REGULATION OF ADRENOCEPTOR RESPONSES IN ISOLATED RAT HEPATOCYTES

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$[Ca^{2+}]_i$	intracellular free calcium ion concentration
α-, β-AR	α-, β-adrenoceptor
α-, β-NF	α-, β-naphthoflavone
AA	arachidonic acid
AIAP	(+)-S-2-amino-5-iodoacetamidopentanoic acid
BSA	bovine serum albumin
cAMP	cyclic adenosine-3'-5-monophosphate
DMSO	dimethylsulfoxide
DTT	DL-dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EET	eicosatetraenoic acid
EGTA	ethylene glycol-bis(b-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
Glc 1*P*	glucose 1-phosphate
GPase	glycogen phosphorylase
GTN	glyceryl trinitrate
HEPES	N-[2-hydroxyethyl]piperazine-N'[2-ethanesulfonic acid]
HETE	hydroxyeicosatetraenoic acid
IBMX	isobutyl methylxanthine
Ins(1,4,5)P_3	*myo*-inositol 1,4,5-triphosphate
MOPS	3-[N-morpholino]propanesulfonic acid
$NADP^+$	nicotinamide adenine dinucleotide (oxidized form)
NADPH	nicotinamide adenine dinucleotide (reduced form)
NO	nitric oxide

NOHA	N^{ω}-hydroxy-L-arginine
NOS	nitric oxide synthase
ODC	ornithine decarboxylase
PLA_2	phospholipase A_2
PLC	phospholipase C
PtdIns	phosphatidyl inositol
PtdIns(4,5)P_2	phosphatidyl inositol-4,5-biphosphate
SIN-1	3-morpholinosyndonimine
SNP	sodium nitroprusside
TGase	transglutaminase
Tris	2-amino-2-hydroxymethylpropane-1,3-diol

SUMMARY

1. Both α_1- and β-adrenoceptors stimulate glycogenolysis in the liver. In the adult male rat, however, the α-response predominates. Under several conditions including primary culture of isolated hepatocytes, there is a switch from the predominantly α- to a β-adrenergic response. The primary goal of this study was to arrest this time-dependent increase in β-adrenergic responsiveness and thus, to elucidate the factors that regulate these changes.

2. Glycogenolysis was measured as an increase in glycogen phosphorylase *a* activity. Initial experiments, showed that cells were responsive to drugs and hormones such as noradrenaline, isoprenaline, glucagon and insulin. The cells were also responsive to Bt_2cAMP and the ionophore, A23187, which act intracellularly.

3. Responses to phenylephrine (α-agonist) and isoprenaline (β-agonist) was respectively inhibited by prazosin (α-antagonist) and propranolol (β-antagonist). B_{max} and K_D for α_1-adrenoceptors, determined by [^{3}H]prazosin binding, were 0.13 ± 0.03 nM and 319.8 ± 18.5 fmol mg^{-1} protein respectively. Values for β-adrenoceptors, determined by [^{3}H]dihydroalprenolol binding, were 1.17 ± 0.22 nM and 56.2 ± 4.6 fmol mg^{-1} protein.

4. The results showed for the first time that the relative expression of the α- and β-adrenergic response depended on the culture medium in which the hepatocytes were kept. In comparison to Krebs-Henseleit buffer, β-responses were greatly enhanced in cells cultured in Williams' E. In Krebs-Henseleit, phenylephrine was ≈7 fold more potent and ≈2.5 fold more effective than isoprenaline. On the contrary, results obtained in cells kept in Williams' E, showed isoprenaline to be equally potent and almost as effective as phenylephrine. Also, the addition of amino acids to Krebs-Henseleit buffer

increased the β-response in the hepatocytes. This observation, therefore, suggests that the amino acid fraction of Williams' E was responsible for the increased β-response.

5. To examine further the role of culture medium on adrenergic responses, individual amino acids were added to Krebs-Henseleit buffer. The effects of eight amino acids (glycine, L-arginine, L-cysteine, L-glutamic acid, L-lysine, L-methionine, L-proline, and L-valine) were investigated. Addition of L-proline (30 mg l^{-1}) to Krebs' increased the β-response in a manner similar to that in Williams' E. Furthermore, L-arginine, L-glutamic acid, and L-methionine caused an increase in the affinity but not the efficacy of isoprenaline. L-Glutamic acid, also, decreased the affinity of phenylephrine.

6. Consistent with previous workers, incubation of hepatocyte suspensions for up to 6 hours led to increased β-adrenergic responsiveness. Increased β-responsiveness was not necessarily accompanied by a complete switch from α- to β-response—in some instances, the α-responses did not decrease with time.

7. Measurement of cyclic AMP accumulation showed no correlation between the generation of cyclic AMP and the activation of glycogen phosphorylase induced by isoprenaline in freshly isolated cells. These, data probably, suggest the involvement of a cAMP-independent mechanism in responses to the β-agonist, isoprenaline.

8. Dimethyl sulfoxide (2%) and sodium butyrate (2 mM), two differentiating agents used successfully to maintain some liver-specific functions, failed to arrest the time-dependent increase in β-response in hepatocyte suspensions. Furthermore, butyrate appeared to accelerate the process. However, both agents suppressed the emergence of the β-response in short-term (24-48 h) monolayer cultures. This may suggest that the actions of DMSO and sodium butyrate

require a much longer period to exert their effects, probably, through the synthesis of new, and not through the modification of preformed, protein(s).

9. Dexamethasone (0.1-1.0 μM) failed to prevent the increased β-adrenergic response in cultured hepatocytes. On the contrary, dexamethasone increased the β-response, presumably, by upregulation of β-adrenoceptors. The effect of dexamethasone was blocked by cycloheximide (2 μM). However, it must be pointed out that cycloheximide, also, inhibited the increased β-adrenergic responses in control (untreated) cells.

10. The ornithine decarboxylase and arginase inhibitor, (+)-S-2-amino-5-iodoacetamidopentanoic acid (200 μM), suppressed the time-dependent increase in adrenergic response in isolated hepatocyte suspensions. Also, putrescine (200 μM), a polyamine and a competitive inhibitor of polyamine biosynthesis, arrested the increased β-adrenergic responsiveness.

11. The cytochrome P450 inhibitors, metyrapone and SKF525A (proadifen), were employed to investigate the possible involvement of cytochrome P450 in the adrenergic (particularly α-adrenergic) signal transduction. Metyrapone (100-200 μM) shifted the concentration-response curves of phenylephrine and isoprenaline to the right. On the other hand, SKF525A (100-200 mM) shifted the curve of phenylephrine to the right but shifted the curve of isoprenaline to the left.

12. To examine further the role of cytochrome P450, rats were treated with the cytochrome P450 inducer, β-naphthoflavone. Pretreatment with β-NF (80 mg kg^{-1}, i.p., daily for 3 days) caused a 2-fold increase in the total microsomal cytochrome P450 content. Also, the microsomal content of CYP1A1 was increased by 3 fold as determined by enzyme-linked immunosorbent assay. Furthermore, there was a significant increase (≈1.5 fold; $P < 0.05$) in CYP2A1 (7α-hydroxylase), a significant decrease in CYP2C11 (16α-hydroxylase), and

no change in CYP3A1/3A2 (6β-hydroxylase) levels- determined by hepatic microsomal metabolism of androst-4-ene-3,17-dione. These changes in the inventory of cytochromes P450 caused a decrease in the α-adrenergic response. Though there was no change in the β-adrenergic response, the decreased α-response meant that isoprenaline was more potent than phenylephrine in the β-NF-treated cells. α-Naphthoflavone, an inhibitor of β-NF-inducible cytochromes P450, antagonized responses to phenylephrine. These results suggest that, though CYP1A1 may play a role in the α-adrenergic response in hepatocytes, changes in CYP1A1 *per se* are not directly involved in the reciprocal changes in adrenergic responses.

13. Nitric oxide donors, glyceryl trinitrate (GTN) and sodium nitroprusside (SNP) activated glycogen phosphorylase in a dose-dependent manner. Furthermore, both agents antagonized the activation of glycogen phosphorylase induced by adrenergic agonists. KT5822, K252a, and K252b, protein kinase inhibitors, had varied and inconsistent effects on the adrenergic responses and on the inhibition of the adrenergic responses caused by the nitric oxide donors.

14. In summary the following conclusions are drawn from the results:

a) the culture medium used may determine the relative expression of α- and β-adrenergic responses in adult male rat hepatocytes; an effect apparently due the presence and composition of amino acids in the medium

b) the differentiating agents, DMSO and sodium butyrate, can suppress the emergence of the β-adrenergic responses and, thus, may justify their use in hepatocytes in which the continued response of the α-response is required

c) polyamine biosynthesis may play a regulatory role in the reciprocal changes in adrenergic responses

d) cytochromes P450 are involved in the α-adrenergic signal and changes in the profile of these enzymes during cell culture could play an important part in the changes in the adrenergic responses.

e) regulation of hepatic glycogenolysis by nitric oxide and for that matter cAMP, is very complex and may involve several other factors not determined in this study.

INTRODUCTION

1.1 BACKGROUND

The use of the isolated hepatocyte preparation as a tool for both pharmacological and toxicological studies has greatly increased over the past few years. One major reason for the increase in popularity is that hepatocytes are more amenable to experimental manipulations than the intact liver or liver slices e.g. hepatocytes from one liver may be used to establish several incubations in a controlled environment. The use of cell-lines or hepatomas is limited because they exhibit only a few functions characteristic of normal liver *in vivo* (Berry *et al.*, 1991). However, despite the numerous advantages, the use of isolated hepatocytes is not without its shortcomings.

Preparations of isolated hepatocyte suspensions are useful for only relatively short-term studies (up to approx. 6 h) because of damage to cells during incubation. The use of primary cultures for intermediate- and long-term studies is limited by morphological and functional alterations of the cells during the first few days of culture. In culture, the cells undergo major changes with time in patterns of gene expression and differentiated functions. Changes include a progressive decrease in various cytochrome P-450 isoforms and particularly a shift towards a more foetal-like state. Several workers have reported increased expression of typical foetal hepatic isoenzymes such as γ-glutamyl transpeptidase and α-fetoprotein and the synthesis of foetal isozymes such as fructose-biphosphate aldolase (Sirica *et al.*, 1979; Guguen-Guillouzo *et al.*, 1983) and decrease in expression of isozymes such as pyruvate kinase L and aldolase B which are characteristic of adult liver.

De-differentiation or 'foetalization' of hepatocytes in culture is not peculiar to enzyme systems. The adrenergic control of glucose metabolism by adult rat hepatocytes which is predominantly an α_1-receptor mediated event, also changes rapidly to a β_2-adrenoceptor mediated event ('foetal state') during short term *in vitro* incubations and in primary culture (Okajima & Ui, 1982;

Nakamura *et al.*, 1984b; Kunos & Ishac, 1987; Ishac *et al.*, 1992). This change in the expression of the adrenergic response is the subject of this study. 'Down regulation' of vasopressin and angiotensin receptors, in isolated hepatocytes, have also been reported (Bouscarel *et al.*, 1990).

1.2 ADRENOCEPTORS IN RAT LIVER

The main subtype of α-adrenoceptor is the α_{1B} (Torres-Márquez *et al.*, 1991; García-Sáinz *et al.*, 1992a) and the main type of β-adrenoceptor is the β_2 subtype (Ishac *et al.*, 1992).

1.2.1 Signal transduction pathways

α and β-adrenoceptors belong to the superfamily of receptors coupled to guanine nucleotide-binding regulatory proteins (G proteins) (Lefkowitz & Caron, 1988; Allen *et al.*, 1991). Receptors of this superfamily contain seven putative transmembrane spanning domains, with an extracellular NH_2-terminal region containing potential glycosylation sites (asparagine residues) and a cytoplasmic COOH-terminus.

1.2.1.1 α_1-Adrenoceptor signalling

Activation of α_1-adrenoceptors result in an increase intracellular calcium by a mechanism involving G proteins-mediated activation of phospholipase C.

In the liver, α_1AR catalytically activates the G proteins of the Gq family-likely G_{11} or G_q (Taylor *et al.*, 1991, Sternweis and Smrcka, 1992). The G protein then dissociates into βγ- and the α_q -subunits which directly stimulate the activity of phospholipase C-β_1 (PLCβ_1). PLCβ_1, a phosphoinositidase, subsequently hydrolyses membrane phospholipids leading to cellular increases in *myo*inositol 1,4,5-triphosphate, which acts in turn to release intracellular calcium stores, and diacylglycerol, which activates protein kinase C (Minneman, 1988). InsP_3 bind to specific receptors located in intracellular vesicles that trap Ca^{2+}; these open Ca^{2+} channels in such membranes and, thus, increase $[Ca^{2+}]_i$

and subsequently activate of Ca^{2+}-dependent processes (Berridge, 1993; Taylor, 1994). The endoplasmic reticulum is the major store for intracellular Ca^{2+} (Burgess *et al.*, 1983). Activation of PKC by DAG is thought to be responsible for the long-term effects e.g., proliferation and differentiation, of α-adrenergic stimulation (Nishizuka, 1986; Asaoka *et al.*, 1992). Activation of PKC (e.g. with phorbol esters) does not mimic the glycogenolytic effect of α_1-adrenoceptors but causes a complete inhibition through phosphorylation of the α_1-receptors (Garcia-Sainz *et al.*, 1985,1986; Lynch *et al.*, 1985; Corvera *et al.*, 1986).

Recently , it has been shown that besides coupling to G_q in the liver, the α-adrenoceptor is also coupled to the G_h family (Wange *et al.*, 1991, Das *et al.*, 1993, Baek *et al.*, 1994). There are some differences in the actions of the nucleotide binding proteins, G_q and G_h, although, both are thought to mediate the actions of α_1-adrenoceptors by activating PLC (Wu *et al.*, 1992; Nakoaka *et al.*, 1994). Whereas $G\alpha_h$ hydrolyses GTP rapidly, the GTPase activity of $G\alpha_q$ is slow, although it is increased by its interaction with its effector PLC-β (Nakaoka *et al.*, 1994). Also, $G\alpha_h$ is a transglutaminase type II (TGase II) (Nakaoka *et al.*, 1994; Huang *et al.*, 1995). Thus, the $G\alpha_h$ protein is unique in that it exhibits two distinct enzyme activities: a) as a guanosine triphosphatase with a signal transduction role and b) as a TGase II, involved with cell growth and activation of phospholipase A_2 (Piacentini *et al.*, 1991; Huang *et al.*, 1995). Recent findings show that PLC-δ_1 is the effector of G_h-mediated signalling (Feng *et al.*, 1996). The functional significance of α-adrenoceptor coupling to two distinct G proteins is yet to be determined.

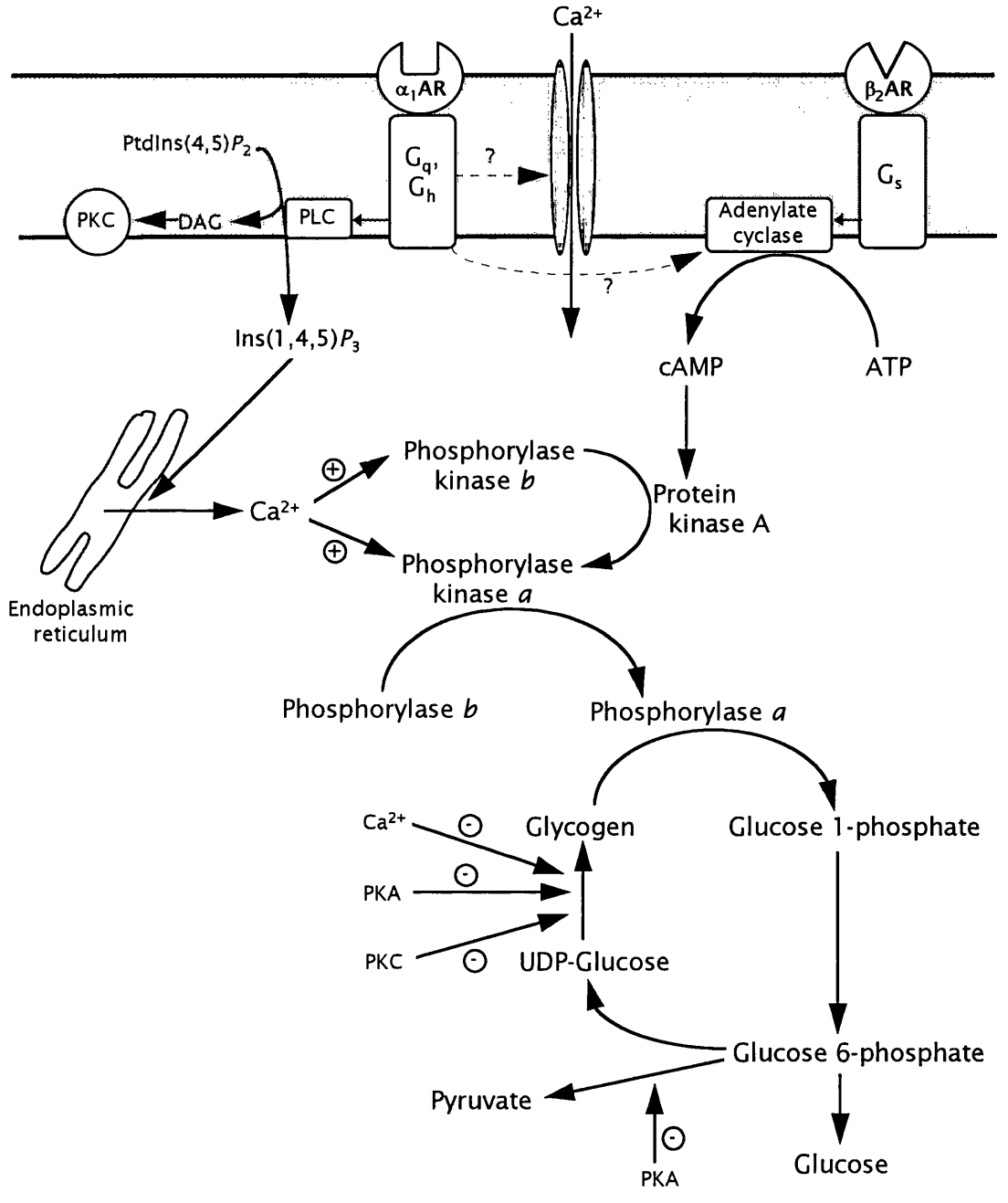


Figure 1.1 Mechanisms of action of α- and β-adrenergic agonist in the liver.

