of inhibition of enzyme activity is different for the two compounds. PMA was found to be capable of inhibiting enzyme activity to 50% of control, whereas DOG only inhibited enzyme activity to 70% of control. The differences in effect between the two compounds in this case again probably reflects the fact that DOG is much more rapidly broken down than PMA, resulting in a low intracellular concentration of DOG.

Phorbol esters are known to be capable of activating PKC independently of calcium (Donelly & Jensen, 1983) and PKC activated in the absence of calcium can still function in the same way as PKC activated via the usual inositol phospholipid turnover pathway (Kishimoto et al,1983). Diacylglycerol, and its synthetic analogues, have a specific requirement for calcium and phospholipid in order to fully

activate PKC (Nishizuka, 1984b).

Our experiments using intact hepatocytes should have contained adequate reserves of intracellular calcium to enhance DOG-mediated activation of PKC, without the need to supplement the medium with calcium, and the most probable explanation for the difference in effect between PMA and DOG is the rapid degradation of DOG within the cell.

4.3(c) Effect of Vasopressin upon hepatic steroid metabolism.

The hormone vasopressin was investigated in our study at both a physiological concentration (100nM) and a supraphysiological concentrations (100μM). Incubation of intact hepatocytes with both concentrations once again produced an inhibition of enzyme activity (figures 20 and 21) in a manner analogous to that seen with PMA (figure 12 & 13).

Analogous effects of PMA and vasopressin have been previously reported in hepatocytes, eg they both block α_1-mediated effects in hepatocytes (Gárciá-Sáinz et al, 1987) and they both have antiketogenic effects (Nomura et al, 1987).

Some of the effects of vasopressin are reported to be mediated by activation of PKC (Blackmore et al, 1986a; Cabot et al, 1988a). It has been reported that vasopressin, upon activation of α_1 receptors stimulates inositol phospholipid turnover, resulting in production of inositol-1,4,5-trisphosphate and diacylglycerol, which cause an elevation of intracellular calcium levels and activation

of PKC respectively (Nishizuka, 1984b). More recent evidence has suggested that vasopressin can also act in a manner which differs to the usual pathway, involving inositol phospholipid turnover. Tijburg et al (1987b) reported that vasopressin can enhance biosynthesis of phosphatidylethanolamine and it has also been proposed that phosphatidylethanolamine can activate PKC (Asaoka et al, 1988). Phosphatidylethanolamine is thought to replace phosphatidylserine in order to activate PKC. Another potential consequence of vasopressin acting upon inositol phospholipid turnover, is that phosphatidylinositol -4,5-bisphosphate (PIP_2), which is produced during the turnover cycle, has also been found capable of activating PKC (Chauhan & Brockerhoff, 1988). It appears that PIP_2 confers a narrower substrate specificity upon PKC than diacylglycerol, and PIP_2 may antecede diacylglycerol as the PKC activator. A difference between vasopressin's and PMA's effects is that both concentrations of vasopressin appear to exert a biphasic effect upon the inhibition of steroid metabolising enzymes activity. Inhibitions of enzyme activity with vasopressin are seen at 20 and 40 minutes, with a return toward control activities in the intervening time periods. This suggests that PKC is subject to a cyclic pattern of activation in the presence of vasopressin. The biphasic effect of vasopressin has been reported previously (Hermon et al, 1986) and it appears that diacylglycerol, produced by inositol phospholipid turnover, is the rate limiting step in the early activation of PKC, whilst calcium, mobilised from intracellular stores by

inositol-1,4,5-trisphosphate, may become the rate limiting step once diacylglycerol is metabolised. This hypothesis fits well with our observations. Both vasopressin and DOG produced inhibitions in enzyme activity at 20 minutes, which quickly wore off. This effect could conceivably be the diacylglycerol-controlled step, the loss of effect representing metabolism of diacylglycerol by diacylglycerol kinase. The later reduction seen with vasopressin at 40 minutes, but not with DOG, could be the calcium-controlled activation step. It is well known that high intracellular levels of calcium can activate PKC, independently of a need for phospholipid (O'Flaherty & Nishihira, 1987), and vasopressin by producing inositol-1,4,5-trisphosphate, could easily elevate calcium levels to the μM concentrations required to activate PKC. Therefore, it appears that vasopressin can exert a dual regulatory control upon PKC in order to inhibit the activity of steroid metabolising enzymes.

4.3(d) Effect of A23187 upon hepatic steroid metabolism.

Incubation of intact hepatocytes with the calcium ionophore A23187 alone (figure 22) showed that this compound had no effect upon the activities of the enzymes metabolising 4-androstene-3,17-dione. A23187 is known to act by increasing intracellular calcium levels and although PKC is known to be activated by high intracellular calcium (Kishimoto et al, 1983), the concentration of A23187 we employed (100nM) may not have been enough to raise intracellular calcium levels sufficiently to activate PKC. The

lack of effect with A23187 has been reported previously in our laboratory (Hussin et al, 1988).

Combining A23187 and PMA together (figure 22) showed that in contrast to A23187 acting alone, the combination of the two compounds produced a marked inhibition of enzyme activity. The degree of inhibition with PMA and A23187 in combination was greater than for PMA acting alone (figure 12). Treatment with the combination produced inhibition to 30% of control, whilst PMA acting alone produced maximum inhibitions to 50% of control. The more pronounced inhibition with the combination of PMA and A23187 suggests that while A23187 alone is incapable of inhibiting enzyme activity, once A23187 is acting in the presence of PMA there is a synergistic effect between the two compounds. Synergistic effects between phorbol esters and ionophores are well documented on diverse cell functions, eg glycogen phosphorylase is synergistically affected by PMA and A23187 (Fain et al, 1984) and interleukin-2 secretion from human peripheral blood lymphocytes is synergistically enhanced by PMA and A23187 (Conradt et al, 1988). The involvement of PKC in these synergistic effects has been implicated by experimental evidence (White and Metzger, 1988; Seiss and Lapetina, 1988) and so it is reasonable to propose that, in our hepatocyte system, PMA acts to cause translocation of PKC from cell cytosol to the plasma membrane, while A23187 increases the intracellular calcium concentration to facilitate PKC activation in the presence of the phorbol ester and endogenous phosphatidylserine. The rise in intracellular calcium levels produced by A23187, although

not high enough to activate PKC alone, would be more than adequate to activate PKC in the presence of the phorbol ester, bearing in mind that one of the functions of PMA upon PKC is to lower its requirement for calcium.

4.3(e) Effect of Angiotensin II upon hepatic steroid metabolism.

The final compound we investigated with proposed actions through PKC, was angiotensin II (Figure 23). Incubation of intact hepatocytes with angiotensin II produced an unexpected, but significant, increase in the activity of the enzymes metabolising 4-androstene-3,17-dione. This was the first, and only, time we saw a stimulation of enzyme activity.

Phorbol esters and angiotensin II have been reported to have similar effects upon hepatic steroid-metabolising enzymes, McAllister and Hornsby (1988) reported that PMA and angiotensin II could inhibit forskolin-induced increases in 3β-hydroxysteroid dehydrogenase and 7α-hydroxylase activities. In contrast to this observation, Gárciá-Sáinz & Hernandez-Sotomayor (1987) reported that, while vasopressin, angiotensin II and PMA all inhibited stimulation of ureogenesis induced by α_1 activation in hepatocytes, the effects of vasopressin and PMA could be blocked by PKC inhibitors while those of angiotensin II were unaffected by PKC inhibitors. This suggests that PMA and angiotensin II can act through both similar and dissimilar pathways to produce the same effect. The fact that we have seen a stimulation of enzyme activity with angiotensin II and an

inhibition with PMA suggests that these different effects are mediated by separate pathways.

Effects of angiotensin II in hepatocytes are mediated by receptor activated processess, unlike phorbol esters which act directly within the cell membrane. Some of these receptor-mediated effects of angiotensin II are proposed to be due to an enhanced mRNA accumulation (Ben-Ari and Garrison, 1988) and so it may be that the stimulatory effects we are seeing with angiotensin II are due to mRNA production.

Bouscarel and co-workers have studied angiotensin II receptors extensively and have proposed the following hypothesis: hepatocytes contain a homogenous population of angiotensin II receptors that are coupled to two different biological effects, apparently mediated by different G proteins (Bouscarel et al, 1988b). The two biological effects of activating angiotensin II receptors are an increase in intracellular calcium levels, which would facilitate activation of PKC, and an enhancement of agonist-induced cyclic AMP accumulation, which would facilitate activation of protein kinase A (PKA). Hence, the two main intracellular signalling pathways appear to be subject to influence from angiotensin II receptors. It has previously been shown in our laboratory that supraphysiological concentrations of cyclic AMP can cause a stimulation in the activity of the enzymes metabolising 4-androstene-3,17-dione in a similar manner and over the same time as we have seen here using angiotensin II (Berry & Skett, 1988b). This would support an effect of

angiotensin II mediated by an enhancment of cyclic AMP accumulation.