Changes in the Ca^{2+} permeability of both intracellular membranes and plasma membranes, evoked by InsP_3, generate a pattern of response whereby the initial Ca^{2+} signal, reflecting Ca^{2+} mobilization, is relatively independent of extracellular Ca^{2+}, the sustained response (after 2-3 min) depending entirely on extracellular Ca^{2+} (Barrit *et al.*, 1981; Manger *et al.*, 1984). Mitochondria sequester Ca^{2+} at the expense of the H^+ gradient during the sustained increase. Thus the elevation is not sustained for more than a few seconds (Williamson *et al.*, 1981). The major steps in the α_1-adrenoceptor transduction pathway in liver are illustrated in Figure 1.1, using the activation of glycogen phosphorylase as an example.

Although the hepatic actions of the α_1-adrenergic agonists and other Ca^{2+}-mobilizing agonists have been associated with their ability to increase the $[Ca^{2+}]_i$ and the subsequent activation of Ca^{2+}-dependent processes (Williamson *et al.*, 1985; Exton, 1988), there is considerable evidence to indicate that α_1-adrenergic agonist activate hepatic functions under Ca^{2+} loading conditions that precluded any further significant increase in $[Ca^{2+}]_i$ (García-Sáinz & Hernández-Sotomayor, 1985; Saz *et al.*, 1989). The latter observation, therefore, suggests the presence of a Ca^{2+}-independent signalling pathway. Recent work by several workers (Butta *et al.*, 1993; Urcelay *et al.*, 1993, 1994; Ciprés *et al.*, 1995), supports the existence of at least two α_1-adrenoceptor signalling pathways in the rat liver that can operate independently- one of them is PKC sensitive and Ca^{2+} independent; and the other one is Ca^{2+}-sensitive and PKC independent (Butta *et al.*, 1996).

α-Adrenergic agonists have also been shown to stimulate transient increases in K^+ of the perfused rat liver (Reinhart *et al.*, 1984; Häussinger *et al.*, 1987). The functional significance of the increased K^+ permeability is not fully understood. However, it has been suggested that the resulting K^+ efflux may play a role in α-adrenergic signal transduction in the liver (Hill & Ajikobi,

1993). There is a possibility that increased K^+ efflux and concomitant membrane hyperpolarization increase Ca^{2+} influx because of the increased driving force on Ca^{2+} and that K^+ fluxes are induced by α-agonists are requisite for maximum effectiveness (Hill & Ajikobi, 1993). This hypothesis is supported by the following evidence:

1. membrane depolarization induced by increases in extracellular K^+ inhibits hormone-stimulated increases in $[Ca^{2+}]_i$ and glycogenolysis in the liver (Altin *et al.*, 1988) and in isolated suspensions of hepatocytes (Savage *et al.*, 1989).
2. depolarization gradients of K^+ do not stimulate Ca^{2+}-dependent glycogenolysis in rat hepatocytes (Hill *et al.*, 1987).
3. presence of a non-voltage-gated Ca^{2+} channel in the rat hepatocyte (Bear, 1990; Bear & Li, 1991).
4. α-adrenergic responses of the perfused rat liver and isolated hepatocytes are inhibited by the K^+ channel blockers, quinidine and 4-aminopyridine (Hill & Ajikobi, 1993).

There is also evidence to suggest that α_1-adrenoceptors may be coupled to a cAMP generating system in isolated hepatocytes from adult male rats (Morgan *et al.*, 1983a, b; Nomura *et al.*, 1991, 1993).

1.2.1.2 β_2-Adrenoceptor signalling

Agonist activation of β_2-adrenoceptors leads to the generation of cAMP by stimulating adenylate cyclase in the plasma membrane (Figure 1.1). This pathway is mediated by the guanine nucleotide binding protein, G_s. Activation of G_s results in the exchange of bound GDP for GTP. G_s acts also as a GTPase because inactivation of G_s results in the hydrolysis of GTP to GDP (Bocckino & Blackmore, 1993). G_s is composed of three subunits, α, β, and γ. The α-subunit (α_s) activates adenylate cyclase. The elevation of intracellular cAMP concentration leads to the activation of cAMP-dependent protein kinase. cAMP-dependent protein kinase is a tetramer composed of two identical monomeric

catalytic subunits that catalyze phosphorylation reactions and a dimeric regulatory subunit that has four binding sites (sites I and II) for cAMP (Bocckino & Blackmore, 1993). After the binding of cAMP to sites I and II on the regulatory subunit, the catalytic subunit dissociates from the regulatory subunits. The catalytic subunit then causes the phosphorylation of a variety of proteins on serine residues. Many of these proteins have been identified as key regulatory enzymes that are involved in the control of hepatic function.

1.3 Reciprocal Expression of α- and β-Adrenoceptors in Isolated Hepatocytes

In the rat liver, adrenergic control of glucose metabolism (glycogenolysis and gluconeogenesis) is by α_1 and β_2-adrenoceptors (Kunos & Ishac, 1987; Ruffolo *et al.*, 1991). In the fetal and neonatal rat, the predominant pathway mediating glucose metabolism is a β_2-adrenoceptor/cAMP pathway (Blair *et al.*, 1979; Scheglmann & Dettmer, 1992; Van Ermen *et al.*, 1992). In the adult rat liver, however, there are differences between the sexes in the relative contribution that each adrenergic pathway contributes to the overall control of glucose metabolism. In the male adult rats, α_1-adrenoceptors play the predominant role in the regulation of glycogenolysis and gluconeogenesis (Exton, 1979; Hems & Whitton, 1980; Kunos & Ishac, 1987). In the female rat, however, both α_1- and β_2-adrenoceptors participate (Studer & Borle, 1982; Studer & Ganas, 1988). Under certain physiological or pathological conditions (see Table 1.1), the pathway for the control of glucose metabolism in the adult male rat is shifted from the predominantly α_1-adrenoceptor/InsP_3 pathway to a predominantly β_2-adrenoceptor/cAMP pathway. A similar shift from α- to β-response is also observed in primary cultures of male rat hepatocytes (Nakamura *et al.*, 1983, 1984; Itoh *et al.*, 1984; Kunos *et al.*, 1984b; Schwartz *et al.*, 1985; Sandnes *et al.*, 1986).

Table 1.1 Pathophysiological conditions causing a shift from α_1- to β_2-adrenergic regulation of glycogenolysis in rat liver.

Condition	References
Adrenalectomy	Exton *et al.* (1976); Guelläen *et al.* (1978); Chan *et al.* (1979); Goodhardt *et al.* (1982); Freunderich & Borle (1988).
Partial hepatectomy	Aggerbeck *et al.*, 1983; El-Refai & Chan, 1986.
Hypothyroidism	Malbon, 1980; Preiksaitis & Kunos, 1979.
Fasting	El-Refai & Chan, 1982
Cholestasis	Aggerbeck *et al.*, 1983; Okajima & Ui, 1984.
Malignant transformation	Christoffersen & Berg, 1975; Refsnes *et al.*, 1986.
Endotoxaemia	Pittner & Spitzer, 1993a.

Also, diabetes has also been reported to alter adrenergic responses in the liver. However, these reports on the effect of diabetes on the activation of adenylate cyclase in response to adrenergic agonists and glucagon are inconsistent. Results obtained by some workers showed increased activity (Soman & Felig. 1978, Allgayer *et al.*, 1982) in contrast to the decrease in adenylate cyclase activity shown by other workers (Yamashita *et al.*, 1980; Dighe *et al.*, 1984). Strictland *et al.*, (1977) observed no change in the responses in comparison those of control rats. Recently, Shima *et al.* (1992) have shown that the duration and/or severity of the experimentally-induced diabetes determines the changes that occurred in the adrenergic responses. In acute (3 days) streptozotocin-induced diabetes, there was an increased sensitivity of adenylate cyclase to isoprenaline without changes in the affinity or numbers of β-adrenoceptors. Increased activity of the b-agonist was accompanied by increased coupling of the receptors to the stimulatory nucleotide-binding

protein, G_s. The chronic (15 days) diabetic state caused a decrease in adenylate cyclase response to hormonal and non-hormonal stimuli with a decrease in the number of α- and β-adrenoceptors.

1.3.1 Possible mechanisms regulating reciprocal changes

This plasticity, which has also been observed in several physiological and pathological conditions, has been extensively studied not only in the liver but also other tissues especially in cardiac muscles (Buczek-Thomas *et al.*, 1992). There is considerable evidence to suggest that increased β-adrenergic responsiveness *in vivo* and *in vitro* is due to one or a combination of the following factors:

1. quantitative and qualitative changes in β-adrenoceptors (Nakamura *et al.*, 1983, 1984b; Refsnes *et al.*, 1983; Bendeck & Noguchi, 1985; Katz *et al.*, 1985; Schwartz *et al.*, 1985).
2. variation in the coupling of $G\alpha_s$ protein with β-adrenoceptors (Kajiyama & Ui, 1994; Kajiyama *et al.*, 1996).
3. alterations in $G\alpha_s$ (Yagami *et al.*, 1994a, b, Rodriguez-Henche *et al.*, 1994)
4. alteration in $G\alpha_i$ (Itoh *et al.*, 1984; Ui *et al.*, 1985).
5. increased amount of adenylate cyclase (Yagami *et al.*, 1994a, b).

Some of the various mechanisms proposed to explain the reciprocal change in a- and b-adrenergic responses are briefly reviewed below.

1.3.1.1 Protein synthesis/receptor numbers

Although protein synthesis and the generation of new β-adrenoceptors have been implicated in the transition from α_1- to β_2-receptor mediated glycogenolysis (Nakamura *et al.*, 1983; Refsnes *et al.*, 1983), these processes take about 1-3 days to fully develop (Nakamura *et al.*, 1984; Schwartz *et al.*, 1985) and the conversion of the receptor response is maximal within 8 h (Okajima & Ui, 1982). In hepatocyte suspensions, the conversion is complete in

4 h (Kunos *et al.*, 1984). Other workers have detected the onset of β-function which is not accompanied by any measurable increase in β-ligand binding sites (Ishac & Kunos, 1987; Tsujimoto *et al.*, 1986; Kajiyama & Ui, 1994). Decrease in α-adrenoceptor numbers (Kajiyama & Ui, 1994; Kajiyama *et al.*, 1996) and changes in the affinities of α and β-adrenoceptors the receptors have also been reported (Kunos *et al.*, 1984; Kunos & Ishac, 1987).

1.3.1.2 Phospholipase A_2/arachidonic acid metabolism:

An increase in phospholipase A_2 mediated release of arachidonic acid has also been suggested as a possible cause in the time-dependent interconversion of the adrenergic receptors involved in glycogenolysis (Kunos *et al.*, 1984). They observed that the addition of arachidonic acid to freshly isolated hepatocytes suppressed the α_1- and enhanced the β-receptor mediated activation of phosphorylase while cyclooxygenase inhibitors in high concentrations inhibited the conversion. Fatty acid-free bovine serum albumin also prevented the shift from α_1 to β_2-response. Kunos and co-workers (1984) suggested that fatty acid-free BSA acted as a 'trap' for fatty acids produced by the cell membranes. The role of phospholipids is also supported by the observation that glucocorticoid deficiency also caused a shift in the receptors (Chan *et al.*, 1979). Glucocoticoids inhibit membrane phospholipase A_2 with a subsequent decrease in the release of arachidonic acid and its metabolites, which are thought to mediate most of the actions of glucocorticoids including cellular differentiation (Edwards *et al.*, 1987). However, despite the successes achieved with glucocorticoids in the maintenance of differentiated functions of hepatocytes in culture (Guguen-Guillouzo & Guillouzo, 1983; Jeffersen *et al.*, 1985; Khan *et al.*, 1993), their use in maintaining differentiated adrenergic phenotype in culture has met with very little or no success (Nakamura *et al.*, 1984; Refsnes *et al.*, 1983). It has been proposed that micromolar concentrations, necessary for the maintenance of certain hepatic-specific functions, may be acting by a

mechanism different from the mechanism with physiological concentrations (Edwards *et al.*, 1987).

1.3.1.3 Guanine nucleotide regulatory proteins:

Ui *et al.* (1985) have suggested the involvement of the inhibitory nucleotide protein, G_i in the conversion of adrenergic responses. They suggested that a gradual loss of the activity of this protein coupled to β_2-receptors, because of ADP ribosylation, accounted for the increase in β_2-activity. An impairment in the stimulatory interaction between the α_s subunit of G_s and the adenylate cyclase catalytic subunit has been reported in cholestasis (Rodriguez-Henche *et al.*, 1994). Furthermore, Yagami and co-workers (1994a, b) have shown that differences in the expression of the β-adrenergic response are due not only to qualitative and quantitative changes in the β-adrenoceptors but also due to alterations in $G\alpha_s$. Two form of $G\alpha_s$, $G\alpha_{s\text{-}S}$ and $G\alpha_{s\text{-}L}$, have so far been identified (Mumby et al., 1986; Jones & Reed, 1987) and it appears the β-adrenergic receptor is differentially coupled to the two forms. The relevance of this to final response is not clear. However, there are differences in the relative amounts of these forms of $G\alpha_s$ in the male and female (Yagami *et al.*, 1994a). Changes are also observed in hepatectomised rats (Yagami *et al.*, 1994b).

1.3.1.4 Loss of post-receptor coupling:

Although hepatocytes contain all the components of the β-adrenoceptor and the adenylate cyclase systems, the response to β-agonists is very low and only seen with high concentrations of adrenaline and isoprenaline (Katz *et al.*, 1985). It has been suggested an 'unmasking' or movement of existent receptor molecules back to or within the membrane could allow the expression of β-receptor activity (Studer and Ganas, 1988).Kajiyama and Ui (1994) have shown that G_s was mostly uncoupled from β-adrenoceptors during cultures that were 2-hour or less old. Subsequently, G_s became promptly coupled to β-adrenoceptors.

1.3.1.5 Loss of cell-cell interactions

The switch from α to β-adrenergic response was inhibited in cultures at high density. This suggests that cell-to-cell contact is essential for preserving the status (Kajiyama & Ui, 1994). Recent studies (Kajiyama *et al.*, 1996) have shown that unsialylated galactosyl termini on the surface of adult hepatoctyes determined the relative development of α_1 and β subtypes of adrenergic responses. This conclusion was based on the following observations:-

1. addition of rat liver membranes to hepatocyte cultures prevented the 'a1 to β subtype switching'
2. the inhibitory effect of the plasma membrane was lost when the membranes were treated with endoglycosidase F or β-galactosidase
3. plasma membranes of young rats did not prevent the switch but became effective on treatment with sialidase.

1.4 Regulation of α_1-Adrenoceptor Gene Transcripts

In Sprague-Dawley rats, transcript destabilization contributes to the decrease in the steady-state levels of β_2-adrenergic receptor mRNA that occurs during early postnatal development in the rat. This process has been associated with a cellular factor (an M_r 85000 protein), identified in adult male rat liver that may interact with the β_2-adrenergic receptor mRNA and may account for the decreased stability of hepatic β_2-adrenergic receptor gene transcripts that occurs during development.(Baeyens & Cornett, 1995). Also, hepatocytes isolated from Sprague-Dawley rats have been shown to express two α_{1B}-adrenergic gene transcripts (Deng & Cornett, 1994). However, the physiological significance of this finding is not clear.

1.5 Adrenergic Control of Hepatic Metabolic Functions

1.5.1 Adrenergic control of glucose metabolism the liver

Catecholamines cause glycogenolysis and in turn the mobilization of glucose output in the liver *via* two main pathways:- (a) a cAMP-dependent β_2-adrenoceptor and (b) a Ca^{2+}/PtdIns-dependent α_1-adrenoceptor pathways (Exton, 1982). Activation of both pathways ultimately leads to the activation of glycogen phosphorylase (1,4-α-D-glucan:orthophosphate α-D-glucosyl-transferase, EC 2.4.1.1).

1.5.2 Adrenergic control of other hepatic functions

1.5.2.1 Effect on synthesis and secretion of lipoproteins

In rat hepatocytes, adrenaline produces an α_1-adrenoceptor mediated inhibition of triglyceride synthesis (Brindle & Ontko, 1986) and a suppression of triglyceride mobilization (Brindle & Ontko, 1988; Woodside & Ontko, 1989). α_1-Adrenoceptor activation also stimulates the oxidation of endogenous fatty acids (Brindle & Ontko, 1988), resulting in a diversion of these substrates from the triglyceride synthetic pathways. In the rat, the ability of adrenaline to produce an acute elevation of total plasma cholesterol is potentiated by phentolamine, suggesting an inhibitory α-adrenoceptor control (Kunihara & Oshima, 1983).

1.5.2.2 Adrenergic modulation of chemical-induced hepatotoxicity

α-Adrenoceptor activation has been shown to decrease hepatocyte glutathione levels. This may result from both an increased efflux and decreased synthetic rate of glutathione (Estrela *et al.,* 1988). Since glutathione plays a key role in the prevention of chemical-induced hepatotoxicity, the adrenergic effects on glutathione levels provide a possible explanation for the ability of α-adrenoceptor antagonists to attenuate the hepatotoxic effect of agents such as

bromobenzene and carbon tetrachloride (Kerger *et al.*, 1989). The α-adrenergic subtype responsible for these actions is not clear (Estrela *et al.*, 1988).

1.5.2.3 Adrenergic control of liver growth

The role of the α-adrenoceptor in the modulation of hepatic regeneration following partial hepatectomy has been well documented (Kost *et al.*, 1992; Michalopoulos, 1990; Fausto & Weber, 1994). Studies using primary cultures of hepatocytes show that α_1-adrenoceptor activation can enhance DNA synthesis (Cruise *et al.*, 1985). Selective blockade by prazosin can inhibit regenerative DNA synthesis following partial hepatectomy (Cruise *et al.*, 1987). A functional role for the α-adrenoceptor in hepatic regeneration is suggested by the ability of hepatic denervation to mimic the effect of prazosin. In hepatocyte cultures, transforming growth factor β (TGF-β) produces a complete inhibition of the increased DNA synthesis induced by epidermal growth factor (EGF). This inhibitory effect of TGF-β can be reversed by norepinephrine (Houck & Michalopoulos, 1989). Male rats were used for these experiments and suggest the involvement of α_1- as opposed to α_2- or β-adrenoceptors. Recent evidence shows that the increase in activity of thymidylate synthetase and thymidine kinase induced by partial hepatectomy involved only an α-adrenoceptor action in male rats, in contrast to a combined α- and β-adrenoceptor action in the female rat (Tsukamoto & Kojo, 1990). Also, β-adrenergic blockade inhibits the growth response after partial hepatectomy and isoprenaline was found to enhance the DNA synthesis of cultures of normal hepatocytes and from regenerating liver (Brønstad & Christoffersen, 1980). Subsequently, Refsnes *et al.* (1992) have shown both α_1 and β_2-adrenergic components to be involved in DNA synthesis in hepatocytes. Finally, Refsnes *et al.* (1992) have shown that catecholamines inhibit the G_1-S transition *via* β-adrenoceptors and may therefore play a role in the termination of hepatic proliferation.

1.5.3 Species heterogeneity of adrenergic control of glycogen phosphorylase activity.

There is no "tissue-specific" pattern of expression since liver cells from different species express different subtypes of α_1-adrenoceptors, i.e., guinea pig express α_{1A}-, rat hepatocytes α_{1B}-, and rabbit hepatocytes α_{1C}-adrenoceptors (García-Sáinz *et al.*, 1992a, b).

Dog (Stevenson *et al.*, 1984) and rabbit (Rufo *et al.*, 1981) hepatocytes display a greater sensitivity to β-adrenergic agonists than do rat hepatocytes. In human liver tissue, cAMP-independent, calcium-mediated agonists do not activate glycogen phosphorylase as much as either glucagon or isoprenaline, both using cAMP as second messenger. Thus human liver tissue resembles rabbit and guinea pig liver tissue, where the cAMP-independent agonists are less efficient than glucagon at activating glycogen phosphorylase (Arinze & Kawai, 1983; Keppens *et al.*, 1993).

1.6 Maintenance of Differentiated Functions in Hepatocyte Cultures

The changes described above lead to the deterioration and death of the hepatocytes within 1 to 2 weeks (Maher, 1988; Berry *et al.*, 1992). Much research effort has been expended to resolve this problem and has involved modification of the cell culture environment with a view to defining appropriate conditions under which hepatocytes will maintain a differentiated phenotype for long periods.

Techniques employed to achieve long-term cultures of adult rat hepatocytes are based on the following:

a) the addition of several soluble factors and solvents to the culture medium

b) on the *in vitro* reconstitution in the *in vivo* cellular environment e.g. co-culture with other epithelial cells or culturing on various substrata

1.6.1 Addition of soluble mediators

This has involved the addition of various substances such as glucocorticoids, trace elements and DMSO in the so-called 'hormonally defined medium' (HDM) (Isom *et al.*, 1985; Jeffersen *et al.*, 1985; Reid *et al.*, 1986) or the addition of pharmacological inducers of differentiation such as DMSO and sodium butyrate.

1.6.1.1 Hormonally defined medium

Several hormones, either alone or in combination, have been used to preserve the differentiated functions of the liver. These include insulin, dexamethasone, hydrocortisone, glucagon and epidermal growth factor (Berry *et al.*, 1991). The commonly used hormones are the glucocorticoids. Glucocorticoids are known to modulate the proliferation and differentiation of various tissues and cell types and dexamethasone in particular has been used to suppress the production of α-fetoprotein (Saad *et al.*, 1993). In our laboratory, the addition of dexamethasone and insulin have been shown to maintain, to some extent, the level of 16α-hydroxylase in primary culture of rat hepatocytes (Khan *et al.*, 1992).

1.6.1.2 Addition of chemicals

The main agents used as inducers of differentiation are dimethyl sulfoxide (DMSO), sodium butyrate and heparin.

Dimethyl sulfoxide (DMSO)

DMSO has been shown to induce differentiation is many cell lines (Higgins and Borenfreund, 1980; Higgins *et al.*, 1983) and it has also been used with some success to maintain the differentiated functions of primary cultures of rat hepatocytes (Baribault and Marceau, 1986; Isom *et al.*, 1985, 1987; McGowan, 1988; Kost and Michalopoulos, 1991). Isom *et al.* (1985) first reported that adult rat hepatocytes in a medium supplemented with EGF and 2% DMSO survived much longer and synthesized albumin much better than cells cultured in standard serum-free medium. They also observed that the

morphology and ultrastructure of DMSO-treated cells resembled those of the normal hepatocytes, though some abnormalities such as misshapen mitochondria and unusual cytoplasmic filaments were also observed. More recently, other workers have reported that when 2% DMSO was added to culture medium after hepatocytes had proliferated, the cells could recover differentiated functions such as albumin and transferrin secretion, and glucose-6-phosphatase activity (Mitaka *et al.*, 1993). The mechanism(s) through which DMSO induces differentiation is not clear. However, it has been shown to trigger the release of Ca^{2+} from intracellular stores; an action independent of phospholipid breakdown (Morley and Whitfield, 1993), and to activate protein kinase C (Yamamoto, 1989). DMSO is also known to affect the expression of several cellular oncogenes whose products are suspected to be the key players in the proliferation and differentiation of many cell types (Lachman & Skoultchi, 1984). DMSO is also thought to protect cells by scavenging hydroxyl radicals and reactive oxygen species that are formed under conventional conditions (Villa *et al.*, 1991). Furthermore, DMSO has been shown to induce the gap junctional protein connexin32 (Cx32) and thus cause the reappearance of extensive gap junctional intercellular communication in adult rat hepatocyte cultures (Kojima *et al.*, 1995). Gap junctional intercellular communication is thought to play a crucial role in cell differentiation and growth control in multicellular organisms (Lang *et al*, 1991; Mesnil and Yamasaki, 1993).

Sodium butyrate

When added to cells in culture and *in vivo,* butyrate has several properties. The main effects of butyrate may be summarized as follows:

a) Arrest of cell proliferation;

b) Alteration of cell morphology and ultrastructure;

c) Alteration of gene expression.

All the effects of butyrate are reversible; shortly after the removal of butyrate from the medium, the cells recover their initial molecular and cellular characteristics (Kruh *et al,* 1995). Butyrate is believed to act by strongly inhibiting histone deactylases, which results in hyperacectylation of histones (Kruh *et al,* 1995). Butyrate has been shown to reduce the growth of several cell types including Chinese hamster ovary (CHO) cells (Wright, 1977), chick fibroblast and HeLa cells (Hagopian et *al.,* 1977) and human breast cancer cells (Guilbaud *et al.,* 1990; Planchon *et al.,* 1991). Addition of butyrate to cell cultures has been shown to lead the arrest of cell growth at the G_1 phase of the cell cycle (d'Anna *et al.,* 1980; van Wijk *et al.,* 1981).

Butyrate is also able to induce the synthesis of several proteins including peptidic and glycoprotein hormones and receptors (Kruh *et al.,* 1995). For example, butyrate induces the synthesis of thyroid hormone receptors in isolated rat hepatocytes, hepatomas and fibroblasts (Matsuhashi *et al,* 1987). Also, the addition of butyrate (0.2-5 mM, final concentration) to culture medium not only increases the number of β-adrenergic receptors but also increases the ability of the receptors to couple with the adenylate cyclase system in HeLa cells and isolated fetal rat hepatocytes (Tallman *et al.,* 1977, 1978; Lin *et al.,* 1979; Kassis, 1985).

Butyrate induces morphological modification of cells. The nature of the modifications vary from one cell type to another. In hepatocytes, butyrate has been shown to have effect the cytoarchitecture and cytoskeletal elements (Gladhaug *et al.,* 1988). Addition of butyrate, two hours after plating, retarded the flattening and maintained the polyhedral shapes of hepatocytes in culture (Gladhaug *et al.,* 1988).

Heparin

It has been shown that heparin, in combination various hormones such as insulin, glucagon and hydrocortisone, which are present in most hormonally

defined media, induces transcription of tissue-specific genes in cultured adult rat hepatocytes (Fujita *et al,* 1986; Spray *et al.,* 1987; Brill *et al.,* 1995). Furthermore, heparin has been shown to regulate the expression of autocrine growth factors both transcriptionally and post-transcriptionally (Zvibel *et al.,* 1991).

1.6.2 Extracellular matrix

In vivo, hepatocytes have a unique cellular and cell-to-cell architecture that helps in the performance of the many functions including exocrine and metabolic functions. Hepatocytes are three-dimensional, polygonal cells with regional specialization of the plasma membrane that facilitates uptake and secretion (Arterburn et al., 1995, Phillips *et al.,* 1987). The basolateral sinusoidal surfaces of the cells face the Space of Disse and are bathed in plasma. These surfaces are specialized for nonspecific and receptor-mediated uptake and section of plasma proteins. Hepatocytes, *in vivo*, aggregate into plates to form trabecular that, together with the increased surface area afforded by the numerous microvilli at the sinusoidal surfaces, result in maximized exposure of the cells to the plasma. The basolateral surfaces form specialized junctions that facilitate adhesion and communication between cells. The apical surfaces are distinctive areas of the membranes between adjacent cells that are sealed by tight junctions and through which bile is secreted into the biliary system. In contrast, monolayer cultures of hepatocytes do not maintain their specialized architecture, but rather express a flattened and often an apolar phenotype (Arterburn *et al.,* 1995).

Cells bind to ECM as a means of attaching themselves, to derive traction for migration and to receive signals from the matrix-bound growth factors (Ruoslathi *et al.,* 1994). The binding of cells to extracellular matrices is mediated by cell surface receptors. The primary class of these receptors is a family of transmembrane proteins known as intergrins.

Extracellular matrices are insoluble structures composed of collagens, various glycoproteins, proteoglycans, hyaluronic acid and elastin. Extracellular glycoproteins interact with cells and this interaction promotes cell adhesion. The adhesive glycoproteins contain various laminins, vitronectins and thrombospondin and von Willebrandt factor. Besides binding for cell attachment, the interactions of cells with the ECM molecules regulate other functions such as cell proliferation, differentiation and migration (Ruoslathi *et al.*, 1987; Streuli *et al.*, 1991).

The ECM of the liver may contain components unique to the liver, suggested by the preferential growth of hepatocytes on matrix prepared from the liver. Hepatocyte proliferation is enhanced by attachment to matrix proteins especially fibronectin (Sawada *et al.*, 1986, Sudhakaran *et al.*, 1986).
The ECM of the liver is composed of at least 5 distinct genetic types of collagen, seven classes of noncollagenous proteins and several proteoglycans and glycoaminoglycans (Rojkind & Greenwel, 1994).

Different types of extracellular matrices have been used successfully to maintain some aspects of liver-specific functions (Bequé *et al.*, 1984; Fraslin *et al.*, 1985; Guguen-Guillouzo, 1986; Kuri-Harcuch & Mendoza-Figueroa, 1989). The use of extracellular matrices such as Matrigel® and collagen in place of the plastic or glass of culture plates has been useful in maintaining liver specific functions. It has been observed that cell-cell and cell-substrate interactions in co-culture and the use of an extracellular matrix play an important role in the development, differentiation and regeneration of multicellular organisms (Saad *et al.*, 1993). The reestablishment of cell-cell contacts between the isolated hepatocytes suppresses growth (observed at low cell densities) and stimulates the expression of liver specific differentiated functions (Mizuno *et al.*, 1993). Also, several workers have successfully established a three-dimensional structure of the hepatocytes in culture and thus maintain the differentiated status

of the cells. These include: culture in a collagen 'sandwich' configuration (Sirica *et al.*, 1979) or culture on or under Matrigel®-a laminin rich collagen and proteoglycan containing extracellular matrix protein from the Engelbeth-Holm-Swarm (EHS) mouse tumor (Schuetz *et al.*, 1988). Isolated hepatocytes entrapped within calcium alginate are reported to retain the ability of *de novo* protein synthesis as well maintain the α-adrenergic response on gluconeogenesis observed *in vivo* (Miura *et al.*, 1988, 1990).

1.6.3 Cell-cell interactions(cocultures)

There is considerable evidence to suggest that cells cultured at high density, which form tight cell-cell contacts, are quiescent for growth and express liver-specific differentiated functions (Nakamura *et al.*, 1983). On the contrary, hepatocytes cultured at low density are receptive to growth signals. It has been proposed that the relationship between hepatocyte growth was regulated by unique plasma proteins (Nakamura *et al.*, 1983, Mizuno *et al.*, 1993). When solubilized and partially purified membrane was added to cells at low density, the cells did not respond to mitogens, as often happens when they were cultured at high cell density. Recently, Kajiyama and coworkers (1994, 1996) have shown that the addition of liver plasma membranes to hepatocytes prevented the time-dependent acquisition of the β-response.

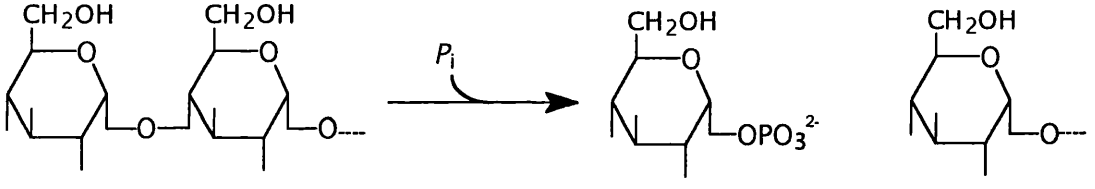
Results from literature suggest that no individual approach may be described as the best culture method since optimum results achieved by co-culturing or the use of complex extracellular matrix is only possible with the addition of hormones and other factor to the medium. Recently a novel system has been employed which benefits both from cell-cell interaction and extracellular matrix (Bader *et al.*, 1996). The cells were cultured in a three-dimensional design reflecting as closely as possible the *in vivo* situation. This was achieved by positioning nonparenchymal cells on top of parenchymal cells enclosed as a monolayer within a collagen sandwich. Cells cultured this way

were cuboidal and formed confluent layers between the nonparenchymal layer and within one week characteristic nonparenchymal cells (endothelial cells, Kupffer cells and Ito cells) had completely covered the second matrix. This system preserved most of the liver-specific functions.

1.7 Glycogen Phosphorylase

1.7.1 Function and regulation

Glycogen phosphorylase plays a central role in the mobilization of carbohydrate reserves in many organisms including bacteria, fungi, plants and insects. It catalyses the first step of the intracellular degradation of glycogen, where P_i removes the non-reducing terminal glucose residue from an α-(1-4)-glycan as α-D-glycopyranosyl phosphate according to the equation:



Glycogen α-D-Glucose 1-phosphate

Glycogen phosphorylase catalyses both the forward and the reverse reaction. Under *in vivo* conditions the phosphate concentration is usually so high that the catalysed reaction is in the direction of glycogen degradation.

Inactive phosphorylase *b* (GPase *b*) can be activated in two ways, namely (a) by covalent alteration through phosphorylation of ser-14 at its terminal tail in response to hormone and neural signals, and b) by allosteric binding to the activator AMP.

The regulation of glycogen phosphorylase and, thus, glycogenolysis by Ca^{2+} and cyclic AMP is schematically shown in Figure 1.2. Calmodulin mediates the Ca^{2+}-dependent regulation of phosphorylase kinase, a site where regulation by hormones and metabolites is integrated in glycogen synthesis and breakdown (Garrison *et al.*, 1984; Cohen, 1992). A subunit of phosphorylase

kinase *b*, a substrate for cyclic AMP-dependent protein kinase (PKA), is Ca^{2+} activated. Calmodulin phosphorylates glycogen phosphorylase, converting it from its *b* (or inactive) to its *a* (or active) form. Phosphorylase kinase also has two forms—nonphosphorylated and phosphorylated. Shift from one form to the other is by a cyclic AMP-dependent protein kinase (and conversely, protein phosphatase). Both forms of phosphorylase kinase are activated by Ca^{2+} and catalyze the phosphorylase of phosphorylase b; the phosphorylated form of phosphorylase kinase b is activated by Ca^{2+} concentrations ≈10 times lower than those needed to activate the nonphosphorylated form (Malencik & Fischer, 1982). In unstimulated cells, cytosolic [Ca^{2+}] is ≈100 nM (Williamson *et al.*, 1985) and at this concentration the phosphorylated form of phosphorylase kinase is active. The rise in $[Ca^{2+}]_i$ after hormonal stimulation activates the nonphosphorylated form. The proteins that are phosphorylated [glycogen phosphorylase (from inactive to active); glycogen synthase (active to inactive) and phosphatase inhibitor protein (inactive to active)] increase glycogen breakdown and decrease glycogen synthesis.

1.7.2 Glycogen phosphorylase isozymes in foetal and adult hepatocytes

There are at least 3 types of glycogen phosphorylase isozyme, namely muscle, brain (or foetal) and liver types, in mammalian organs (Sato & Sato, 1980; Newgard *et al.*, 1989). Although all of these forms can be interconverted as stated above, they are differently regulated by allosteric effectors in a way that reflects their physiological role. The major difference is that, the muscle and brain dephosphorylated forms (*b* or inactive form) exhibit their activities in the presence of AMP, whereas liver phosphorylase *b* is inactive even in the presence of this nucleotide and requires further SO_4^{2-} for the full activity (Stalmans & Hers, 1975).