Bouscarel et al (1988a) further suggested that down-regulation of angiotensin II receptors induced by their agonist is homologous and does not involve the inhibitory guanine-nucleotide binding protein, Ni, calcium or PKC. It appears that the angiotensin II receptor can regulate two different messengers, calcium or cyclic AMP, depending upon the mode of activation of the receptor.

We propose that in our hepatocyte model, in this particular study, angiotensin II receptor activation selectively produces cyclic AMP within the cell, which then acts to stimulate the activity of the enzymes metabolising 4-androstene-3,17-dione. This effect may also involve de novo protein synthesis, as it has been shown that high concentrations of cyclic AMP produced their stimulatory effect upon enzyme activity in a protein-synthesis-dependent manner (L. Berry, Ph.D. thesis, 1988). It may be that the production of cyclic AMP in some way represses the calcium limb of the angiotensin II response, thus preventing PKC activation and accounting for the absence of any inhibitory effect of angiotensin II upon 4-androstene-3,17-dione metabolising enzymes. It is known that cyclic AMP can inhibit the action of phorbol esters in some cells (Goto et al, 1988).

It has been reported (Summers et al, 1988) that activation of PKC can increase the initial rate of cyclic AMP synthesis and so another possible hypothesis for angiotensin II effects is that activation of PKC by

angiotensin II could enhance cyclic AMP synthesis and production of cyclic AMP by the two pathways regulated by angiotensin II could cause a synergistic effect and produce the marked stimulation in enzyme activity which we observe.

4.3(f) Conclusions to section 4.3.

Activation of protein kinase C in intact rat hepatocytes, was found to cause a time-dependent inhibition of the activity of the enzymes metabolising the steroid substrate 4-androstene-3,17-dione, apart from the hormone angiotensin II. The reductions in activity seen at 20 and 40 minutes suggest that PKC has a biphasic effect upon enzyme activity, which is regulated by diacylglycerol production initially with the later effect more dependent upon calcium. The loss of effect with vasopressin after 40 minutes is probably due to calcium sequestration back into intracellular stores, while the loss of effect with PMA is probably due to a down-regulation of PKC.

4.4 Activation of protein kinase C by 4β-Phorbol-12-myristate-13-acetate.

Protein kinase C (PKC) is reported to be the intracellular receptor for phorbol esters in a variety of tissues and cell lines, eg brain (Baraban, 1987; Shearman et al, 1987), spleen, retina, thymus and intestine (Yoshida et al, 1988), hepatocytes (Gárciá-Sáinz et al, 1986), BC3H-1 myocytes (Cooper et al, 1987) and pituitary cells (Ramsdell et al, 1986).

Before we can attribute the inhibitory effect of

4β-phorbol-12-myristate-13-acetate (PMA) upon hepatic steroid metabolism to an activation of PKC, it was necessary to confirm that PKC could become activated in the presence of PMA in our hepatocyte model. The method of Pelech et al (1986) was employed for this purpose. PKC is known to phosphorylate a variety of protein substrates which are used as intracellular markers of PKC activation. One of the commonest proteins used to identify phosphorylation by PKC are the histones (Jakes et al, 1988a; Brocklehurst & Hutton, 1984; Ono et al, 1988a) and the assay we used determines phosphorylation of histone H1. The assay showed (figure 24) that as the concentration of phorbol ester was increased, a translocation of PKC occured, reflected by a decrease in activity of PKC in the soluble fraction, which represents the cell cytosol, and an increase in activity in the particulate fraction, which represents the cell membrane, to which PKC becomes bound in order to become activated. Translocation of PKC from the soluble to the particulate fraction is taken as an indication that the enzyme has become activated (Portilla et al, 1988; Vegesna et al, 1988a). The maximum activity of PKC in the particulate fraction was seen with 10nM PMA and this concentration correlates well with concentrations of PMA which we earlier found were able to reduce steroid metabolism (section 4.3: 50nM and 1nM). The loss of activity of PKC from the soluble fraction and the concomitant increase in activity in the particulate fraction, suggests that the majority of activated PKC found in the particulate fraction has come from the soluble fraction. The soluble fraction represents

the storage area for PKC in the quiescent cell.

The above results indicate that PMA is capable of activating PKC in our hepatocyte model and it is presumably via activation of PKC that the inhibitory effects upon steroid metabolism are mediated. The optimal concentration of PMA required for activation of PKC in this assay was 10nM.

4.5 The role of protein kinases and protein synthesis in mediating the effects of 4β-Phorbol-12-myristate-13-acetate upon steroid metabolism.

Activation of protein kinase C (PKC) with either phorbol esters or agonists is known to result in the phosphorylation of a variety of protein substrates which then mediate the intracellular effect of PKC. Two proteins used as intracellular markers of PKC activation are an 80 Kd protein (Malvoisin et al,1987; Blackshear et al, 1987) and a 40 Kd protein (Chida et al, 1988a; Vila and Weber, 1988). PKC is also known to phosphorylate certain liver enzymes such as glycogen synthetase (Arino and Guinovart, 1986) and pyruvate kinase (Hsu et al, 1987). In addition to phosphorylating specific proteins and hepatic enzymes, Pyerin et al (1987) reported that a variety of rat liver cytochrome P450 isozymes were phosphorylated by PKC. The same group also reported that activation of the cyclic AMP-dependent protein kinase (PKA) resulted in phosphorylation of cytochrome P450 isozymes, and there was some interaction between PKC and PKA in mediating phosphorylation of these cytochrome P450 isozymes. The role

of protein kinases in mediating effects upon hepatic steroid metabolism was, therefore, carefully examined.

Phorbol esters, because of their mitogenic properties, can exert some of their actions via an enhancement of gene expression (Wu et al, 1988; Deutsch et al, 1988; Lindstein et al, 1988) with these effects occuring at both the transcriptional and translational level. The possibility that protein synthesis may be involved in the effect of PMA upon hepatic steroid metabolism in the present study was therefore, also investigated.

4.5(a) Agents

(i) Protein kinase inhibitors.

PKC inhibitors have been used previously but these inhibitors have suffered from problems of selectivity and side-effects. Staurosporine is a widely used inhibitor of PKC (Matsumoto and Sasaki, 1989; Sako et al, 1988) but staurosporine is relatively non-specific and also inhibits tyrosine kinases as well as PKC (Smith et al, 1988). Another inhibitor of PKC is the endogenous phospholipid sphingosine (Merrill & Stevens, 1989; Kolesnick & Clegg, 1988) but this again suffers from selectivity problems (Krishnamurthi et al, 1989). A recently developed class of kinase inhibitors, the K252 compounds, have been found to show a great potential as protein kinase inhibitors.

K252 compounds are a group of substances derived from microbial origin, from the species Nocardiopsis of strain K252 (Kase et al, 1986). The first compound isolated was K252a, which was found to be an indole carbazole compound.

Initially, K252a was shown to be active against PKC, and it inhibited PKC with an IC_{50} of 32.9nM and a Ki of 25nM (Kase et al,1986). K252a was subsequently reported to be capable of inhibiting both cyclic AMP-dependent protein kinase (PKA), with a Ki of 18nM, and cyclic GMP-dependent protein kinase (PKG), with a Ki of 20nM (Kase et al, 1987). It appears then that K252a is a general, non-selective inhibitor of both cyclic nucleotide-dependent and cyclic nucleotide-independent protein kinases.

Nakanishi and his co-workers (1986) found that a related microbial strain of Nocardiopsis, K290, also possessed the ability to inhibit PKC, and K290 gave rise to a series of compounds termed K252b, c and d. These compounds were all found to possess PKC blocking activity, but K252b was found to be the most potent, with an IC_{50} of 38.3nM. The IC_{50} for K252c and K252d were 214nM and 337nM respectively. Upon structural analysis of K252b, it was discovered that it was the 9-carboxylic acid derivative of K252a, previously identified by Kase et al (1987). K252b differed from K252a in that it was relatively specific against PKC, with minimal actions toward PKA and PKG. K252b has a Ki for PKC of 20nM, whereas its Ki for PKA is 90nM and Ki for PKG is 100nM (Kase et al, 1987). We used K252b at a concentration of 20nM so that it would act selectively to block PKC and have minimal activity against PKA and PKG.

Upon chemical modification of K252a, two further derivatives were discovered, both of which possessed kinase blocking activity. KT5720 was found to be the 9-hexyl ester derivative of K252a and with a Ki of 60nM for PKA is a

selective inhibitor of this kinase (Kase et al, 1987). The other derivative is KT5822, which was found to be the 9-methoxy derivative of K252a, and with a Ki of 2.4nM was found to selectively inhibit PKG (Kase et al, 1987).

All of these protein kinase inhibitors act by competing with intracellular ATP for the active site on the respective kinase (Kase et al, 1987) and this action is identical to that reported for the inhibitory action of the PKC inhibitors staurosporine, quercetin and the isoquinoline sulfonamide derivative, H7, (Nakadate et al, 1988). One drawback to using the K252 compounds is that they become relatively non-selective at high concentrations and to take account of this, we employed the inhibitors at a concentration equivalent to their Ki for their respective kinases. To eliminate any possible direct effect of the kinase inhibitors against hepatic monooxygenase activity, test samples were compared to control samples which had been treated with inhibitor alone. It was found that the kinase inhibitors themselves exerted little or no direct effect upon the hepatic monooxygenases, when the inhibitor-treated cells were compared to untreated control cells.

Kase et al (1987) established the inhibitory potencies of K252 compounds using in vitro models and they did not determine the effects of the inhibitors upon intact cells. Our preliminary studies showed that K252 compounds were inactive in our hepatocyte model but studies with electropermeabilised cells established that once the cells were permeabilised, the K252 compounds were highly effective (L. Berry, personal communication). The electroperm-

eabilisation parameters we employed (1000V for 5 pulses) have been previously standardised and have been found to allow access of compounds into the cell without causing adverse effects upon cell viability or cytochrome P450 levels (L. Berry, Ph.D. thesis). The problem of cell access with the K252 compounds may be one of the disadvantages to using these compounds to investigate physiological processess.

(ii) Cycloheximide.

Cycloheximide is a glutaramide antibiotic and it inhibits protein synthesis at the translational level. Peshka (1971) reported that cycloheximide may act by inhibiting both the initiation of protein synthesis and polypeptide chain elongation by interaction with the 60S ribosomal subunit.

A disadvantage to the use of cycloheximide in investigating hepatic monooxygenase activity is that general non-selective inhibition of protein synthesis will occur and this may result in a loss of the total cell cytochrome P450 level. The loss of cytochrome P450 would have profound effects upon the activity of monooxygenases. To combat this effect of cycloheximide, we compared test cells treated with cycloheximide to control cells which had received cycloheximide alone, thereby overcoming the potential effect of cycloheximide upon hepatic enzyme turnover.

The effect of cycloheximide was investigated in electropermeabilised cells, again to ensure that cycloheximide would penetrate the cell and any effect of

cycloheximide was, therefore, a consequence of an intracellular action.

4.5(b) Effect of K252a.

The effect of the non-selective kinase inhibitor K252a is shown in figure 29. It can be seen that PMA acting alone was able to inhibit steroid metabolism in its usual manner, which we have seen and described earlier (section 4.3(a)).

When PMA and K252a were added together, it could be seen that PMA was still capable of causing inhibition of hepatic steroid metabolism in the same way as PMA acting alone. This suggests that in our isolated rat hepatocyte system, K252a was not able to inhibit the action of PMA, which is acting presumably through protein kinase C (PKC).

The lack of effect with K252a was unexpected, as we were using the compound at its Ki and also the cells had been electropermeabilised to ensure access of K252a into the hepatocyte. Due to the lack of effect with K252a we are unable to provide any definite theories as to the action of this compound upon phorbol ester-mediated inhibition of hepatic steroid metabolising enzyme activity.

4.5(c) Effect of KT5720.

KT5720, the selective inhibitor of PKA, produced the effect shown in figure 30. KT5720 was found to have no detectable effect upon the action of PMA upon hepatic steroid metabolism. PMA was equally effective both in the presence and absence of KT5720.

This confirms our previous suspicion that activation of

PKA plays no role in mediating PMA effects upon steroid metabolism. The inhibitory effect of PMA appears to be exerted independently of cyclic AMP production and PKA activation.

4.5(d) Effect of K252b.

Addition of the selective PKC inhibitor K252b, as shown in figure 31, was found to cause a virtually complete blockade of PMA effects and in the presence of K252b, the activities of the enzymes are returned to control levels. It would appear, therefore, that the actions of PMA upon hepatic steroid metabolism are exerted directly through activation of PKC.

The results show that some of the enzymes metabolising 4-androstene-3,17-dione seem to be more susceptible to modulation by PKC than others. PMA was still able to cause slight inhibitions in activity in the presence of K252b, although not as efficiently as for PMA acting alone.

K252b appears to be selective for PKC in our hepatocyte model, and this effect is in keeping with the idea that PKC is the intracellular receptor for phorbol esters (Niedel et al, 1983).

4.5(e) Effect of cycloheximide.

The use of cycloheximide (figure 28) indicates that, as well as having a dependency upon PKC, the effect of PMA also has a requirement for protein synthesis. Cycloheximide was found to effectively block the actions of PMA upon all of the enzymes metabolising 4-androstene-3,17-dione.

The effect of cycloheximide was rapid in onset, the actions of PMA could be blocked within 10 minutes, suggesting that the protein inhibited by cycloheximide is subject to rapid turnover and its synthesis appears to be completely and irreversibly blocked by cycloheximide. The search for such a protein, with a rapid turnover and fast onset of action, has been conducted and there are a few possible candidates.

Angel et al (1987b) reported that phorbol ester-inducible genes contain a common cis element which is recognised by a PMA-modulated trans-acting factor. Angel and his group proposed that this element is transcription factor AP-1. Transcription factor AP-1 appears to be at the receiving end of a complex pathway responsible for transmitting the effects of phorbol ester tumor promoters from the plasma membrane to the transcriptional machinery of the cell. It is possible that cycloheximide is inhibiting the synthesis of transcription factor AP-1 in our hepatocyte system, and blockade of synthesis of this protein causes the blockade of PMA effect.

Another possible candidate for cycloheximide action are the c-myc and c-fos proteo-oncogenes. It is known that PKC activation, with phorbol esters, can stimulate the accumulation of mRNA for both c-myc and c-fos (Blackshear et al, 1987; Stumpo et al, 1988), and most studies seem to implicate the c-fos gene as the more important transcript. A c-fos binding factor has been proposed as the essential mediator of c-fos expression by PKC (Stumpo et al, 1988) and it is possible that the c-fos gene is necessary for the

effect of PMA upon hepatic steroid metabolism and this protein is inhibited by cycloheximide.

The results with cycloheximide suggest that the action of PMA upon hepatic steroid metabolism are dependent upon protein synthesis. Although we have not determined the identity of the actual protein inhibited by cycloheximide, the literature suggests that either transcription factor AP-1 or the c-myc/ c-fos proteo-oncogenes may be potential candidates. The role of the genes responsible for phorbol ester-mediated effects are still being investigated and extensive work, outwith the scope of this thesis, is necessary before we could confidently predict the protein responsible for mediating PMA effects upon hepatic steroid metabolism.

4.5(f) Conclusion to section 4.5.

Using selective protein kinase inhibitors and the protein synthesis inhibitor cycloheximide, we have shown that the effects of the phorbol ester PMA upon hepatic steroid metabolism are mediated solely by activation of PKC, no other kinases appears to be affected by PMA. This result enhances the idea that PKC is the only intracellular receptor for phorbol esters. The effect of PMA also requires protein synthesis, although the identity of the protein or proteins remains to be established.

It is possible that once activated, PKC has subsequent effects upon gene expression and these genes produce the effects upon hepatic steroid metabolism. This idea is further expanded in section 4.9.

4.6 Mechanisms of action of 4β-Phorbol-12-myristate-13-acetate.

Having determined that the active phorbol ester PMA could exert inhibitory effects upon hepatic steroid metabolism, we next attempted to elucidate the underlying mechanism of this action. The first area of investigation was upon PMA action on various components of the NADPH/ cytochrome P450 enzyme system. The key enzymatic components of this mixed function monooxygenase system are the flavoprotein NADPH-cytochrome c(P450) reductase and cytochrome P450 itself. NADPH-cytochrome c reductase has been reported to be a phosphorylatable substrate of PKC, activated by PMA (Tamoto et al, 1989) and phosphorylation of specific cytochrome P450 isozymes by PKC has also been reported (Pyerin et al, 1987). We hoped to extend, or at least confirm, these observations in our own study.