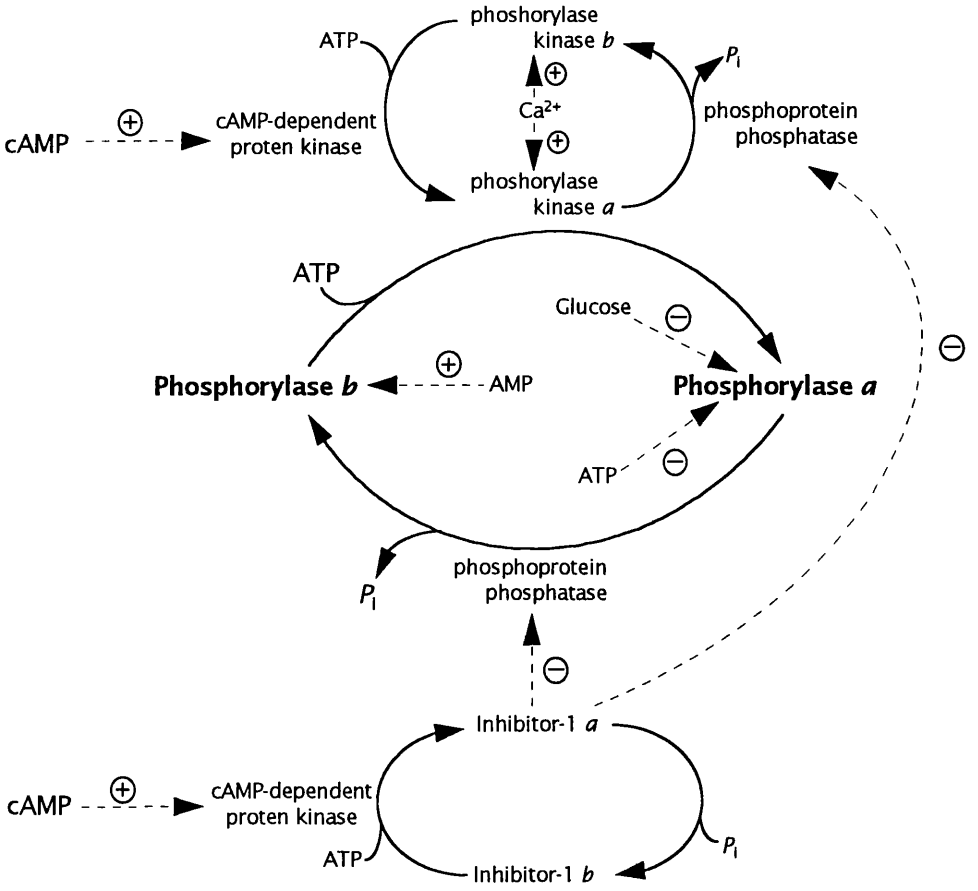


Figure 1.2 Covalent activation of glycogen phosphorylase

The major physiological role of the liver form is to ensure a constant supply of glucose for extrahepatic tissues, especially the central nervous system which relies on glucose as its major source of fuel. This is in contrast to the function of the muscle phosphorylase isozyme, which is primarily to supply energy for the sole utilization of the tissue in which it is found. The role of the brain enzyme is the supply of emergency glucose during stressful periods (Newgard *et al.*, 1989).

A shift from adult to foetal isoenzyme expression has been described for several enzymes, including glycogen phosphorylase, involved in carbohydrate metabolism in hepatomas and during liver regeneration (Sato & Sato, 1980; Weinhouse, 1982). However, there has been no reported work on glycogen phosphorylase isozyme in cultured hepatocytes.

Such alterations have several implications and it is believed that such alterations account for the loss of the control mechanisms of glycolysis e.g. the foetal liver, despite exhibiting a glucagon-dependent rise in cAMP concentration and activity of cAMP-dependent protein kinase similar to that found in adult hepatocytes, lacks the hormonal response induced by glucagon on pyruvate kinase, 6-phosphofructo-2-kinase (PFK-2) and fructose 2,6- biphosphatase that is present in adult tissue (Martin-Sanz *et al.*, 1987).

1.8 Nitric Oxide and Hepatic Metabolism

Nitric oxide (NO) is a cellular mediator with physiological activities including vasodilatation, neurotransmission, regulation of platelet aggregation and cytotoxicity (Nathan, 1992; Bredt & Snyder, 1992; Moncada & Higgs, 1993). NO was initially discovered in endothelial cell as being synonymous with endothelium-derived relaxing factor (EDRF) (Palmer *et al.*, 1987; Ignarro *et al.*, 1987). In mammalian cells, NO is formed through the oxidation of one of the guanido nitrogens of L-arginine at the expense of NADPH and molecular oxygen through an intermediate, N^{ω}-hydroxy-L-arginine (Figure 1.3).

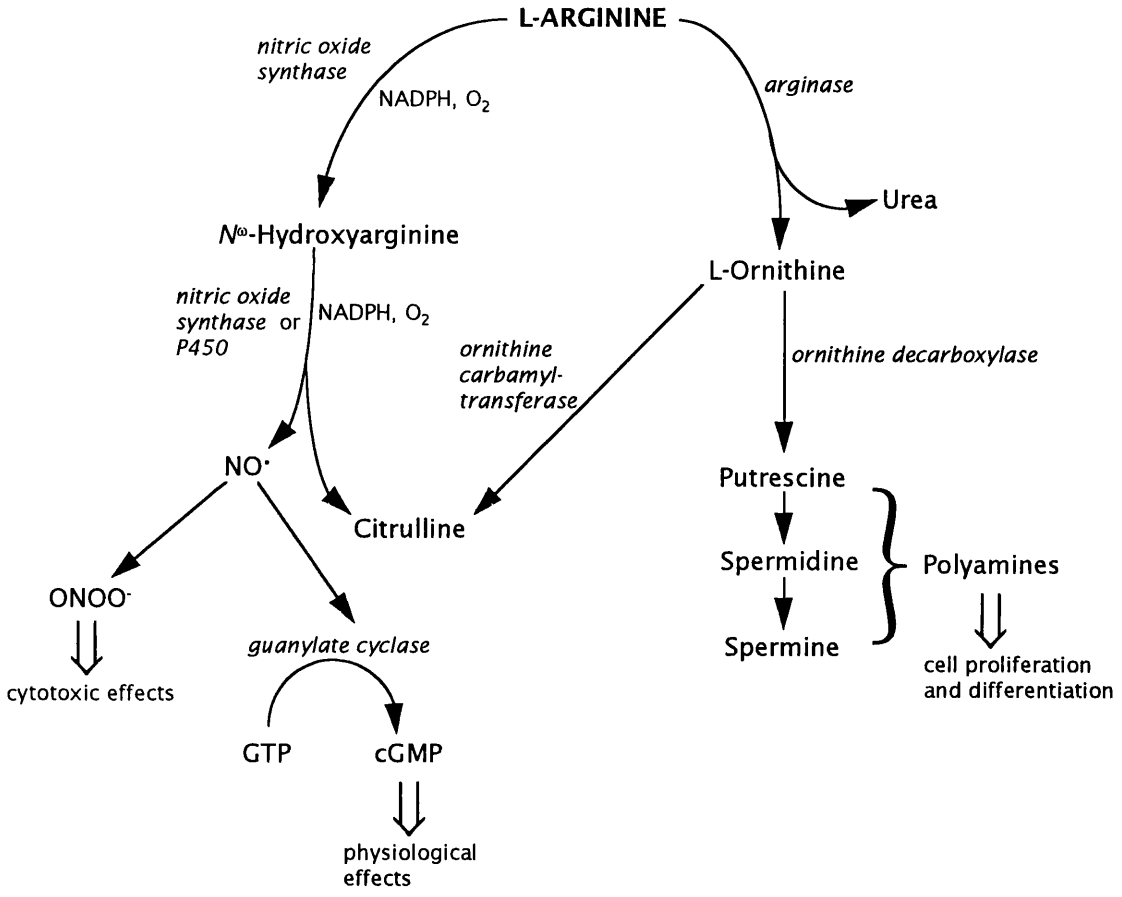


Figure 1.3 Metabolism of arginine by nitric oxide synthase and arginase in the liver.

The formation of nitric oxide from arginine requires four cofactors (FMN, FAD, haem and tetrahydrobiopterin) and the presence of calmodulin (Stuehr & Griffith, 1992; Knowles & Moncada, 1994). Figure 1.3 also shows the relationship of the NOS pathways to the urea cycle and the biosynthesis of polyamines. The two pathways are closely related to each other and the significance of this is the subject of intensive investigation (Stadler *et al.*, 1995).

There are three distinct isoforms of NO synthase (Knowles & Moncada, 1994). The first is nNOS; originally identified in neuronal tissues, is constitutively expressed and is Ca^{2+}-dependent. The second isoform eNOS was first identified as being constitutive in vascular endothelial cells and is also Ca^{2+}-dependent. Finally, iNOS first identified as an enzyme which was inducible in macrophages and liver cells by endotoxin and cytokines. This isoform is not dependent on calcium concentrations in the physiological range.

It is commonly believed that NO mediates most of the hepatic dysfunction observed during sepsis or treatment with endotoxin (Milbourne & Bygrave, 1995, Stadler *et al.*, 1995). NO has been shown to inhibit protein synthesis (Billiar *et al.*, 1990; Curran *et al.*, 1991) and the activity of cytochrome P450 (Stadler *et al.*, 1994). The effects of NO on carbohydrate metabolism appears to be inconsistent. It has been shown that hepatic glycogenolysis is indirectly influenced by NO-mediated vasodilation (Moy *et al.*, 1991) and directly inhibited by NO-donors (Brass *et al.*, 1993).

NO is cytoprotective in *in vivo* models of endotoxin or *C. parvum*-induced hepatic necrosis (Billiar *et al.*, 1990; Renaud *et al.*, 1993).

1.9 Polyamines and Cell Functions

Ornithine decarboxylase is the first and key enzyme in polyamine biosynthesis (Figure 1.4). Turnover of this enzyme is rapid (half-life, 10-30 min) and it is and one of the most highly regulated enzymes known. The enzyme is induced by many kinds of growth stimuli and its suppression by specific

inhibitors or mutation inhibits cellular growth and differentiation (Tabor & Tabor, 1985; Hayashi & Murakami, 1995).

Several stimuli cause a rapid increase of 10 to > 200-fold in the level of ODC, both *in vivo* and in cultured mammalian cells. (Tabor & Tabor, 1984). Some of the most effective stimuli include hepatectomy and hormones such as growth hormones, corticosteroids, and testosterone. In the liver, inhibitors of ODC not only prevent the accumulation of putrescine and spermidine but also substantially reduces the stimulation of DNA synthesis induced by partial hepatectomy (Danzin & Mammon, 1987).

Recent work has shown that β-adrenoceptors play a role during brain development by controlling ODC activity (Wagner *et al.*, 1994, 1995). β-adrenoceptors stimulate the synthesis of new ODC through a cAMP-mediated induction of the protooncogene c-*fos* (Wagner *et al.*, 1994). Also the β-adrenergic agonists, isoprenaline and terbutaline, stimulate the synthesis of c-fos mRNA in neonatal and fetal livers (Slotkin *et al.*, 1995). These results could possibly explain the effects of β-adrenoceptors on liver growth. Also, polyamine depletion prevents the induction of the immediate early genes c-*fos*, c-*jun* and c-*myc* by mitogens, viral products, or tumour growth (Celano *et al.*, 1989, Wang *et al.*, 1993; Schulz-Lohoff *et al.*, 1994; Tabib & Bachrach, 1994). Putrescine, spermidine, spermine are competitive inhibitors for ODC (Bey *et al.*, 1987).

Polyamines (putrescine, spermine, and spermidine) are ubiquitous polycations that have numerous and unique interactions in eukaryotic cells. Length of backbone, net charge at physiological pH and charge distribution of the polyamines favour their interaction with large anionic molecules such as DNA, RNA, and phospholipids (Tabor & Tabor, 1984; Marton and Morris; 1987; Schuber; 1989). Interaction of polyamines with the molecules above causes several perturbations in the normal cell functions.

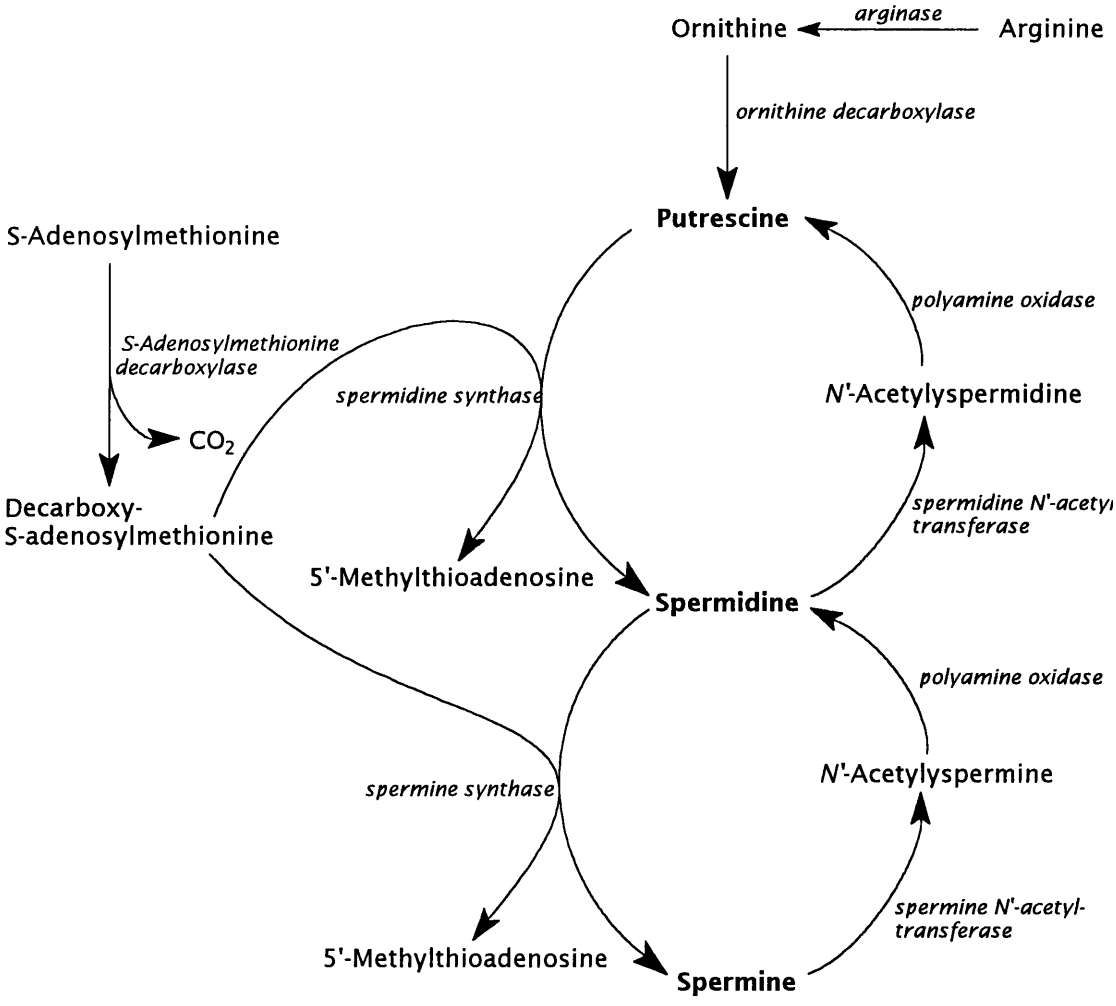


Figure 1.4 Polyamine metabolism

1.10 Cytochrome P-450 Metabolism of Arachidonic Acid

The term 'cytochrome P450' refers to a family of heme proteins, present in all mammalian cell types except mature red blood cells and skeletal muscle cells, which are important in the oxidative, peroxidative, and reductive metabolism of numerous endogenous compounds such as steroids, bile acids, fatty acids, prostaglandins, leukotrienes, and biogenic amines (Okita & Masters, 1992; Nelson *et al.*, 1993). Furthermore, many of these enzymes metabolize a wide range of foreign chemicals (xenobiotics) including drugs, environmental pollutants, natural plant products, and alcohols. The metabolism of xenobiotics can frequently produce toxic metabolites, of which some have been implicated as agents that may be responsible for tumor initiation, promotion, and tumor progression.

The term 'cytochrome P450' originated from its spectral properties for the CO-adduct of the reduced form (Sato & Omura, 1961). However, the Nomenclature Committee of the International Union of Biochemistry (NC-IUB) recommends the term "heme-thiolate protein" instead of "cytochrome" for P450 (Palmer & Reedijk, 1989).

1.10.1 Metabolic pathways

Three types of cytochrome P450 mediated reactions have been identified with arachidonate as substrate (see Figure 1.5).

1. Hydroxylation at or near the terminal sp^3 carbon (ω and ω-1 oxygenase reactions i.e. position 20 and 19 of arachidonic acid (Capdevila *et al.*, 1990a) and at positions 16, 17, and 18 (Falck et al., 1990). These reactions have been attributed to *CYP*1A1, *CYP*1A2 and *CYP*2C11 (Falck *et al.*, 1990; Capdevila *et al.*, 1990b) as well as, *CYP*2E1 and *CYP*3A2 (Tanaka *et al.*, 1990).

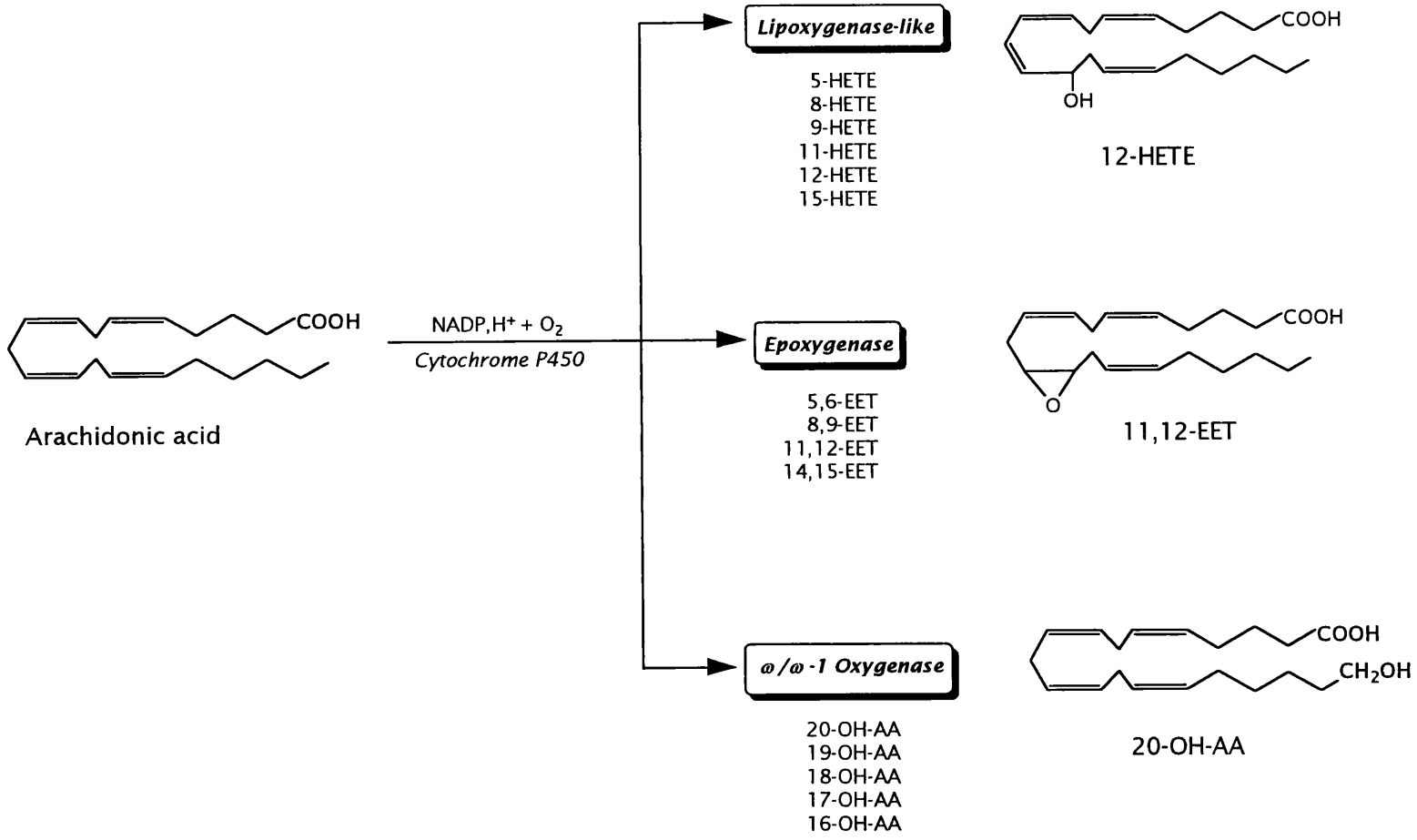


Figure 1.5 Reactions catalyzed by microsomal P450 during the metabolism of arachidonic acid (Capdevila *et al.*, 1995).