4.6(a) Effect of PMA upon the cofactor NADPH.

One of the mechanisms by which PMA may be acting is by affecting the availability of the cofactor NADPH. NADPH is essential in cytochrome P450-mediated reactions, and it acts as the electron donor for hepatic monooxygenases. The activity of the cytochrome P450 enzyme system in isolated hepatocytes depends upon an *in vivo* supply of NADPH and so we tested the possibility that the changes in enzyme activity which occur with PMA were due to a change in the supply of NADPH.

Figure 25 shows the effect of PMA acting in the presence of excess NADPH. It can be seen that

homogenisation of the hepatocytes, following incubation with PMA, and the addition of excess cofactor, NADPH, had no effect on either the rate or magnitude of PMA's inhibition of steroid metabolism. PMA still acted in the same way as when added alone to the hepatocytes (figures 12 and 13). This indicates that the inhibitory effects of PMA are not dependent upon the availability of NADPH.

As the availability of NADPH did not appear to be involved in the effect of PMA, we tested the possibility that the rate of degradation of NADPH may be important in controlling PMAs effects. NADPH degradation by NADPH oxidase may be a limiting factor in controlling the rate of cytochrome P450-mediated reactions, and it is possible that PMA acting upon NADPH oxidase may affect the activity of cytochrome P450-dependent reactions. The results indicate, however, that the rate of degradation of NADPH in both control and PMA-treated samples remains at a constant level and there is no significant difference between the rate of loss of NADPH between the two samples (figure 26). It appears then, that NADPH oxidase is not affected in any way by PMA and consequently, the rate of loss of NADPH is not affected by PMA.

The above result indicates that neither the availability nor the rate of loss of the cofactor NADPH are involved in PMA's inhibition of hepatic steroid metabolism.

4.6(b) Effect of PMA upon NADPH cytochrome c (P450) reductase.

NADPH cytochrome c (P450) reductase is a flavoprotein present in the microsomal fraction of the liver and it acts by transferring the necessary reducing equivalents from NADPH to cytochrome P450 during drug metabolising reactions as determined by the equation

$$\text{NADPH} \xrightarrow{e^-} \text{NADPH cytochrome c reductase} \xrightarrow{e^-} \text{Cytochrome P450}$$

NADPH cytochrome c reductase has been reported as a substrate of activated PKC (Tamoto et al, 1989; Steele and Virgo, 1988) and phosphorylation of the enzyme is reported to cause activation. We measured the activity of NADPH cytochrome c reductase because by determining the reduction of cytochrome c, we can directly determine the reduction of cytochrome P450 itself, on the principle of the above equation. Reduction of cytochrome P450 directly is difficult to measure experimentally, but by measuring reduction of cytochrome c we can obtain an estimate of the reduction of cytochrome P450 itself. Cytochrome c is used as an artificial electron acceptor in this instance.

The principal of the method is that both the oxidised (ferric) and reduced (ferrous) form of cytochrome c have characteristic absorption spectra. The difference between the ferrous and ferric spectra is that the reduced (ferrous) form absorbs at 550nm, while the oxidised (ferric) form does not. Hence, activity of NADPH cytochrome c reductase can be conveniently determined by measuring

absorption at 550nm as a function of time (Philips and Langdon, 1962).

The results show (figure 27) that treatment of hepatocytes with PMA had no effect upon the activity of NADPH cytochrome c reductase. The activity of the enzyme declined gradually after 20 minutes, but both control and PMA-treated samples showed a decline in activity at the same rate suggesting that this was not a direct effect of PMA.

The above findings imply that in the isolated rat hepatocyte, NADPH cytochrome c reductase is not a target for PMA and from this, we can infer that PMA does not affect the rate of reduction of cytochrome P450 in order to exert its inhibitory effect upon hepatic steroid metabolism.

4.6(c) Effect of phosphorylation of cytochrome P450.

It has been previously reported (Pyerin et al, 1987) that various cytochrome P450 isozymes are substrates for phosphorylation by both PKC and cyclic AMP-dependent protein kinase (PKA) *in vitro*. Some of the isozymes appeared to be more susceptible than others to phosphorylation and the kinases themselves appeared to selectively phosphorylate different isozymes. Rat cytochrome PB3a (P450IIB1) was reported (Pyerin et al, 1987) to be a target for phosphorylation by both PKA and PKC, rat cytochrome P450 MC-1b (P450IA1) and rat cytochrome P450 UT-1 (P450IIA1) were reported not to be phosphorylated by either kinase and rat cytochrome P450 PB2c (P450IIC11) was reported to be phosphorylated only by PKC.

We investigated a more physiological, *in vivo*, system

to try to determine whether or not any of the cytochrome P450 isozymes which were proposed substrates for these kinases, underwent phosphorylation in the presence of PMA to try to determine if phosphorylation of cytochrome P450 may be a mechanism by which PMA inhibited steroid metabolism in isolated rat hepatocytes.

When we examined incorporation of ^{32}P into cytochrome P450 PB 2c (P450IIC11), the isozyme thought to be responsible for up to 90% of the hydroxylation of 4-androstene-3,17-dione at the 16α position (Waxman, 1984), we found that we were unable to detect any incorporation of ^{32}P into immunoprecipitated cytochrome P450 PB2c (P450II C11). Neither the control hepatocytes nor those incubated for 1 hour with 50nM PMA (figure 33) showed any incorporation of ^{32}P. Cytochrome P450 PB3a (P450IIB1), a reported substrate for PKC, and cytochrome P450 MC-1b (P450I A1) and cytochrome P450 UT-1 (P450II A1), all failed to show any incorporation of ^{32}P, again after 1 hour treatment with 50nM PMA prior to immunoprecipitation (figure 33). The immunoprecipitated cytochrome P450s were compared to whole cell samples, which did show ^{32}P incorporation into both control and PMA-treated samples.

The above findings imply that direct phosphorylation of cytochrome P450 isozymes by PKC is not a physiological effect, which contradicts the earlier findings of Pyerin et al (1987).

It could be argued that our immunoprecipitation technique was at fault and we were not actually precipitating cytochrome P450 isozymes, but some other proteinaceous

component. Further experimental evidence, however, tends to refute this hypothesis;

(a) The supernatant used for immunoprecipitation was assessed spectrophotometrically before being treated with the antibodies, and we were able to detect cytochrome P450 in the supernatant, by the method of Omura and Sato (1962b).

(b) The presence of a large, solitary protein band of apparent molecular weight 40,000 - 50,000D on the SDS gels indicates that the isolation process has yielded a protein with a comparable molecular weight to that of cytochrome P450.

(c) Cytochrome P450 is a haem-containing flavoprotein and by assessing the amount of haem present in each precipitate (Table 17) we can see that haem is present in our immunoprecipitates and consequently it is probable that cytochrome P450 itself is also present, as haem is an integral component of cytochrome P450. The lower values for the amount of haem present in the isozymes compared to the whole cell samples is a reflection of the fact that the immunoprecipitates contain only one form of cytochrome P450, while the whole cell samples express the complete range of cytochrome P450 isozymes.

This evidence implies that we have successfully immunoprecipitated the relevant cytochrome P450 isozymes, using our methods.

As a further confirmation that there was no ^{32}P incorporation into the cytochrome P450 immunoprecipitates, the precipitates were directly counted. The whole cell samples showed a high level of counts (Table 18) although there was no significant difference between PMA-treated and control samples, suggesting that PMA had not enhanced ^{32}P incorporation into the cells. The immunoprecipitates showed very low counts in comparison to the whole cell samples, but once again there was no difference between control and PMA-treated samples.

As well as our own data with the phosphorylation of cytochrome P450 isozymes, additional evidence exists which implies that phosphorylation of cytochrome P450 isozymes may not be the underlying mechanism of PMA-mediated, PKC-dependent inhibition of steroid metabolism:

(i) Pyerin et al (1987) reported that cytochrome P450 UT-1 (P450IIA1), which mediates 7α-hydroxylation of 4-androstene-3,17-dione (Waxman et al, 1987; Nagata et al, 1986) was not phosphorylated by either PKA or PKC. We have reported that activation of PKC with PMA causes an inhibitory effect upon the activity of this enzyme in isolated hepatocytes and it has also been reported that activation of PKA with cyclic AMP also produced an inhibition of activity of this ensyme (L. Berry, Ph.D. thesis, 1988).

(ii) In our earlier discussion (section 4.3) we reported

that all of the enzymes metabolising 4-androstene-3,17-dione were inhibited in the presence of PMA. The enzymes 5α-reductase and 17-hydroxysteroid dehydrogenase do not contain cytochrome P450 as integral components (Golf and Graef, 1978) and yet they are inhibited in exactly the same manner as the cytochrome P450-containing enzymes, 7α-hydroxylase, 6β-hydroxylase and 16α-hydroxylase. Enzyme inhibition, therefore, cannot be attributed to phosphorylation of cytochrome P450 for these cytochrome P450-independent enzymes.

4.6(d) Conclusion to section 4.6.

In the present study, we have shown that the inhibition of hepatic steroid metabolising enzymes, mediated by activation of protein kinase C with the phorbol ester, PMA, appears to be mediated independently of any change in the availability or rate of loss of the cofactor NADPH, without a direct effect upon the reduction of cytochrome P450 itself or by a direct phosphorylation of cytochrome P450 isozymes. PKC can apparently mediate its inhibitory effect entirely without the involvement of the NADPH cytochrome P450 drug metabolism enzyme system.

4.7 Protein Phosphorylation.

Many of protein kinase C's (PKC) intracellular actions are reported to occur as a consequence of protein phosphorylation. A variety of proteins have been identified as PKC substrates in a diverse range of cells, eg the PKC intracellular marker proteins 40 Kd, 47 Kd and 80 Kd are

phosphorylated in BC3-H1 myocytes (Vila et al, 1989), a 42 Kd Class 1 heavy chain protein is a substrate in human B lymphoblastoid cells (Peyron and Fehlman, 1989), and the EGF receptor (Davis, 1988). These phosphorylations typically occur at serine and threonine residues within the protein (Jakes et al, 1988a; Soderling et al, 1986).

We investigated phosphorylation of proteins within isolated rat hepatocytes, to try to identify a precise protein or proteins which may be responsible for the effect of the phorbol ester upon hepatic steroid metabolism.

The phosphorylation study utilised (γ^{32}P)ATP as a radiolabel, which was preincubated with the hepatocytes for 15 minutes at 4°C to allow intracellular pools of ATP to become equilibrated with the label. Two main studies were conducted, the first of which was a time course study to investigate changes in phosphorylation with time, and the second study employed the specific PKC inhibitor K252b (section 4.5 (d)) to see if inhibition of the kinase affected protein phosphorylation.

The time course study, results of which are depicted in figure 35, showed that although we could detect a vast array of proteins on the SDS gel, analysis of the corresponding autoradiogram failed to show any incorporation of ^{32}P into these proteins. Modification of experimental technique and repetition of the experiment still failed to show any incorporation of ^{32}P into any protein.

Investigation into the incorporation of ^{32}P into proteins in the presence of the PKC inhibitor, K252b, also

failed to show any incorporation of ^{32}P into any protein. This implies that any protein phosphorylation which may be occuring happens outwith the time period of our assay.

These results suggest that phosphorylation of proteins may not be involved in mediating the effects of phorbol esters upon hepatic steroid metabolism. This hypothesis seems unlikely when we consider that phorbol esters act via activation of PKC and the sole action of PKC is to cause protein phosphorylation.

An alternative, and more credible, hypothesis is that any protein phosphorylation which may be occuring is occuring very rapidly, in the time before we have taken our first sample at 5 minutes. Phorbol esters are known to be capable of phosphorylating proteins within seconds, through activation of PKC, (Blackshear, 1988; De Graan et al, 1988) and these proteins are also rapidly dephophosphorylated. It may be that this rapid phosphorylation/dephosphorylation has occurred during the first 5 minutes of the time course and the intracellular protein profile has returned to normal by the time we begin to determine phosphorylation patterns. To test this hypothesis, a shortened time course study should have been conducted, but such an experiment was not performed here.

A third reason why we may not be able to detect any change in phosphorylation could be proposed on the knowledge that phosphoproteins exist within the cell. Of the 1000 or so phosphoproteins which appear to exist, 120 are proposed

to be phosphorylated in response to active phorbol esters, such as PMA (Blackshear, 1988). Exactly how many of these phosphoproteins are true substrates for PKC or are the result of secondary phosphorylation is not clear.

On the strength of the above results we can only speculate as to the precise mechanism by which PMA and subsequent activation of PKC, inhibits steroid metabolism through protein phosphorylation. It is possible, although not proven, that PMA activates PKC very rapidly, within seconds, and the activated PKC then induces phosphorylation of the proteins responsible for mediating effects upon steroid metabolism. This potential phosphorylation may occur too rapidly for us to detect with our techniques, but although the actual phosphorylation may be rapid, the effect upon steroid metabolism appears to be sustained for up to 1 hour, perhaps via a secondary effect of the rapidly phosphorylated protein.

The integral details of PKC-mediated effects, therefore remains to be determined, but it may involve a form of cascade system which is regulated by the rapidly phosphorylated substrate proteins for PKC.

4.8 Approaches to the study of protein kinase C involvement in intracellular effects.

We have employed the tumor-promoting phorbol ester PMA in this study, assuming previous evidence to be correct, and that phorbol esters act exclusively through protein kinase C (PKC). Although phorbol esters activate PKC both *in vivo*

and in vitro, because of their mitogenic properties and because they do not necessarily reflect a true physiological situation, several criteria must be fulfilled and borne in mind when using phorbol esters to probe PKC-mediated effects within an experimental system.

4.8(a) Can we attribiute a biological phenomenon to activation of protein kinase C?

For certain short term responses, the answer to this question is almost certainly yes. For example, incubation of 3T3-L1 adipocytes upon exposure to PMA or dioctanoylglycerol, both of which activate PKC, for 30 minutes was found to enhance mRNA accumulation of the c-fos proteo-oncogene (Blackshear, 1988), PMA can inhibit tumor-necrosis factor activity within 15-60 minutes of treatment (Johnson & Baglioni, 1988) and also, PMA preincubation for 30-180 seconds inhibits EGF-stimulated production of inositol-1,4,5-trisphosphate and subsequent increases in intracellular calcium (Johnson & Garrison, 1987).

For these short term studies, it is generally accepted that, if a process can be stimulated by phorbol esters with the appropriate stereospecificity for PKC and also by appropriate diacylglycerols (synthetic or natural), then the biological phenomenon which is observed is a consequence of activation of PKC.

The studies we have performed were of no more than 120 minutes duration and we observed similar effects with biologically active PMA, the synthetic diacylglycerol

1,2-dioctanoylglycerol and also vasopressin, which acts to stimulate inositol phospholipid hydrolysis to produce endogenous diacylglycerol. As we see the same effect regardless of the manner in which we activate PKC, we have therefore fulfilled the criteria for activation of PKC within a short term incubation to produce a biological phenomenon.

The issue becomes more complicated, however, when longer term effects of phorbol esters are studied. The problem arises in that long-term treatment of cells with phorbol esters results in down-regulation of PKC and this down-regulation typically takes between 3 and 24 hours (Cherqui et al, 1987; Bouscarel et al, 1988c; von Reucker et al, 1988).

The phenomenon of down-regulation appears to be reversible, at least if a phorbol ester is used which can be eliminated from the cells by extensive washing. Phorbol dibutyrate is such a phorbol ester and although incubation with phorbol dibutyrate causes a 90% decrease in specific PKC activity in both soluble and particulate fractions of the cell, washout of phorbol dibutyrate and reincubation in normal culture medium restores PKC activities in both fractions to near pretreatment levels after 48 hour incubation (Blackshear, 1988).

The effect of down-regulation does not appear to involve significant changes in the levels of PKC mRNA (Makauske et al, 1986). Most evidence suggests that exposure of cells to phorbol esters leads to a translocation of PKC to the particulate fraction, followed by an increased

rate of destruction of the bound PKC. The rate of biosynthesis of mRNA or protein is not strikingly affected and these continue at a slow rate to replace PKC if the phorbol ester stimulus is removed.

The problems in assessing long term activation of PKC are summarised below and should be borne in mind when conducting such a study;

(1) Down-regulation of PKC occurs in many cells in response to phorbol esters, therefore, are the long-term effects of phorbol esters a consequence of PKC activation or a consequence of its disappearance?

(2) Synthetic diacylglycerols are metabolised rapidly by most cells and phorbol esters themselves can be broken down eventually, especially by hepatocytes and liver cells.

(3) Phorbol esters appear to be toxic to some cells, eg K562 and leukemic HL60, in long-term incubation studies.

(4) Active phorbol esters cause an "unnatural" permanent association of cytosolic PKC to the particulate fraction.