2. Olefin epoxidation (epoxygenase reaction) This is the predominant mode of P450-mediated metabolism of arachidonic acid with the formation of four regioisomeric epoxyeicosatrienoic acids (EETs). Cytochromes P450 involved are *CYP*2B1, *CYP*2B2, *CYP*2C11 and to a lesser extent *CYP*1A2 (Capdevila *et al.*, 1990b; Falck *et al.*, 1990).
3. Allylic oxidation of the sp^2 carbons with the formation of six regioisomeric hydroxyeicosatetraenoic acids (HETEs), mediated by CYP1A1 and CYP1A2 and to a lesser extent CYP2C11 (Capdevila *et al.*, 1990b; Falck *et al.*, 1990).

In the rat, cytochrome P450 epoxygenases constitute the major component of the hepatic cytochrome P450 system (Karara *et al.*, 1989). It has also been shown that EETs represent two-thirds, and HETEs almost one-third, of eicosanoids generated by microsomes obtained from the liver of adult male rats not treated with enzyme inducers (Capdevila *et al.*, 1990b). Induction of hepatic cytochromes P450 by β-naphthoflavone (β-NF) and phenobarbitone, alter the composition of eicosanoids generated by hepatic microsomes. Treatment with β-NF increases the production of HETEs (19- and 20-HETEs) by hepatic microsomes, while reducing the formation of EETs (Falck *et al.*, 1990). Cytochrome P450 induction with phenobarbitone altered the streoselectivity of microsomal epoxygenases favouring the formation of 11(S)-, 12(R) rather than 11(*R*)-,12(*S*)-EET the principal product before the administration of phenobarbitone (Capdevila *et al.*, 1990b). Furthermore, the administration of phenobarbitone increased the synthesis of total EETs by hepatic microsomal enzymes (Capdevila *et al.*, 1990b).

EETs are also present as endogenous constituents of rat liver and rabbit kidney. In rat liver, more than 92% of endogenous EET pools are esterified in the *sn*-2 position of several cellular glycerophospholipids (Karara *et al.*, 1991). Analysis of fatty acids from the rat liver revealed the presence of 8,9-, 11,12-, and 14, 15-EETs in all three classes of phospholipids, with 55% of the total

EETs in phosphatidylcholine, 32% in phosphatidylethanolamine, and 12% in phosphatidylinositols (Karara *et al.*, 1991).

1.10.2 Physiological and pharmacological actions of epoxygenase metabolites

EETs display a variety of potent biological activities. Among these activities include:

- vasoactive properties
- effects on ion transport
- peptide hormone release

The 5,6- and 14,15-EETs act as vasodilators at the systemic level and in tissue microcirculation (McGiff, 1991). In contrast, 8(*S*),9(*R*), the major enantiomer circulating in the plasma (Karara *et al.*, 1992), appears to be a powerful and stereoselective renal vasoconstrictor (McGiff, 1991). Furthermore, the 5,6- and 14,15-EETs have been shown to increase cytosolic Ca^{2+} concentrations in several cell preparations including pituitary cells, hepatocytes and kidney mesangial and proximal tubule cells (McGiff, 1991). Also, EETs serve as potent *in vitro* secretagogues for several brain, pituitary, and pancreatic hormones such as somatostatin, luteinizing hormone, growth hormone, vasopressin, prolactin, insulin and glucagon (McGiff, 1991).

Many of the actions of EETs may probably be due to their ability to become incorporated into cellular lipids (Capdevila *et al.*, 1992) and, consequently, to alter cell membrane permeability and/or fusogenic properties and cause changes in ion fluxes or peptide hormone secretion (McGiff, 1991; Capdevila *et al.*, 1992). When added exogenously, EETs modify the intracellular concentrations of ions such as Ca^{2+}, Na^{+}, K^{+}, and the permeability of cell membranes to water or peptide hormones (Fitzpatrick & Murphy, 1988; McGiff, 1991; Capdevila *et al.*, 1992).

It has been suggested that the biosynthesis of endogenous pools of phospholipids containing esterified EET moieties in rat liver, kidney, brain, and plasma and in human kidney and plasma (Capdevila *et al.*, 1992; Karara *et al.*, 1992) may indicate new and potentially important functional roles for cytochrome P450 (Capdevila *et al.*, 1995). As a participant of the arachidonic acid cascade, microsomal cytochrome P450 may play a central role in the biosynthesis of unique cellular glycerolipids and, thus, in the control of membrane physicochemical properties and/or the generation of novel lipid-derived mediators. The presence of endogenous EETs in phospholipids will provide, in contrast to other eicosanoids, the potential for the cellular generation of preformed bioactive EETs via hydrolytic reactions, thus obviating the need for AA oxidative metabolism.

1.11 Aims and Objectives

In rats, stimulation of α_1 and β_2-adrenoceptors lead to glycogenolysis. However, in the adult male rats, α_1-adrenoceptors play the predominant role despite the presence of β_2-receptors as well on the liver membranes. Under certain conditions, *in vivo*, the predominantly α–response is progressively replaced by a β–response. This shift from α to β response occurs rapidly in isolated hepatocyte suspensions (2-4 h) and in monolayer cultures (8-12 h). Several factors have been identified as being responsible for this shift, however, the underlying mechanism(s) are still obscure. The primary aim of this project is to attempt to prevent these time-dependent changes in adrenergic response in hepatocyte cultures, with the view to understanding more clearly the factor(s) that regulate the α and β response. To realize this objective, the series of experiments listed below were performed.

1. A series of experiments were performed to validate the glycogen phosphorylase assay since the activation of glycogen phosphorylase was used as the measure of adrenergic responses.
2. The relative expression of α- and β-adrenoceptor responses in Krebs-Henseleit buffer and in Williams' E. The experiments were performed, because, during the preliminary experiments an observation was made suggesting that the type of culture medium used could modify the α and β responses.
3. The effects of dexamethasone and the differentiating agents, DMSO and sodium butyrate on the time-dependent changes in α- and β-adrenoceptor responses. All these agents have used successfully to prevent the loss of some liver-specific functions in the isolated hepatocytes.
4. The effects of polyamine biosynthesis on the time-dependent changes in adrenoceptors. A proliferative state appears to the underlying cause of adrenoceptor changes in several conditions. Polyamine biosynthesis, a key, early step in cellular proliferation was blocked with (+)-S-2-amino-5-iodoacetamidopentanoic acid (AIAP).
5. The role of cytochrome P450 in the transduction of the adrenergic signal. The effects of the cytochrome P450 inhibitors, SKF525A and metyrapone and β-NF-inducible cytochromes P450 on the adrenergic responses were investigated.
6. The effects of NO on adrenergic responses using the NO-donors, sodium nitroprusside and glyceryl trinitrate. Earlier workers have shown that endotoxaemia (presumably NO-mediated) caused time-dependent changes in hepatocytes. The effects of protein kinase inhibitors on the actions of GTN were also examined.

MATERIAL AND METHODS

2.1 Experimental Animals

Adult male Wistar rats (250-300 g) or females (185-210 g), bred in the Department of Pharmacology or the Central Research Facility (University of Glasgow), were used for the experiments. The rats were allowed free access to water and standard rat chow and kept under regulated lighting (0800-2000 on) and temperature (20 °C).

2.1.1 Induction of cytochrome P450 1A1

The systemic administration of some polycyclic aromatic hydrocarbons (PAH) is known to cause the induction of multiple members of the cytochrome P450 1A gene family (Nerbert *et al.*, 1989). The main PAHs used in the study of cytochrome P450 are 3-methylcholanthrene, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), and β-naphthoflavone (β-NF).

In our experiments β-NF was used as the inducing agent and it was administered as described by Gibson & Skett (1994). The rats were given an intraperitoneal injection of β-NF 80 mg/kg (dissolved in corn oil) once daily for 3 days, before the isolation of hepatocytes. Control animals were given corn oil (equivalent to the test group) as an intraperitoneal injection according to the same schedule.

2.2 Isolation Of Hepatocytes

Hepatocytes were isolated by a modification of the two-step collagenase perfusion of Seglen, (1976). The Ca^{2+}-free conditions are necessary for the disruption of the tight junctions, which intimately connect the parenchymal cells. However, the use of the Ca^{2+}-free medium is paradoxical in view of the Ca^{2+}-requirement for the action of collagenase. The purpose of the second Ca^{2+}-free buffer is to wash out traces of the calcium chelator, EGTA, which otherwise would inhibit subsequent collagenase activity.

The solutions were gassed for about 20 min with O_2/CO_2 (19:1 v/v) before the perfusion to meet the oxygen demands of the metabolically active parenchymal cells. This is important, since the avoidance of hypoxia is necessary in the preparation of hormone-sensitive cells (Zahlten & Stratman, 1974). Other precautions taken were good temperature control and the use of the shortest exposure of the liver to collagenase (Berry *et al.*, 1991). The liver was perfused in situ.

2.2.1 Perfusion apparatus

Figure 2.1 illustrates the perfusion apparatus used in the isolation of hepatocytes. The reservoir, a 100-ml water jacketed organ bath is connected to the warming coil by silicone rubber tubing. Through a Model 502S peristaltic pump (Watson-Marlow, Cornwall, England) the liver was perfused with the buffers at a rate of 35 ml min^{-1}. The length of the tubing between the coil and the liver is kept as short as possible to minimize heat losses. We found that setting the heater at 42°C delivers the perfusate to the liver at 37°C (the required perfusion temperature). A polypropylene Y-piece serving as the bubble trap was placed between tubing B and C (see Figure 2.1). One end of tubing C was fitted with a male Luer-type lock to ease coupling to the inflow cannula. Lengths of tubing A, B, and C are 20, 28, and 60 cm respectively.

The surgical table was made from a polypropylene tray (43 x 33 x 2 cm) fitted with a spout at one corner for drainage.

2.2.2 Surgery and perfusion

The rat was anesthetized with 4% (v/v) halothane in oxygen/nitrous oxide (0.8 l/0.8 l min^{-1}) and was transferred to the surgical tray and placed on its back after it became unconscious (loss of reflex when pressure is applied to the paws). The limbs of the rat were secured to the tray with adhesive tapes and the rat kept under anaesthesia throughout the dissection. The chest and abdominal areas were wiped with 70% (v/v) ethanol to keep loose hair from the surgical area and to

provide some degree of sterility. The abdomen was opened by making a V-shaped incision from the pelvic area to the diaphragm and the gut was displaced to the right to expose the hepatic portal vein (see Figure 2.2). The connective tissue surrounding the vein was cleared and a loose ligature tied around the midpoint of the portal vein with a piece of braided silk suture. An 18-gauge, 51-mm long Quick-Cath cannula (Baxter Healthcare, Norfolk, England) was inserted in the portal vein just below the ligature which was then tightened with a double knot. Immediately after the cannula had been secured in place the pump was started and the cannula was connected to the inflow tubing and the liver was first perfused with 400 ml of a Ca^{2+}-free HEPES-buffered Krebs-Henseleit solution (see Appendix A) containing 10 mM EGTA. The liver blanches within seconds as the perfusion buffer washes out the blood. The vena cava was cut at 2 cm below the kidney to allow the perfusate to flow out of the liver. Another outlet for the perfusate was made by snipping the diaphragm and then cutting the vena cava just above the diaphragm. In some preparations, the liver lobes were gently massaged to effect complete washout of the blood.

A short wash with 100 ml of EGTA-free buffer followed the Ca^{2+}-free buffer. Perfusion was then switched over to 100 ml of Krebs-Henseleit solution containing 4.2 mM $CaCl_2$ and 40 mg of collagenase (collagenase A from *Clostridium histolyticum*; specific activity, 0.3-0.6 U mg^{-1}). This perfusate was returned to the reservoir and recirculated. The perfusion was stopped after 12 min, by which time the liver appeared swollen and showed signs of breakage, and the liver carefully removed and placed in 50 ml of washing buffer. The Glisson's capsule was disrupted with forceps and the cells carefully dispersed with a blunt spatula. The suspension of cells was then filtered through a double layer of gauze into a 50-ml polythene centrifuge tube. This filtration separates connective tissue and undigested liver from the cells. Parenchymal cells were then harvested after washing and centrifugation twice at 50 *g* for 2 min at 5°C.

This process removes constituents that have leaked from intact or damaged cells, cell debris and damaged cells, also most of the non-parenchymal cells (Berry *et al.*, 1991). The sedimented cells were suspended in the culture medium and kept on ice for 1 hour before use.

2.2.3 Cell viabilty

Cell viability was assessed by the ability of the cells to exclude trypan blue. The method is based on the principle that live (viable) cells do not take up certain dyes, whereas dead (non-viable) cells do.

A hundred-microliter sample of the cell suspension(1-1.3 x 10^6 cells ml^{-1}) was transferred to a test tube and diluted with 0.9 ml washing buffer. Two hundred and fifty (250) microlitres of a 0.4% w/v trypan blue solution was added to the diluted sample and mixed thoroughly. The cells were left for 3-5 min and then examined under a light microscope on a hemocytometer. Cell viabilty was calculated as:

$$\textit{Cell viability (\%)} = \frac{\textit{total viable cell (unstained)}}{\textit{total cells (stained and unstained)}} \times 100$$

Hepatocyte preparations with a viabilty above 85% were used for all experiments.

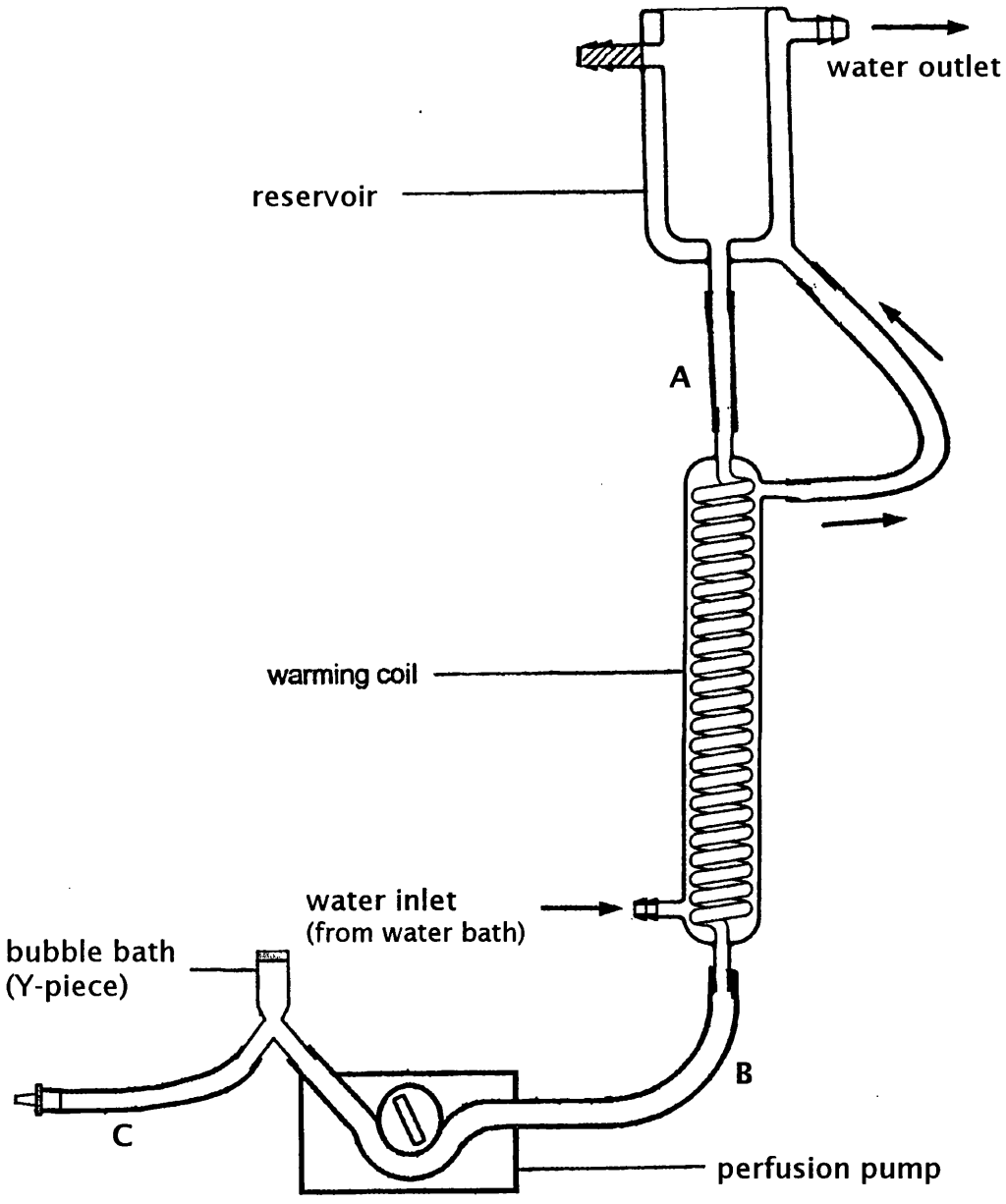


Figure 2.1 Illustration of the perfusion apparatus.