(5) Long term treatment with phorbol esters may lead to both down-regulation and constitutive activation of any remaining PKC.

4.8(b) Does a specific agonist activate protein kinase C in an intact cell?

Two major criteria have been used to demonstrate that protein kinase C (PKC) is being activated in intact cells;

(i) Protein phosphorylation.

The increased phosphorylation of specific substrates of PKC within cells is a widely used approach to investigate the involvement of PKC in an observed effect. Recognised substrates include the EGF receptor (Davis, 1988), an 80 Kd protein regarded as a major intracellular marker of PKC action (Brooks et al, 1987; Rodriguez-Pena et al, 1986; Erusalimsky et al, 1988) and a 40 Kd protein, also an intracellular marker for PKC (Kroll et al, 1988; Nishizuka et al, 1988).

Within the past ten years, a vast body of evidence has accumulated concerning potential substrates for PKC. Nishizuka and his colleagues have extensively researched PKC phosphorylatable substrates (Nishizuka, 1984a; Nishizuka, 1984b; Takai et al, 1984) and their data is widely used as the basis for determining whether protein phosphorylation is a consequence of PKC activation.

In this study, we attempted to determine whether or not protein phosphorylation was occuring to try to identify a protein which may be related to the effect we observed upon hepatic steroid metabolism subequent to activation of PKC. However, all our attempts to show protein phosphorylation were unsuccessful, despite modification of experimental technique and extensive repetition of the experiments. This

may imply that protein phosphorylation is not an important factor in mediating the effect of activated PKC, but this is unlikely as discussed earlier (section 4.7).

Although protein phosphorylation is reported to be the most effective method of investigating PKC action, several problems do occur when conducting protein phosphorylation experiments. One problem is that it is important to ensure that protein phosphatases are inhibited, to prevent any non-specific phosphorylation. We used β-glycerophosphate to ensure any phosphatases would be inhibited, so we have accounted for this effect in our cells. Another factor which must be considered, but which is more difficult to prevent, is that secondary or tertiary kinases have become activated once PKC is activated. Finally, although a protein may be a substrate for PKC in cell-free systems, this does not necessarily imply that the protein will be phosphorylated in intact cells, which will be subject to the action of phosphatases and other kinases as mentioned above.

Hence, although protein phosphorylation is a useful method for investigating the action of PKC in an intact cell, there are still an array of difficulties which can complicate the interpretation of results.

(ii) Protein kinase C translocation.

Another method used to evaluate the involvement of PKC in an effect is to look for evidence that "translocation" of PKC has occurred from the soluble to the particulate fraction of the cell. Translocation usually refers to

agonist-induced association of the kinase with the whole cell particulate fraction, although it very occasionally is used to refer to a decrease in membrane-associated kinase and an increase in cytosol-associated kinase. Translocation of kinase from the cell membrane to the cell nucleus has been demonstrated (Cambier et al, 1987).

In this study, we successfully showed (section 4.4) that in the presence of the active phorbol ester PMA, there was a dramatic shift in the location of PKC, with an increase in membrane-associated activity and a decrease in the soluble kinase activity. Therefore, we feel satisfied that activation of PKC has occurred in the presence of an agonist, and that activation of PKC is responsible for the effect upon hepatic steroid metaboliism.

4.8(c) Which effects of an agonist are mediated through protein kinase C-dependent and which by protein kinase C-independent events ?

To answer this question, it would be advantageous to possess cell lines which lack protein kinase C (PKC), but such cell lines do not exist. The closest to a PKC-free cell is when a cell has had its levels of intracellular PKC down-regulated by prolonged treatment with phorbol esters. Even if PKC-free cell lines were available, they would not be useful for evaluating PKC-independent processes in other cell lines.

The two main approaches to determine if a process is dependent upon PKC are to use inhibitors of PKC or to induce down-regulation of the kinase. As we did not perform

long-term studies, down-regulation of PKC was not investigated in this study. PKC inhibitors, however, were employed.

Inhibitors of kinase action in the past have been plagued by problems of selectivity, which we appear to have been able to overcome by the use of the relativly specific set of inhibitors derived from Nocardiopsis, according to the methods of Kase and his group (1987). The use of these inhibitors suggests that the actions of phorbol esters upon hepatic steroid metabolism are exerted entirely through activation of PKC. No other kinase or combination of kinases appears to be involved in the phorbol ester effect.

The inhibitors we employed here will hopefully become more widely used in the future to aid determination of kinase involvement in different intracellular effects.

4.8 (d) Conclusion to section 4.8.

The purpose of this section of the discussion was to bring to light some of the problems which are associated with the investigation of protein kinase C involvement in signal transduction. It can be seen that various criteria must be fulfilled in order to implicate PKC with an observed biological phenomenon. We have managed to satisfy some of these criteria in this study, with respect to activation of protein kinase C during a short-term incubation, the ability to translocate the kinase upon exposure to a known agonist from the soluble to the particulate fraction of the cell and, with the use of inhibitors, we have further evidence that activation of protein kinase C is a major factor in

causing inhibition of hepatic steroid metabolism. The one criterion we have failed to fulfill is that of demonstrating protein phosphorylation, but reasons for this discrepancy have been proposed.

4.9 Mechanism of action by which activated protein kinase C may regulate steroid metabolism at the intracellular level.

This study into the control of hepatic steroid metabolism has shown that there appear to be two consequences of activating protein kinase C (PKC). The first and major effect is that activation of PKC through the use of biologically active phorbol esters, synthetic diacylglycerols or hormones, produces a marked inhibition in the activity of the enzymes responsible for metabolising the steroid substrate 4-androstene-3,17-dione, an effect which is apparently dependent upon protein synthesis. The other effect, which does not appear to be as important as the inhibitory effect, was seen only with the hormone angiotensin II and this was a stimulation in the activity of the enzymes metabolising 4-androstene-3,17-dione. The method by which each effect occurs will be considered separately.

4.9 (a) Inhibition of hepatic steroid metabolism.

An interesting finding from this study is that activation of PKC, which was probed by the use of the tumor-promoting, biologically active phorbol ester 4β-phorbol-12-myristate-13-acetate (PMA), appears to inhibit hepatic steroid metabolism in a manner which is independent

of any effect or direct involvement of the NADPH/cytochrome P450 enzyme system. Although this effect appears unusual when we consider that most regulation upon hepatic monooxygenases is through this system, the idea becomes more acceptable when we realise that there are very few reports about inductive or degradative effects on cytochrome P450 levels in isolated rat hepatocytes, mediated by activation of PKC. Inductive effects upon cytochrome P450 levels have been widely reported for cyclic AMP acting through protein kinase A (Dokas & Kleinsmith, 1971; Johnson & Allfrey, 1972) and the effect of cyclic AMP is thought to be due to a stabilisation of post-translational events in an analagous manner to the effects reported for phenobarbitone and 3-methylcholantherene. Degradative effects of PKC upon cytochrome P450 are unknown at this time, although there is evidence that PKC may play a role in inhibiting induction of basal levels of cytochrome P450 (Steele & Virgo, 1988).

Direct effects of activating PKC upon levels of cytochrome P450 were not determined in this study, but the lack of reported effects of activated PKC upon cytochrome P450 implies that this is not the main way by which PKC affects hepatic function.

Activation of PKC, by phorbol esters, has been reported to cause the inhibition of some hepatic enzymes, such as pyruvate kinase (Soderling et al, 1986), glycogen synthetase (Ahmed et al, 1984) and the glucose transporter (Allard et al, 1987). These inhibitions of enzyme activity appear to occur as a consequence of phosphorylation of the enzymes, although enzyme inhibition can also occur as a consequence

of inhibition of gene expression. Activation of PKC was found to inhibit the gene expression of phosphoenolpyruvate carboxykinase (Chu et al, 1987; Chu & Granner, 1986). The absence of any detectable phosphorylation after activation of PKC in this study suggests, that the effect of activated PKC to inhibit steroid metabolism may occur through an effect upon gene expression.

The half-life of the enzymes metabolising 4-androstene -3,17-dione are approximately 48 hours and it is unlikely that these enzymes are subject to acute effects upon gene expression effects. The effect upon steroid metabolism which we observe is a rapid effect, inhibition of enzyme activity can be seen after 5 minutes of treatment with PKC agonists. This implies that a rapidly turning over protein is the site of action for gene expression modulation.

As mentioned earlier (section 4.5 (e)) the use of cycloheximide suggests that inhibition of protein synthesis is occurring. We proposed several candidates for this inhibition by cycloheximide, including trans-acting factor AP-1 and the c-myc and c-fos proteo-oncogenes. A role for PKC in mediating an effect upon gene transcription to ultimately inhibit steroid metabolism seems more realistic when we consider a recent report by Deutsch and his group (1988). This group further extended the concept that activation of both protein kinase A (PKA) and PKC could stimulate gene expression by way of interactions of specific proteins with DNA control elements.

It is known that many genes are transcriptionally regulated by cyclic AMP and these genes contain a conserved

sequence in the 5'-flanking region that is identical or highly similar to the palindromic octamer TGACGTCA, the so-called cyclic AMP-responsive element (cAMP-RE)(Silver et al, 1987). A very similar symmetrical heptameric motif, TGACTCA (the PMA-responsive element, PMA-RE), or close relatives thereof, has been identified in many genes transcriptionally activated by phorbol esters (Angel et al, 1987a; Angel et al, 1987b; Lee et al, 1987). It was shown (Montminey et al, 1986) that a protein called CREB bound preferentially to the 8 base pair (bp) cAMP-RE, while a nuclear protein AP-1, mentioned above as a possible target for cycloheximide action, with a molecular weight of 44-47kD on SDS-PAGE, could bind to the related 7 bp PMA-RE (Angel et al, 1987b; Lee et al, 1987). It is likely, therefore, that the transcriptional activities of the cAMP-RE and the PMA-RE consensus sequences are mediated by protein phosphorylation, initiated by the respective signal transduction events of adenylate cyclase activation to produce cyclic AMP and inositol phospholipid hydrolysis to produce diacylglycerol.

Because of the close similarity in consensus sequence between the two base pair motifs, the parallels in mechansim of action and the fact that the two transduction systems can interact at several levels (Imagawa et al, 1987; Emoto et al, 1988; Choi & Toscano, 1988), the capability of either consensus motif to respond to either second messenger analog was investigated (Deutsch et al, 1988). It was found that the functional properties of the related DNA elements could be dissociated by the addition or subtraction of a single base pair and that different DNA binding proteins interact

with the 8bp or 7bp sequences.

It was found that PMA and cyclic AMP could respond synergistically on the 7bp motif (PMA-RE) but not on the 8bp motif (cAMP-RE). This implies that the two signal transduction systems converge at one of several levels, some of which have the following precedents;

(i) Activation of PKC (for example by phorbol esters) enhances cyclic AMP accumulation (Ritvos et al, 1987) perhaps by way of phosphorylation of an inhibitory G protein (Pyne et al, 1989; Gordeladze et al, 1988), phosphorylation of a stimulatory G protein (Summers & Cronin, 1988) or phosphorylation of the catalytic subunit of adenylate cyclase (Choi & Toscano, 1988; Summers et al, 1988).

(ii) Cyclic AMP activates PKC or translocates it to the nucleus (Cambier et al, 1987)

(iii) PKA and PKC phosphorylate the same protein, for example AP-1

(iv) Different proteins at the end of the PKA- and PKC-mediated phosphorylation cascades, for example CREB and AP-1, can bind to the 7bp motif (PMA-RE).

The ability of cyclic AMP alone to activate the 7bp motif (PMA-RE) but not for PMA to activate the 8bp motif (cAMP-RE) favours mechanisms i,ii and iii above. It has been reported, however, that the 7bp and 8bp motifs appear

to bind different proteins (Deutsch et al, 1988) and this favours model iv, although distinctly phosphorylated versions of the same protein may have different molecular masses. Clearly, these two potent DNA motifs, differing by 1bp have different transcriptional and protein binding properties, but they share the property of cyclic AMP responsiveness, reflecting the complex interaction between these parallel signal transduction pathways.

Hence, an effect upon gene expression appears to be the most satisfactory explanation for activation of PKC causing inhibition of hepatic steroid metabolism. Based on the above literary evidence, we propose the following hypothesis for PKC-mediated inhibition of hepatic steroid metabolism.

Activation of PKC, either by phorbol esters, synthetic diacylglycerol or vasopressin, promotes a rapid activation of PKC as a consequence of translocation of the kinase to the cell membrane. Once activated, PKC causes a very rapid protein phosphorylation, which could possibly be a site-specific phosphorylation upon the trans-acting factor protein AP-1, which has been identified as a substrate for PKC. This phosphorylation is so rapid that it has occurred before we have begun to take samples in our phorphorylation study, and is probably the reason why we did not observe a change in the phosphorylation profile of the cell in the presence of phorbol ester.

Phosphorylation of AP-1 causes it to become activated , in a way which is unclear at the present time, but the activation prompts the binding of AP-1 to the 7bp motif TGACTCA, the so-called phorbol ester-responsive element

(PMA-RE). Once the AP-1 factor and PMA-RE are coupled together, protein translation occurs, and the resulting proteins then act upon the enzymes metabolising 4-androstene-3,17-dione to inhibit the activity of these enzymes. The synthesis of these unidentified proteins appears to be the site of action for cycloheximide, and it seems that cycloheximide acts in intact cells at a post-translational site to modify proteins produced from translation induced by AP-1. This hypothesis for the site of action of cycloheximide is supported by the evidence that the induction response of the 7bp motif (PMA-RE) to PMA is resistant to cycloheximide (Imbra & Karin, 1986; Imbra & Karin, 1987; Angel et al, 1987a) and also the increase in AP-1 activity is resistant to cycloheximide (Angel et al, 1987b). The translation proteins which are produced by activation of PKC do not appear to be cytochrome P450 itself or the enzymes responsible for mediating steroid metabolism. They appear to be an, as yet, unidentified protein or set of proteins which can exert inhibitory actions upon the activity of hepatic steroid metabolising enzymes. The precise manner by which this inhibition is produced remains to be elucidated.

4.9 (b) Stimulation of hepatic steroid metabolism.

The only time stimulation of hepatic steroid metabolism was observed was when we employed the hormone angiotensin II. Angiotensin II is known to exert intracellular effects via activation of PKC (Gárciá-Sáinz et al, 1986; McAllister & Hornsby, 1988; Pfeilschifter, 1988) and also independently

of PKC (Gárciá-Sáinz & Hernandez-Sotomeyor, 1986; Bouscarel, 1988a). Some of the effects of angiotensin II are thought to be mediated by changes in cyclic AMP accumulation and it has been reported that high intracellular levels of cyclic AMP can cause a stimulation of hepatic steroid metabolism (L. Berry, Ph.D. thesis, 1988) in a similar manner to that which we have seen here with angiotensin II.

Angiotensin II has also been reported to act by increasing mRNA accumulation within the cell (Ben-Ari & Garrison, 1988).

It is conceivable that the increase in steroid metabolism which we observe with angiotensin II is due to an induction of cytochrome P450 levels. However, this hypothesis seems unlikely when we remember that 4-androstene-3,17-dione is metabolised by both cytochrome P450-dependent and cytochrome P450-independent enzymes, and the activity of all the enzymes metabolising 4-androstene-3,17-dione are affected in a similar manner by angiotensin II. Also, induction of cytochrome P450 was only seen with supraphysiological concentrations of cyclic AMP, and the concentration of angiotensin II which we employed (100nM) would not elevate intracellular cyclic AMP to anywhere near these concentrations, because cyclic AMP would be rapidly degraded by cyclic AMP phosphodiesterase.

The effect of angiotensin II to stimulate hepatic steroid metabolism can be most satisfactorily explained, again, by an effect upon gene expression.

It will be remembered from the above section (4.9(a)), that cyclic AMP can bind to both the 7bp motif (PMA-RE) and

the 8bp motif (cAMP-RE) and it can have synergistic actions when it binds to the 7bp motif. As angiotensin has effects upon both cyclic AMP levels, which will activate PKA, and calcium levels, which will facilitate activation of PKC, the marked stimulatory effect of angiotensin II can be proposed to occur as follows.

Occupation of angiotensin II receptors can activate either PKA or PKC alone or possibly both together, in a manner which is regulated by the receptors themselves in a complex, and unexplained manner, based on the evidence of Bouscarel et al (1988b). The activated kinases then phosphorylate their selective transduction proteins, either CREB (Montminey et al, 1986) for PKA or AP-1 (Angel et al, 1987b) for PKC. These phosphorylated, and activated proteins, then bind to their respective base pair motifs and induce protein translation. The proteins produced in the presence of angiotensin confer a stimulatory effect upon the enzymes metabolising 4-androstene-3,17-dione. Whether or not the synthesis of these proteins can be inhibited by cycloheximide was not determined in this study.