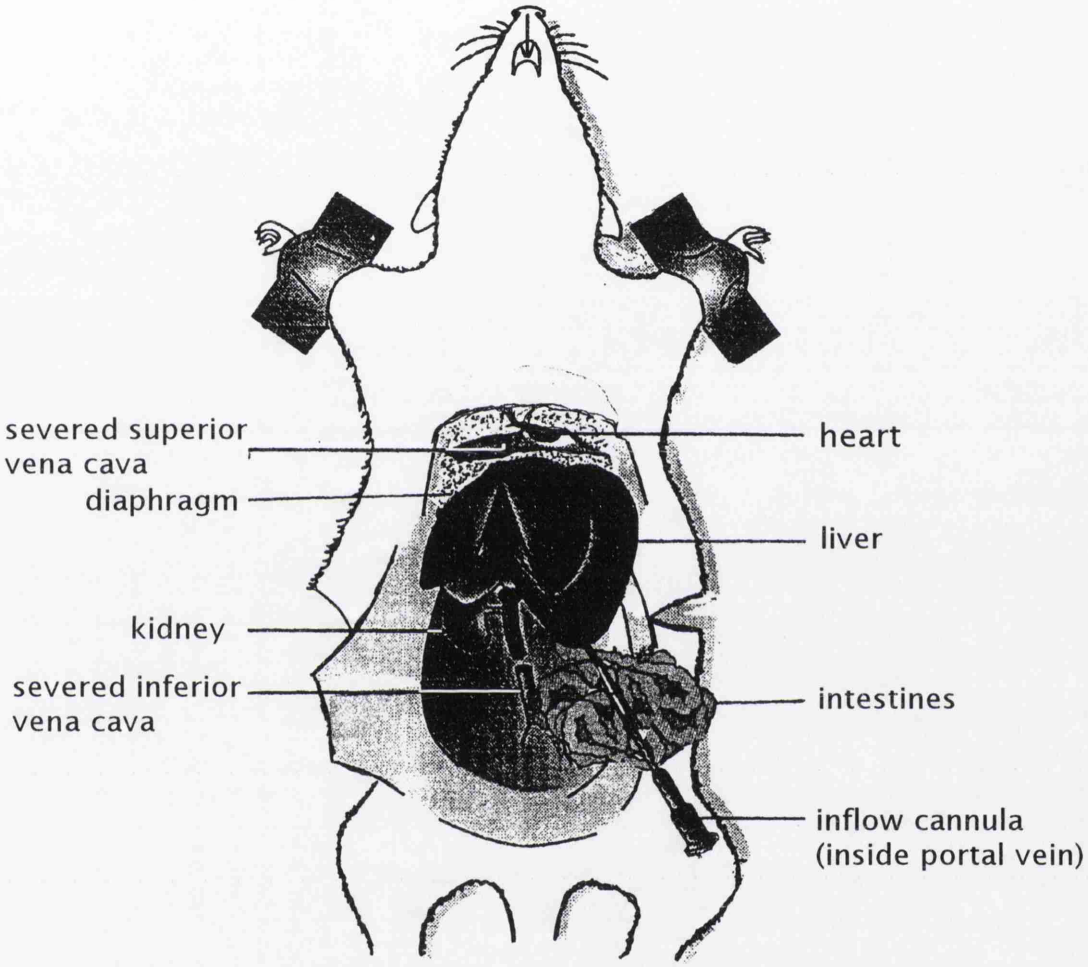


Figure 2.2 Diagram of the liver perfusion (Adapted from Berry *et al.*, 1991).

2.3 Culture Of Hepatocytes

The isolated hepatocytes were kept either as suspensions for short-term studies (up to 6 h) or as monolayers for longer term studies.

2.3.1 Hepatocyte suspensions

The hepatocytes were adjusted to 3-5 x 10^6 cells ml^{-1} with the appropriate medium. Two-milliliter aliquots of the suspensions were then placed in 20-ml plastic screw-capped vials, gassed with O_2/CO_2 (19:1) for 15 s and incubated with constant shaking (80 cycles/min) at 37 °C. The vials were 'regassed' at 2-hour intervals. In these conditions, cells maintained their viability above 85% for up to 6 h.

2.3.2 Monolayer culture

Cells were plated at a density of 1.7 x 10^5 cm^{-2} in 100 x 15 mm Nunclon Petri dishes (Life Technologies, Roskilde, Denmark). The medium was changed after 4 h by aspirating as completely as possible the old medium and adding 9 ml fresh medium per dish. The medium was subsequently changed at 24-h intervals. A 14-gauge, 60-mm long stainless steel needle (sterilized by flaming in a Bunsen flame at regular intervals) was used in aspirating the medium.

2.4 Glycogen Phosphorylase Assay

The enzyme activity was determined by the filter paper assay method of Thomas *et al.* (1968) as modified by Stalmans and Hers (1975).

2.4.1 Principle

Glycogen phosphorylase catalyses the reversible reaction:

$$(\alpha\text{-1,4-glucoside})_n + P_i \rightleftharpoons (\alpha\text{-1,4-glucoside})_{n-1} + \text{Glc1}P$$

Although the equilibrium constant of this reaction at physiological pHs strongly favors glycogen synthesis, phosphorylase works in the glycogenolytic direction *in vivo* because the ratio of P_i to glucose-1-*P* concentration exceeds the equilibrium constant (Newgard *et al.*, 1989). The high ratio of P_i to glucose-1-*P*

is maintained in large part by the enzyme phosphoglucomutase, which rapidly converts glucose-1-*P* to glucose-6-*P* in a reaction whose equilibrium is strongly in the direction of glucose-6-*P* *in vivo*.

In this method, glycogen phosphorylase *a* is assayed in the 'unphysiological' direction, i.e. employing the reaction between D-Glc-1*P* and glycogen. This is achieved by performing the reaction at a pH of 6.5 and providing high substrate concentrations. Glycogen, being insoluble in alcohol, is precipitated and adsorbed onto the filter paper squares which are counted for radioactivity.

The addition of caffeine, which inhibits specifically phosphorylase *b* (Stalmans & Hers, 1975), renders the assay specific for phosphorylase *a*. To assay the total glycogen phosphorylase activity *i.e.* phosphorylase *a* + phosphorylase *b*, caffeine in the radioactive mixture was replaced with AMP and Na_2SO_4 (see *Appendix C*). Although phosphorylase *b* is inactive, the addition of AMP and sulfate increases it activity dramatically (Stalmans & Hers, 1975; Newgard *et al.*, 1989). NaF and EDTA were added to the disruption buffer to inhibit phosphoprotein phosphatase and protein kinase respectively (Stalmans & Hers, 1975; Blackmore & Exton, 1985).

The enzyme activity was measured as the amount (in moles) of [^{14}C]glucose-1-*P* incorporated into glycogen.

2.4.2 Validation of the glycogen phosphorylase a assay

The assay protocol was designed based on the results of preliminary experiments - kinetics of the glycogen phosphorylase *a* reaction and the time course of drug action. The possibility of damage to receptors and/or signal transduction pathway(s) during the isolation process was investigated by studying the response of the cells to hormones and other agents known to activate glycogen phosphorylase.

2.4.3 Preparation of samples for the assay

A summary of the key steps in the assay protocol is shown diagrammatically in Figure 2.3.

***The effect of drugs on phosphorylase* a.**

After the preincubation period, 450-μl samples of cell suspensions were transferred to 1-ml microcentrifuge tubes that contained 50 μl of agonist at various concentrations or vehicle (control). The mixture was briefly shaken and incubated at 37°C on a dry heating block (Cherlyn Electronics, Cambridge, England). After 4 min, the incubations were stopped by rapidly freezing the tubes in liquid nitrogen and the samples were stored at -80°C until glycogen phosphorylase *a* activity was determined. The samples maintained the enzyme activity for up to 72 h at this temperature; inactivation of the enzyme was observed after this period. Each concentration and control were assayed in duplicate.

Preparation of crude glycogen phosphorylase

An equal volume (500 µl) of ice-cold disruption buffer (100 mM MOPS, 200 mM NaF, 30 mM EDTA, and 10 mM DTT; pH 6.5, see *Appendix C*) was added to the frozen samples obtained from above and the samples allowed to thaw on ice and was then sonicated in a Transonic T310 ultrasonic bath (Camlab, Cambridge, England) for 60 seconds. This procedure breaks up the cells. The lysate was then briefly vortexed and centrifuged for 10 min at 2,500 *g* at 4°C in a DPR-6000 centrifuge (Damon-IEC, Dunstable, Beds., England). The supernatant contains the enzyme and was used for the next step.

The temperature of the samples was maintained at 0-4°C since higher temperatures inactivate the enzyme.

2.4.4 Assay procedure

Fifty-microlitre aliquots of the supernatant were transferred to 1-ml microcentrifuge tubes that contained 50 μl of radioactive assay mixture (see

Appendix C). The tubes were briefly vortexed and incubated in a water bath for 20 min at 37°C. The reaction was stopped by removing 30 μl aliquots of the mixture and spotting on paper pieces (Whatman 3MM chromatography paper, 10 mm^2), previously marked with a pencil and dropping in a beaker containing 66% (v/v) ethanol in water. Blank samples were prepared by mixing 50 μl of disruption buffer with 50 μl of assay mixture.

The filter paper squares were subsequently washed three times for 40 min in the 66% ethanol on a magnetic stirrer, replacing the ethanol with a fresh mixture after each wash. The stirring bar was screened from the papers with an aluminium mesh to prevent disintegration of the filter papers. After the final wash, the ethanol was decanted and the papers washed for 1 min in acetone. The papers were air-dried and placed in 2 ml of Ecoscint A scintillation fluid (National Diagnostics, Manville, NJ, U.S.A.) and counted in a Tri-Carb 2000CA scintillation counter (Packard, Pangbourne, Berks., England) for radioactivity. The total amount of radioactivity in the assay mixture was measured by spotting 50 μl of the reaction mixture onto a filter paper and counting the paper as described above.

Calculation of glycogen phosphorylase a activity

Enzyme activity was calculated from the equation below and expressed as nmol of ^{14}C-glucose 1-phosphate incorporated into glycogen min^{-1} mg^{-1} protein.

$$\textit{Enzyme activity} = \frac{DPM_{sample} - DPM_{blank}}{DPM_{total}} \times \frac{[S]}{(P \times t)} \times \frac{10{,}000}{3}$$

where,

DPM = disintegration min^{-1}

[S] = substrate concentration (50 mM in all assays)

P = protein conc. of supernatant

t = incubation time (min)

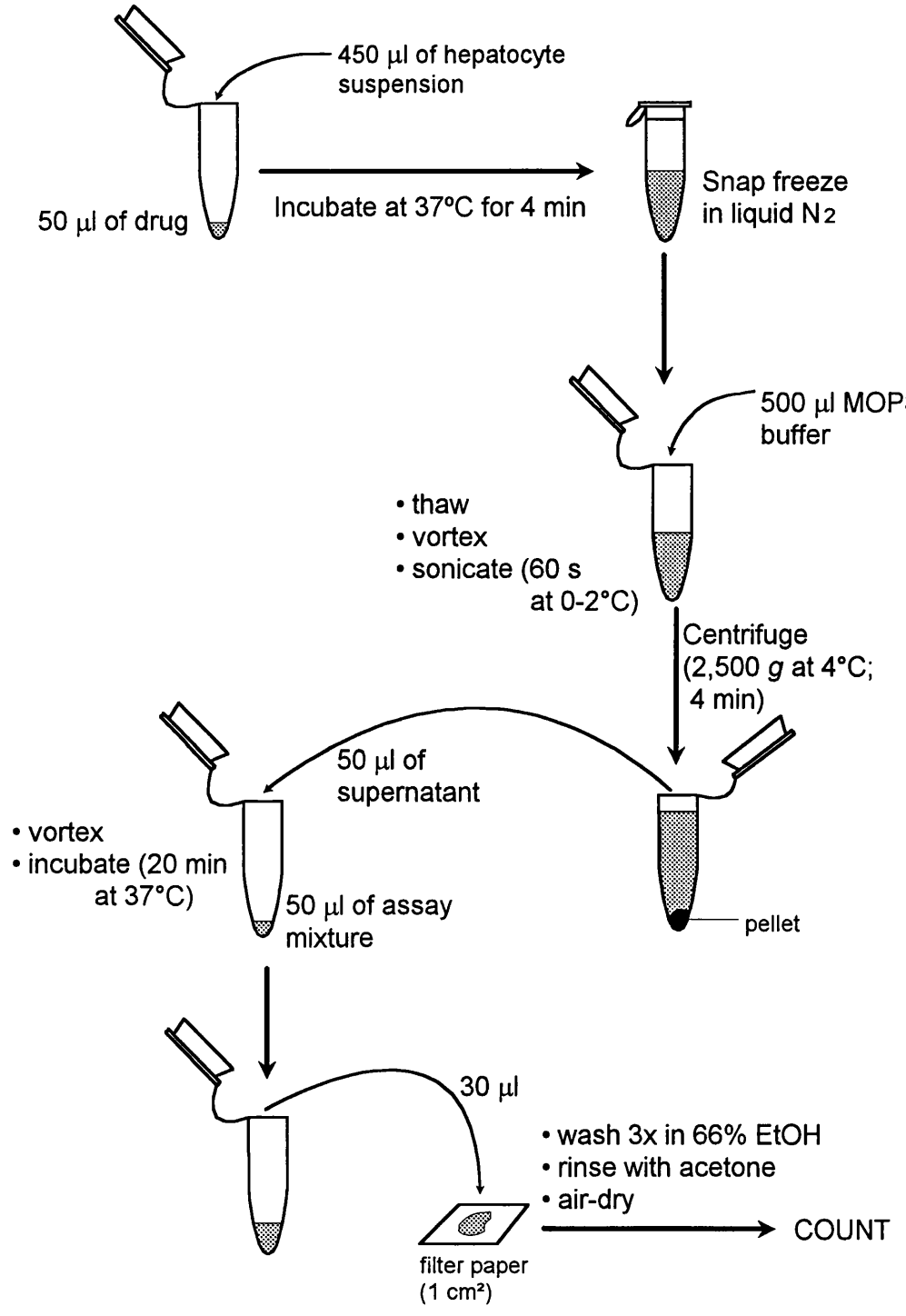


Figure 2.3 Protocol for glycogen phosphorylase *a* assay.

2.5 Cyclic AMP Assay

A RIANEN® cAMP [^{125}I] radioimmunoassay kit (Du Pont Company, Billerica, MA, U.S.A.) was used to assay cAMP generated by the agonists/drug indicated in the text. The assay is a double-antibody radioimmunoassay based on the general procedure of Harper & Brooker, (1975) and Brooker *et al.*, (1979). Standards and test were acetylated to increase the sensitivity of the assay.

2.5.1 Principle

The principle of the assay is the competition between a radioactive and a nonradioactive antigen for a fixed number of antibody binding sites (Figure 2.4). Separation of the bound from free antigen is achieved by a prereacted antibody-secondary antibody complex.

2.5.2 Preparation of samples

Hepatocyte suspensions (3-5 x 10^6 cells ml^{-1}; final volume 2 ml) were incubated in Williams' E medium containing 2 mM IBMX (a phosphodiesterase inhibitor) for 30 min. Samples (450 μl) were transferred to microcentriguge tubes that contained various concentrations of agonists and incubated for 4 min at 37°C. The reaction was stopped by adding to each tube 0.5 ml of 2 M HCl and immediately immersing the tubes in hot water (100°C) for 1 min. The tubes were the spun at maximum speed (3,500 rpm) in a MicroCentaur centrifuge (Fisons, Loughborough, Leics., England) for 3 min. The amount of cAMP generated was determined in the supernatant.

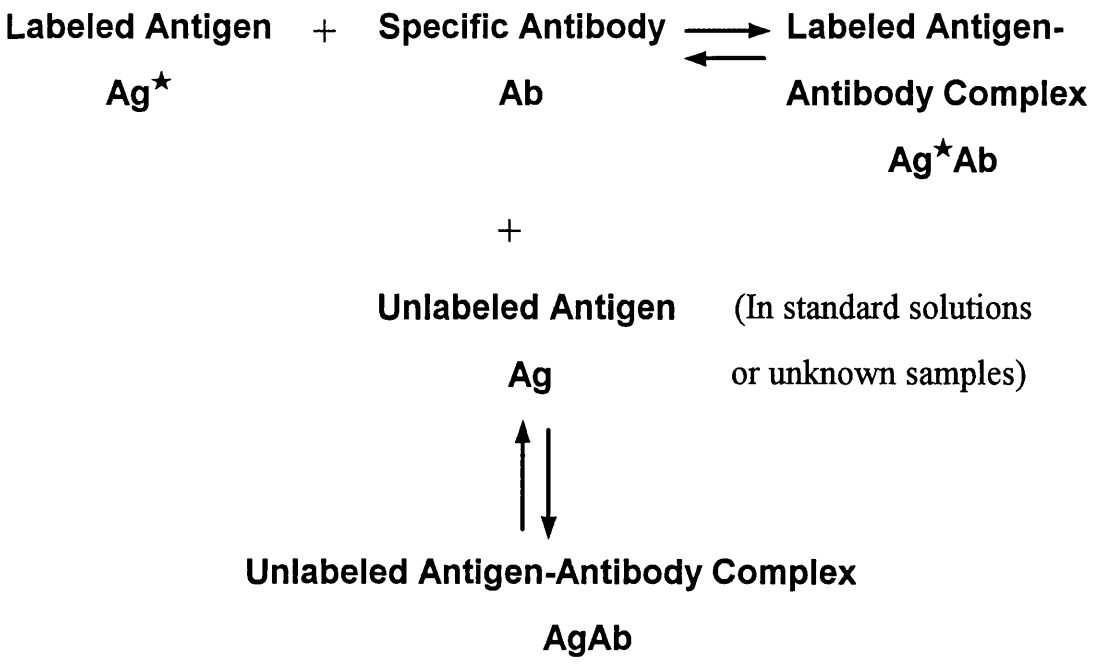


Figure 2.4 Principle of cAMP radioimmunoassay.

2.5.3 cAMP Assay Procedure

The assay was carried out as described in the instruction manual accompanying the kit using half the recommended quantities of the reagents. The quantities used did not affect the sensitivity of the assay.