The reason we see such a marked stimulation with angiotensin II may be accounted for, based on the fact that angiotensin II induces the production of cyclic AMP, which has the ability to bind to either the 8bp cAMP-RE or the 7bp PMA-RE. If cyclic AMP binds to both motifs at once, a synergistic effect upon protein transduction will occur, possibly as a result of both motifs phosphorylating AP-1, and ultimately producing the marked stimulation which we observe.

An investigation into the effect of angiotensin II acting in the presence of the selective PKA inhibitor KT5720 (section 4.5(c)) would be advantageous in order to probe an involvement of PKA in angiotensin II action to stimulate hepatic steroid metabolism.

It is equally possible that PKC activated by angiotensin II selectively produced proteins which stimulated the activity of the hepatic steroid metabolising enzymes, whilst activation of PKC by phorbol esters, synthetic diacylglycerols or vasopressin induces a selective production of inhibitory proteins. Such a selective production of inhibitory or stimulatory proteins through the actions of the different DNA motifs remains to be estabilished.

4.9 (c) Conclusion to section 4.9

In this section we have tried to elucidate the precise intracellular mechanism by which activation of PKC can either inhibit or stimulate steroid metabolism in isolated rat hepatocytes. By dissecting out some of the various ways by which PKC is known to act, such as protein phosphorylation and enhancement of gene expression, we arrived at the hypothesis that activation of PKC produces proteins, through an enhancement of gene transcription, which appear to possess the ability to modulate the activity of hepatic steroid metabolising enzymes, in a manner which remains to be clarified.

The issue becomes more complicated in the case of the hormone angiotensin II, which appears to possess the ability

to modulate steroid metabolising enzyme activity through a pathway which involves an interaction between both PKA and PKC cascade systems. The processes by which such a cascade interaction may be regulated can only be speculated upon at the present time.

The proteins produced by an enhancement of gene expression do not appear to be the enzymes metabolising 4-androstene-3,17-dione themselves, cytochrome P450 or other proteinaceous components of the hepatic monooxygenase system. To determine the nature of these proteins is an exciting future prospect.

4.10 Physiological implications

The results of this study have shown that activation of the phosphorylating enzyme, protein kinase C (PKC), by hormones known to induce inositol phospholipid turnover can produce marked inhibitory or stimulatory effects upon hepatic monooxygenases. The involvement of PKC in these effects is implicated by the fact that a variety of compounds, which are known to act either completely or at least partly, through activation of PKC all produce the same effect. The predominant effect upon activation of PKC is to cause inhibition of the activity of hepatic monooxygenases.

In vivo effects of various hormones have already been suggested to be a consequence of activating PKC. Vasopressin can act through PKC to inhibit ureogenesis in hepatocytes (Gárciá-Sáinz & Hernandez-Sotomayor 1987), it can inactivate glycogen synthetase (Blackmore et al, 1986) and it can induce the hydrolysis of various cellular

glycerophospholipids (Cabot et al, 1988). The involvement of PKC has been implicated in the actions of other hormones such as thyroid stimulating hormone (Ginsberg et al, 1988), gonadotropin releasing hormone (McArdle et al, 1988), parathyroid hormone (Yamaguchi et al, 1988), adrenocorticotrophic hormone (ACTH) (Reisine & Zatz, 1987), insulin (Acevedo et al, 1989; Cooper et al, 1987), the interleukins (Baumann et al, 1988), and prolactin (Buckley et al, 1988).

The inhibitory effects of vasopressin mentioned above suggest that activation of PKC and its subsequent intracellular effects are one of the main ways of controlling hepatic monooxygenase activity within the liver.

Direct inhibition of hepatic monooxygenases by the activation of PKC has not been widely reported and such reports which exist are contradictory. Inhibitory effects upon hepatic monooxygenases in the past have been attributed to changes in cyclic AMP levels either directly or indirectly (Irvine et al, 1986; Heyworth et al, 1984c).

Glucagon, however, has previously been reported (Hussin et al, 1988) to mediate stimulation of phosphatidylinositol-4,5-bisphosphate (PIP_2) hydrolysis, which results in activation of PKC, and this is thought to be the process by which glucagon inhibits steroid metabolism. Inhibitory effects upon hepatic monooxygenases due to hormonal activation of PKC will hopefully be closely investigated in the future.

Although reports in the past have suggested that cyclic

AMP and protein kinase A (PKA) are important for mediating effects upon hepatic monooxygenases, this study and previous work in our laboratory implies that inhibition of hepatic steroid metabolism can be directly affected by activation of PKC (present study) and also susequent to activation of PKC by activated PKA (L. Berry, Ph.D. thesis, 1988). It appears that cyclic AMP alone is unable to mediate inhibitory effects upon hepatic steroid metabolism and PKA has to activate PKC in order to mediate an inhibitory effect. Whether or not PKA directly activates PKC to cause this effect or perhaps has some unknown effect upon PKC remains to be determined.

Another finding from this study was that activation of PKC by different agonists (section 4.3) had no effect upon the sexually differentiated enzyme profile from either male or female rats and the effect upon inhibition of all steroid metabolising enzymes occurred non-specifically in rats of either sex. This implies that activation of PKC is not the mechanism by which the postulated in vivo feminising factor, now thought to be growth hormone (Guzelian et al, 1988) exerts its effects. Indeed, growth hormone is thought to act directly upon hepatocytes to feminize the cytochrome P450 phenotype, without a requirment for any transduction system. The action of growth hormone to feminise cytochrome P450 phenotype is thought to require hormonal influence and it has recently been shown that a combination of growth hormone, thyroxine and dexamethasone can mediate feminisation of male rat steroid metabolism (P. Gulati, 1989 in press). The combination of these hormonal factors is the

probable cause of feminisation, but the ultimate effect appears to be independent of PKC (French et al, 1989).

Although PKC is a recognised mediator of intracellular hormonal effects, both in the liver and throughout the body, its role in mediating changes in steroid metabolism has not been previously reported. The prospect of fully elucidating the mechanism for regulation of hepatic monooxygenases by PKC is one to look forward to.

4.11 Pharmacological implications.

Although inductive effects of compounds such as phenobarbitone (Hutterer et al, 1975) and 3-methylcholanthrene (Byus et al, 1976) have been attributed to an enhancement of cyclic AMP accumulation and/or activation of protein kinase A (PKA), such a role for protein kinase C (PKC) activation and turnover of inositol phospholipids has not been proposed. As the two cascade systems are subject to interregulation, it may be that an involvement of PKC in induction is being masked by an overriding action of PKA, or it may be that PKA and PKC act in close concert to produce subsequent effects upon induction.

It has been reported that phenobarbitone can inhibit PKC activation in rat brain (Chauhan & Brockerhoff, 1987) and it is possible that PKC acts *in vivo* to suppress induction of cytochrome P450, but once PKC is inhibited,

induction then occurs as a consequence of activation of PKA. The relevance of such a suppression of induction of cytochrome P450 remains to be determined.

The evidence of Pyerin and his group (1987) indicating that different isozymes of cytochrome P450 can be preferentially phosphorylated by either PKA or PKC is also another area in which a more extensive investigation is required.

4.12 General conclusions.

This study has shown, that in isolated rat hepatocytes, stimulation of the signal transduction system which ultimately activates the enzyme protein kinase C (PKC), produces a distinctive effect upon the activity of hepatic steroid metabolising enzymes. Activation of PKC with a variety of different agonists produces the same effect upon steroid metabolising enzymes, implying that activation of PKC is an important *in vivo* modulator of hepatic monooxygenase activity. The effect produced on the steroid metabolising enzymes is either a predominant inhibition of enzyme activity or a less important stimulation. The ultimate effect appears to depend upon the forces controlling signal transduction.

Inhibition occurs as a consequence of directly activating PKC and inhibition of steroid metabolising enzyme

activity appears to be a property of agonists which mediate their intracellular actions solely through the PKC transduction pathway. The inhibitory effect upon steroid metabolising enzymes may be produced after modulation of gene expression by activated PKC.

Stimulation of steroid metabolising enzymes occurs in the presence of a hormone which can exert actions through both transduction systems, angiotensin II being the example we have used. In order to produce a stimulation of steroid metabolising enzyme activity, a complex interaction system appears to be at work between the two cascade systems and it is this interaction, which is as yet unclarified, which determines how the stimulation occurs. It is probable that gene expression is also a controlling factor in producing this stimulation.

We propose that activation of the signal transduction pathway responsible for controlling the activation of the enzyme protein kinase C is an important intracellular mechanism by which hormones can modulate the activity of hepatic monooxygenases.

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