The assay kit contained

- Sodium acetate buffer, pH 6.2
- cAMP standard (5000 pmol ml^{-1})
- cAMP antiserum complex
- cAMP [^{125}I] Tracer (Succinyl cAMP Tyrosine Methyl Ester [^{125}I])
- cAMP carrier serum
- Acetic anhydride and triethylamine
- cAMP precipitator

Acetylation of standards and samples

Standard curve

The working standards were prepared as described in the accompanying instruction manual. The cAMP stock standard reagent was diluted serially to give a 40 pmol ml^{-1} solution. 200 μl of the 40 pmol ml^{-1} solution was placed in a marked test tube and acetylated by adding to it 10 μl of freshly prepared acetylating reagent (triethylamine:acetic anhydride; 2:1). The reaction was allowed to proceed for at least 3 min at room temperature (20°C) and then 1.8 ml of the assay buffer was added to the mixture (final cAMP conc., 4 pmol ml^{-1}). The 4 pmol ml^{-1} solution was diluted appropriately with assay buffer(containing 50 μl of acetylating reagent/10 ml of buffer) to give a range of standards, 0.1, 0.25, 0.5, 1.0, 2.0 and 4.0 pmol ml^{-1}.

Samples

The acid extracts were diluted 5 times to put the cAMP concentration in the range of the standard curve. The dilution factor was determined in a previous

experiment and corresponds with a protein concentration of 0.3 to 0.6 mg ml^{-1}. One hundred-micoliter aliquots of the diluted samples were put into separate labeled test tubes and acetylated by adding 5 μl of acetylating reagent. The mixture was immediately vortexed and allowed to incubate at room temperature for at least 3 min at the end of which 900 μl of assay buffer was added.

Assay

Fifty-microlitre aliquots of each acetylated standard and acetylated sample were placed into separate labeled glass test tubes. To each of the tubes was added the following in that order :

1) 50 μl of working tracer solution (equal volumes of the cAMP[^{125}I]-tracer and cAMP carrier serum),
2) 50 μl of antiserum complex.

Two tubes were included for the estimation of nonspecific binding (blanks) and two tubes for the estimation of total bound cAMP. The blank tubes contained 50 μl of the modified assay buffer, 50 μl of working tracer solution, and 50 μl of antiserum complex. The tubes for the total bound cAMP contained 50 μl of working tracer solution. The tubes were covered and incubated overnight (16-18 h) at 2-8 °C. The tubes for the total counts were set aside and 250 μl of cold (2-8 °C) cAMP precipitator added to all the tubes. The tubes were vortexed and centrifuged at 2-8°C for 15 min at 1200 *g*. The supernatant was decanted by gently inverting the tubes into a sink suitable for the disposal of radioisotopes. The tubes were kept inverted and placed on filter papers for blotting of the drops.

The tubes were counted in a Cobra B5003 γ-counter (Packard, Pangbourne, Berks., England) for 1 min The average net counts for the standards and samples were calculated by subtracting from each the average blank counts.

The percent bound (%B/B_o) values were calculated and expressed as a percentage of the average net counts for the zero standard

A standard curve was obtained by plotting %B/B_o of each standard against the corresponding concentration of cAMP in pmol ml^{-1} (Figure 2.5). A nonlinear curve was fitted using the computer software InPlot Version 3.14 (GraphPad Software, San Diego, CA, U.S.A.) and the concentration of cAMP in the samples determined by interpolation of the standard curve.

2.5.4 Validation of assay

Two experiments were performed to establish the validity of the experiments (Brooker *et al.*, 1979). In the first was the tissue extract dilution curve (Figure 2.6). The graph shows a linear relationship between the amount of cyclic nucleotide and the concentration of the acid extract. This indicates a lack of nonspecific inhibitory effect of the extract (in the range of extract concentrations used experimentally).

Each tube was spiked with 250 fmoles of cAMP and assayed. The resultant curve was linear and parallel to the original unspiked extract dilution curve. The value at each point is equivalent to the amount of cAMP added. This verifies that there are no inhibitory or interfering substances in the tissue extract and that the measured extract value was an accurate representation of the true cAMP concentration in the extract.

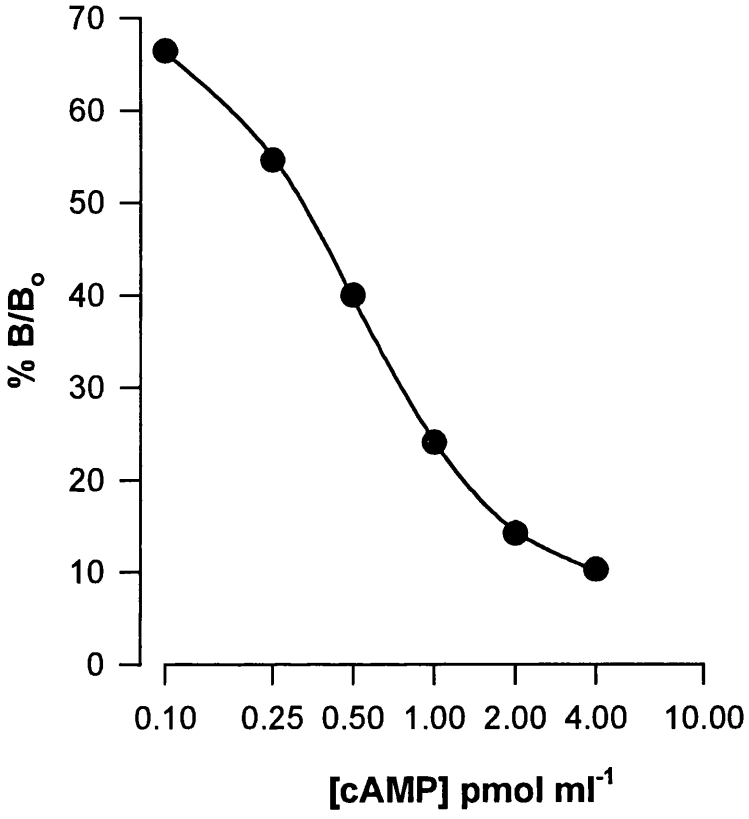


Figure 2.5 Standard curve for cyclic AMP assay.

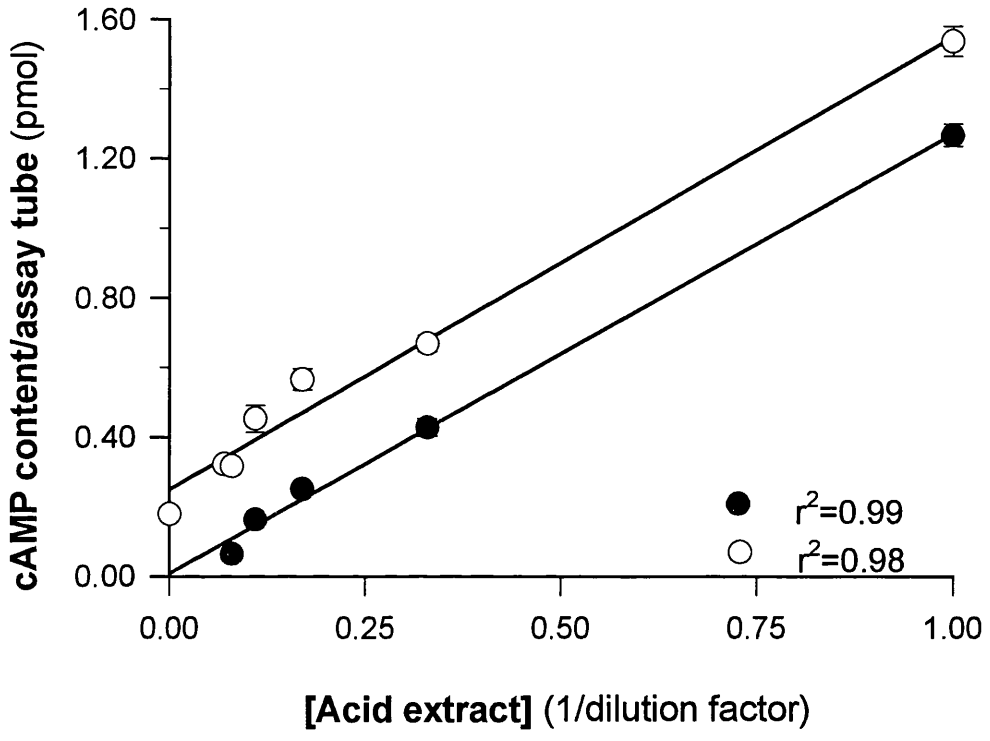


Figure 2.6 Validation of the radioimmunoassay for cyclic AMP. Various dilutions of the acid extract of hepatocytes were assayed for cyclic AMP (●). Also, each extract dilution was spiked with 250 fmoles of cyclic AMP and assayed for cyclic AMP (○). Each point represents mean ± s.e. of a triplicate determination

2.6 ARGINASE ASSAY

The assay for arginase activity was based on the method of Schimke (1970) as modified by Corraliza *et al.* (1994).

2.6.1 Principle

$$L-Arginine + H_2O \xrightarrow{arginase} L-ornithine + urea$$

Arginine is hydrolyzed to ornithine and urea. The reaction requires Mn^{2+}-ions for activation of arginase (Corraliza *et al.*, 1994). Urea is measured colorimetrically after stopping the reaction with H_2SO_4:H_2PO_4:H_20 (1:3:7) mixture. In the presence of the acids urea gives a pink color with α-isonitropropiophenone (INPP) with a maximal absorbance at 540 nm.

2.6.2 Sample preparation

After incubation for appropriate periods, 1-ml aliquot of the cell suspension (3 x 10^6 cells ml^{-1}) was placed in microcentrifuge tubes and centrifuged at 150 *g* for 3 min. The culture medium was removed and the pellet washed 2x with 1 ml ice-cold phosphate-buffered saline (PBS, pH 7.4) with centrifugation at 150 *g* for 3 min. The pellet was resuspended in 1 ml of ice-cold 50 mM Tris HCl, pH 7.5. The cells were lysed by freeze-thawing and sonication as described in section 2.4. The lysate was centrifuged at 1500 *g* for 10 min and the supernatant obtained used for the assay.

All the buffers contained 0.1 mM PMSF as a protease inhibitor.

2.6.3 Assay

Before use the supernatant solution was diluted to give a final protein concentration of 0.1-0.2 mg ml^{-1}. One-milliliter aliquots of the diluted solution were activated by incubating at 55 °C in the presence of 10 mM $MnCl_2$ for 5 minutes. Arginine hydrolysis was carried out in 10-ml glass metabolism tubes and was initiated by the addition of 125 μl of 0.5 M arginine hydrochloride to a 125 μl aliquot of the previously activated lysate. The mixture was incubated at

37 °C for 60 min and the reaction stopped by the addition of 2 ml of an acid mixture containing H_2SO_4, H_3PO_4 and H_2O (1:3:7). The urea formed was colorimetrically quantified at 540 nm after the addition of 250 μl of 9% w/v INPP (dissolved in absolute ethanol) and heating at 100 °C for 45 min. After 10 min in the dark the absorbance was recorded on a CE303 UV/visible spectrophotometer (Cecil Instrument, Cambridge, England).

Calibration curve. A calibration curve was prepared with increasing concentrations of urea between 10 and 30 μg ml^{-1}. To 500 μl of urea solution at the appropriate concentrations, 2 ml of the acid mixture and 250 μl INPP were added, and the procedure followed as described above. The curve was linear in the range 0 to 30 μg ml^{-1} (Figure 2.7).

Arginase activity, expressed as the number of moles of urea in each sample was calculated from the following equation:

$$\textit{Arginase activity} = \frac{4 \times C \times 10^{-6}}{m.wt.\ of\ urea \times t \times P} \times \textit{dilution factor}$$

where,

C = urea concentration (μg ml^{-1}) of sample

t = incubation time (min) for the hydrolysis

P = protein concentration (mg ml^{-1})

The molecular weight of urea is 60.01.

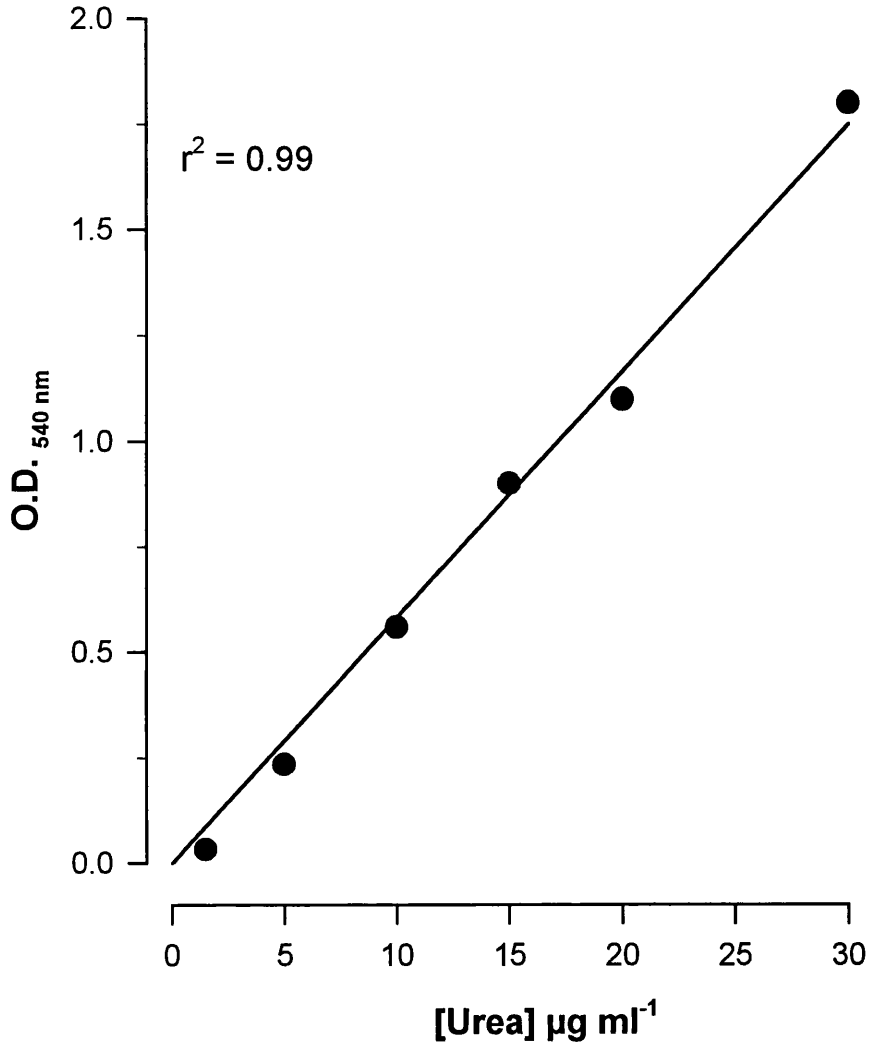


Figure 2.7 Calibration curve for arginase assay.

2.7 CYTOCHROME P450

2.7.1 Preparation of hepatic microsomes

Microsomes were prepared from hepatocytes from normal and β-naphthoflavone-treated(see section 2.1.1) rats. Isolated hepatocytes (10^7 cells ml-1; 20 ml) were washed with PBS, pH 7.4, suspended in 10 mM Tris-HCl buffer, pH 7.4 and homogenized by 20 strokes of a Dounce homogenizer. The homogenate was centrifuged at 150 *g* for 5 min at 4°C in a DPR-6000 centrifuge and the pellet homogenized again. Homogenates were pooled and centrifuged at 12,500 *g* for 15 min at 4°C in a Sorval RC-5B centrifuge (Dupont Instruments, Wilmington, DE, U.S.A.) to pellet intact cells, cell debris, nuclei and mitochondria. The resultant supernatant (the post-mitochondrial supernatant) was carefully decanted.

Microsomal fractions were prepared from the post-mitochondrial supernatant by the calcium precipitation method (Gibson & Skett, 1994). The method is based on the calcium-dependent aggregation of endoplasmic reticulum fragments and subsequent 'low speed' centrifugation of the aggregated microsomal particles. Aliquots (approximately 10-12 ml) of post-mitochondrial supernatant were mixed with 88 mM $CaCl_2$, such that 0.1 ml, 88 mM $CaCl_2$ was added per ml of supernatant (final $CaCl_2$ concentration is 8 mM) and left to stand on ice for 5 min, with occasional gentle swirling. The mixture was then centrifuged at 27,000 *g* for 15 min, the supernatant discarded and the pellet resuspended by homogenization in 5 ml of 0.1 M Tris buffer, pH 7.4 containing glycerol (20% v/v). The microsomal suspension was stored at -80 °C till use.

2.7.2 Determination of total cytochrome P450 in microsomes

The cytochrome P-450 content determined by the method of (Omura & Sato, 1964). Cytochrome P450, a hemoprotein, is characterized by the presence of an absorbance band at 450 nm for the CO-adduct of the reduced form.

Microsomal samples were diluted in 0.1 M Tris HCl buffer, pH 7.4 containing 20% (v/v) glycerol to a concentration of 1-1.5 mg of protein/ml (final volume 6 ml). Two (2) ml of the diluted sample was then added to both matched sample and reference cuvettes of a UV-visible spectrophotometer (Shimadzu, Tokyo, Japan) and a baseline recorded between 400 and 500 nM. A few grains of sodium dithionite was added to both sample and reference cuvettes with gentle stirring and the sample cuvette was gently bubbled with carbon monoxide for approximately 20 s. The spectrum was then rescanned from 400 to 500 nm. The change in absorbance at 450 nm relative to 490 nm was measured and converted to the concentration of cytochrome P450 by using the molar extinction coefficient 91 cm^{-1} mM^{-1}.

Calculation of the cytochrome P450 concentration

$$\textbf{nmoles cytochrome P450} = \frac{(A_{450} - A_{490})}{91 \times [P]} \times 1000$$

[*P*]= microsomal protein concentration (mg ml^{-1}) in the cuvette.

2.7.3 Androst-4-ene-3,17-dione metabolism

Several hepatic microsomal enzymes act on androst-4-ene-3,17-dione to produce monohydroxysteroid metabolites (Figure 2.8). Some of the regio- and stereospecific hydroxylations of the steroid nucleus serve as the sensitive fingerprints for the identification of specific cytochrome P450 isoenzymes; examples include testosterone 6β-, 2α-, 7α-, and 15α-hydroxylases and androstenedione 16β-hydroxylase are, respectively, specific indicators of rat cytochromes P450 3A, 2C11, 2A1, 2A2 and 2B1 expression (Arlotto *et al.*, 1991; Waxman, 1991a,b; Pampori & Shapiro, 1994).

A modification of the thin layer chromatographic method described by Berg and Gustaffson (1973) was used for the analysis of androstenedione and its metabolites in the experiment described below.

2.7.3.1 Incubation

The assay was carried out in 1.5-ml microcentrifuge tubes. Each tube contained:

- 100 μl of microsomal suspension
- 5 μl of androst-4-ene-3,17-dione (500 μg, dissolved in acetone)
- 10 μl of [4-^{14}C]androst-4-ene-3,17-dione (1.85 Mbq, dissolved in 500 μl acetone)
- 190 μl of Tris buffer, pH 7.4

Five replicate incubations were done for each microsomal sample. The reaction was started by adding 10 μl of an NADPH-generating cofactor solution (Appendix D). The mixture was incubated at 37 °C on a heating plate for 10 min. The reaction was stopped by the addition of 500 μl of Folch's solution (chloroform:methanol; 2:1) and 100 μl of normal saline. Each sample was vigorously vortexed and kept in the dark (to prevent the formation of light-induced oxidation products) overnight.

2.7.3.2 TLC and measurement of radioactivity

The organic and aqueous phases of each sample were separated by centrifugation (2500 *g* for 5 min) and the aqueous phase (upper layer) was aspirated and discarded. The organic phase was transferred to a clean microcentrifuge tube with care - to avoid transferring residual aqueous phase. The organic phase was then evaporated to dryness under a stream of nitrogen. The residue was redissolved in 100 μl of chloroform and vortexed. The samples were applied as individual spots at the origin, drawn 2 cm up from the bottom, of precoated preparative TLC plates (silica gel, 10 x 20 cm x 0.25 mm; 60 F_{254} , Merck, Darmstadt, Germany). Five samples were spotted on each plate, leaving 1-cm gaps between the spots and the edge of the plate. The TLC plates were developed (ascending, one-dimensional) in glass tanks, preequilibrated with chloroform:ethyl acetate (4:1) for at least 1 h. Migration distance and running

time were 18 cm and 1 h respectively. The plates were removed, air-dried at room temperature and the metabolites located by autoradiography.

Autoradiography: The location of the labeled metabolites on the chromatograms was accomplished by placing the TLC plates in contact with X-ray films for 7 days at room temperature, after which the films were removed and immersed in LX-40 X-ray liquid developer (Kodak, Paris, France) for 1 min. The films were then removed from the developer solution and immersed in FX-40 X-ray liquid fixer for 5 min after which they were immersed in running water at least 30 min and the films air-dried at room temperature. Figure 2.9 illustrates a typical chromatogram.

Identification of metabolites. Individual metabolites have previously been identified by cochromatography of authentic samples in our laboratory. The individual radioactive spots on the TLC plates were marked in pencil with squares, by superimposition using the autoradiograph as a template. The silica gel was carefully scraped into 20-ml plastic scintillation vial, suspended in 5 ml of Ecoscint scintillation solution (National Diagnostics, Manville, NJ). The radioactivity in each sample was measured in a Tri-Carb 2000CA scintillation counter (Packard, Pangbourne, Berks., England).

Calculation of enzyme activity. The activities of the enzymes were calculated from the formula below and were expressed as the amount (pmol) of the metabolite formed min^{-1} mg^{-1} protein.

$$\textbf{Enzyme activity} = \frac{S \times DPM_{metabolite}}{DPM_{total} \times t \times P \times m.wt}$$

where

DPM = disintegration min^{-1}

S = amount of substrate added ()

t = incubation time

P = protein conc. of microsomes

$m.wt$ = molecular weight of the substrate (286)

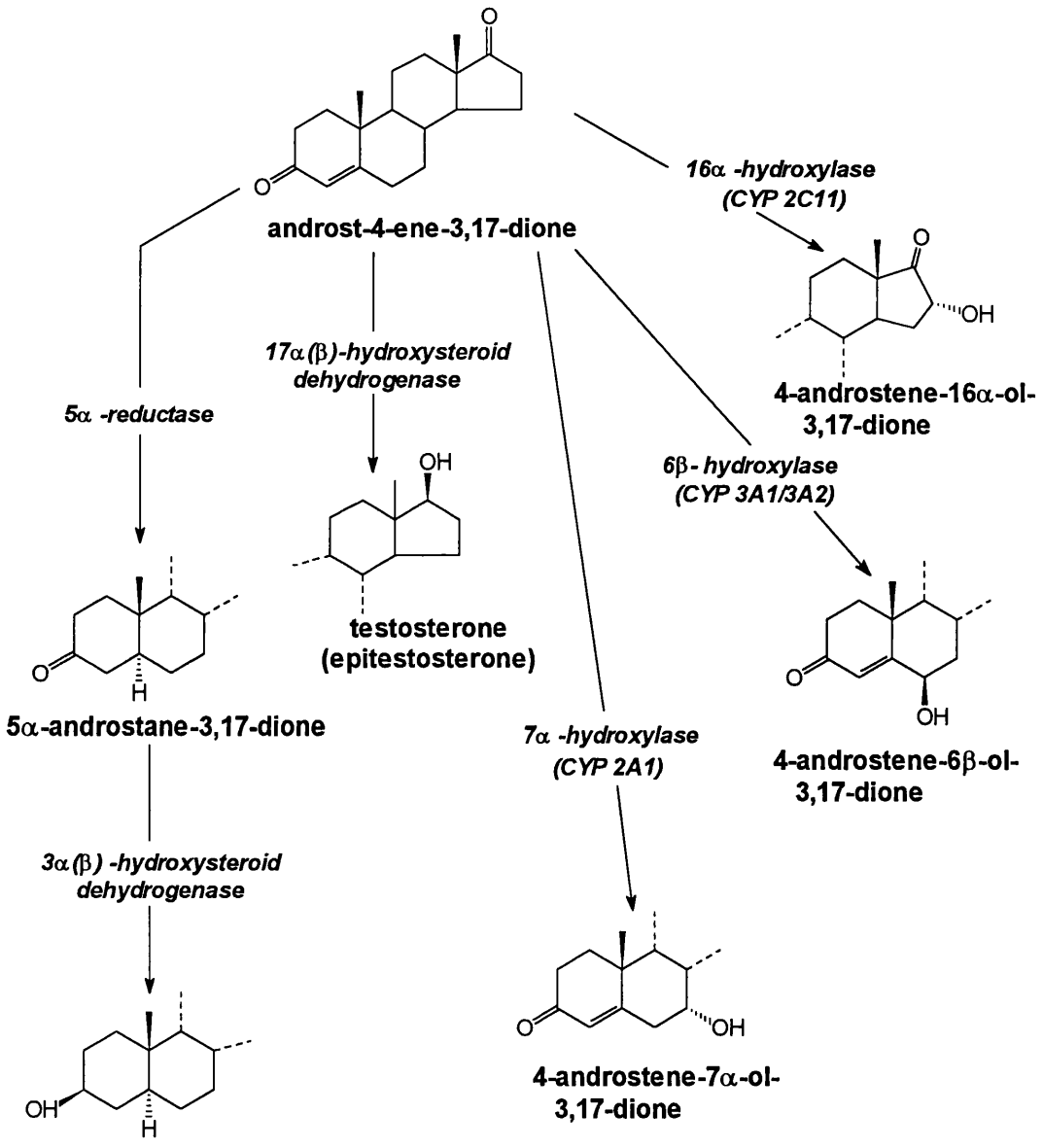


Figure 2.8 Phase 1 metabolism of androst-4-ene-3,17-dione in the liver. Specific cytochrome P450 isoenzymes thought to be involved are given in parentheses.

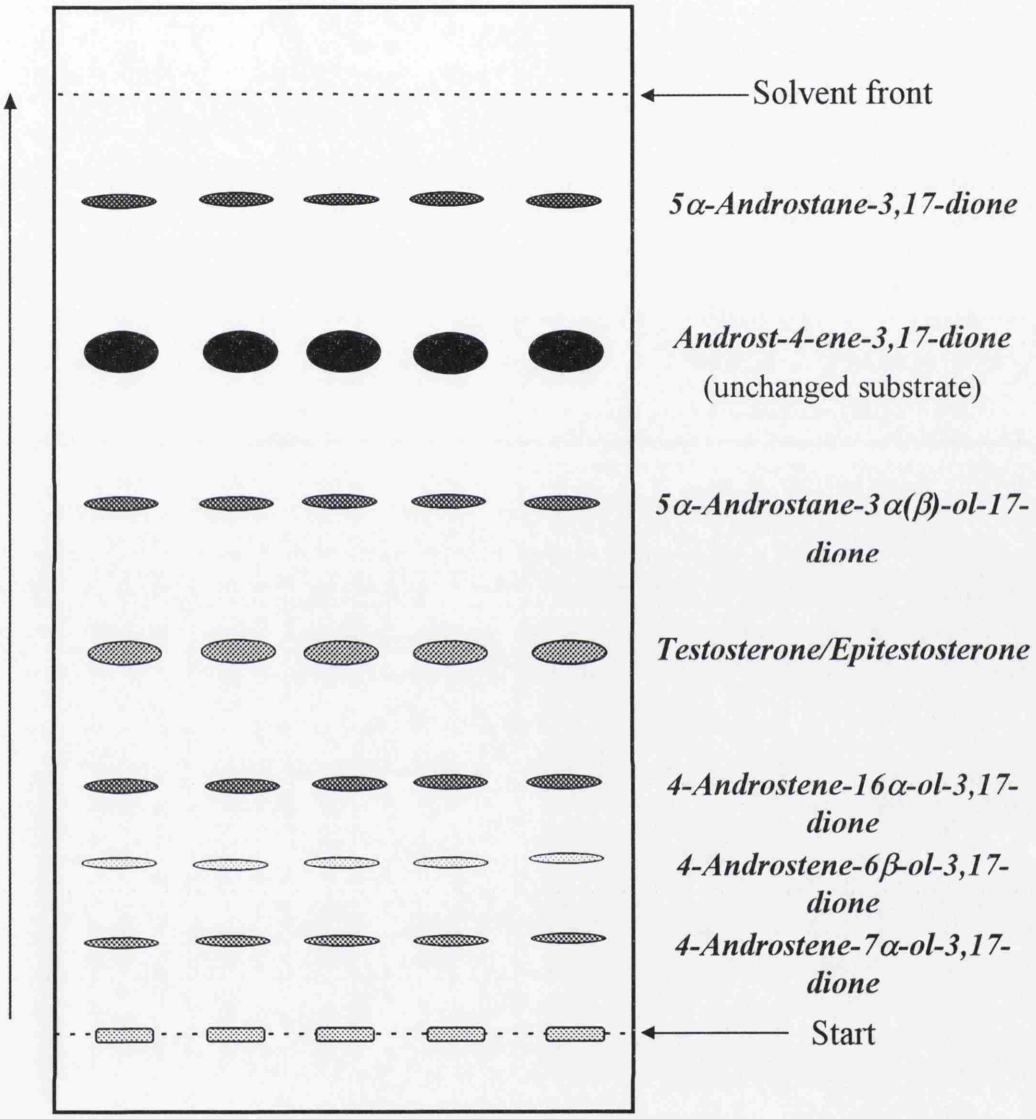


Figure 2.9 Schematic representation of the autoradiography of a thin-layer chromatogram of androst-4-ene-3,17-dione metabolites.

2.7.4 Enzyme-linked immunosorbent assay of cytochrome P450 1A1

Presence of *CYP*1A1 in microsomes obtained from hepatocytes in control and β-naphthoflavone-treated rats was detected by an enzyme-linked immunosorbent assay (ELISA).

2.7.4.1 Principle

Antibodies specific to a protein antigen are applied to an indicator enzyme such as horseradish peroxidase(HRP) and alkaline phosphatase. After binding of the peroxidase-coupled antibody to the antigen, the peroxidase can be used to generate a colored product that is measurable and whose concentration is related to the amount of the antigen in the sample. In HRP-based assays, cleavage of H_2O_2 (the natural substrate) is coupled with the oxidation of a hydrogen donor which changes color during the reaction. Sensitivity of the assay is increased by using an unlabeled 1° antibody and then detecting the antigen-antibody complex with a labeled 2° antibody that binds to the 1° antibody.

2.7.4.2 Assay procedure

The essential steps in the assay are described below.

Antigen coating

Microsomes were diluted to 1-5 μg ml^{-1} in 0.1 M carbonate/bicarbonate buffer, pH 9.5. 100 μl was placed in each well of a 96-well polystyrene microtitre plates (Life Technologies, Roskilde, Denmark) and allowed to stand for 2 h at room temperature (22 °C). This allows the solids (antigens) to attach to the wells.

Washing

Each well was then washed three times with phosphate-buffered saline, pH 7.5, containing Tween-20 (0.1% v/v) by filling each well from a wash bottle, and then emptying.

Blocking of nonspecific binding sites

After washing, nonspecific binding sites were blocked with 200 μl per well of bovine serum albumin (1% w/v) in phosphate-buffered saline pH 7.5 for 1 h at room temperature. This step is necessary to ensure the antibodies do not become nonspecifically bound to the wells of the microtitre plates if there are free binding sites that have not been coated with the antigen of interest.

Addition of the 1° antibody

The *CYP*1A1 antibody (Borlakoglu *et al.*, 1993a & b), was diluted 2000 times with PBS/Tween, pH 7.5 and 100 μl of the diluted antibody was placed in each well and incubated for 1 h. The wells were then washed three times as described above.

Incubation with horseradish peroxidase(HRP)-labeled 2° antibody

The secondary antibody was HRP-labeled rabbit Ig (from donkey), obtained from the Scottish Antibody Production Unit, Carluke, Lanarks., Scotland, was diluted 3000 times and 100 μl of the diluted antibody added to each well and incubated for 1 h.

Detection with 3,3',5,5' Tetramethylbenzidine (TMB)

Hundred (100) microliters of 0.4 mM TMB with 0.004% H_2O_2 in acetate buffer pH 5.6 was added to each well and incubated further for 10 min and the reaction stopped by the addition of 100 µl of 2M H_2SO_4.

The absorbance of the solution in each well was read at 450 nm in an MR5000 microtiter plate reader (Dynatech Lab., Guernsey, Channel Islands, U.K.).

2.8 Radioligand Binding Studies

2.8.1 Preparation of plasma membranes

Preparation of crude membranes. Freshly isolated hepatocytes (10^6 cell ml^{-1}; 20 ml) were washed with PBS, pH 7.4 and then suspended in 10 mM Tris-HCl (pH 7.4) containing 10 mM EDTA and 0.1 PMSF. The cells were homogenized by

20 strokes of a Dounce homogenizer. The suspension was centrifuged at 150 *g* for 5 min and the supernatant was recentrifuged at 1500 *g* for 20 min. The pellet was washed twice with 20 ml of the same buffer with centrifugation at 3000 *g* for 20 min, and finally resuspended in 50 mM Tris-HCl containing 1 mM EDTA ,$MnCl_2$ and 0.1 mM PMSF. The crude membranes were stored at -70 °C till they were used.

Purification of crude membranes

The crude membranes were purified by the method of Belsham *et al.* (1980) as modified by Nakamura *et al.* (1984).

Two-milliliter aliquots of the crude membrane suspension were dispersed in 10 ml of isoosmotic Percoll solution composed of 7 volumes of Percoll, 1 volume of 2 M sucrose-80 mM Tris-HCl buffer containing 8 mM EDTA and 32 volumes of STE solution (0.25 M sucrose in 10 mM Tris-HCl buffer (pH 7.5) containing 2 mM EGTA). The mixture was centrifuged at 10,000 *g* for 60 min and the clear band of membranes just below the surface was collected, washed by dilution with 5 volumes of 50 mM Tris-HCl buffer (pH 7.5) containing 2 mM $MgCl_2$ and 1 mM EDTA and centrifuged at 10,000 *g* for 10 min. The pellet was washed 2 times with 20 ml of the same buffer with centrifugation at 3000 *g* for 20 min, resuspended in 50 mM Tris-HCl containing 1 mM EDTA , 2 mM $MnCl_2$ and 0.1 mM PMSF. A Sorvall RC-5B centrifuge (Dupont Instruments, Wilmington, DE, U.S.A.) was used for all the centrifugation steps.

The membranes were stored at -70 °C till they were used.

2.8.2 Preliminary binding studies

The following experiments were carried out to determine appropriate conditions for binding assays:

The effect of protein concentration on binding of the ligands

Two hundred picomole concentrations of the ligands were incubated with protein concentrations ranging from 0.04 to 0.32 mg/ml for 1 h at 37 °C in the presence and absence of 10^{-6} M phentolamine or propranolol.

2.8.3 Assay of [^{3}H]Prazosin and [^{3}H]Dihydroalprenolol Binding

2.8.3.1 Kinetic studies

Purified membranes (200 μg ml^{-1}) were incubated with either [^{3}H]prazosin (100 pM) or [^{3}H]dihydroalprenolol (1 nM) at 37 °C in 250 ml Erlenmeyer flasks. After 30 min, a large excess (10 mM) of phentolamine or propranolol was added to the mixture to initiate the dissociation reaction. At designated times the reactions were stopped by filtration through Whatman GF/C glass fiber filters (2.5 cm).

Analysis of results

Results were analyzed as described by Landi *et al.*, 1992. The association reaction was linearised by plotting $\ln[B_{eq}/(B_{eq}-B_t)]$ *vs.* time. Similarly, the dissociation reaction was linearised by plotting $\ln(B_t/B_{eq})$ vs. time. B_{eq} and B_t are the amount of ligand bound at equilibrium and at time t respectively. The apparent rate constant for the association kinetics, k_{ob} was calculated by linear regression analysis. The pseudo-first-order rate constant, k_1 was determined by the formula

$$k_1 = (k_{ob} - k_2)/[ligand]$$

where k_2 is the first-order dissociation constant for the dissociation reaction.

2.8.3.2 Saturation and competition studies

Binding was assayed by a modification of the method of Nakamura *et al.* (1983, 1984). Purified plasma membranes (100-200 µg of protein) were incubated with various concentrations of [*furanyl*-5-^{3}H]prazosin (0.01-2.50 nM) or l-[*propyl*-2,3-^{3}H]dihydroalprenolol (0.01-2.5 nM) in 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM $MgSO_4$ and 0.1 mM PMSF in a total volume of 1 ml for 30 min at 30°C. The reaction was stopped by filtration under reduced pressure through Whatman GF/C glass fiber filters (2.5 cm) which had been previously moistened with the buffer. Each filter was immediately washed twice

with 5 ml of ice-cold 50 mM Tris buffer (pH 7.5) and placed in 5 ml of Ecoscint A scintillation fluid (National Diagnostics, Manville, NJ, U.S.A.) and counted in a Tri-Carb 2000C (Packard, Pangbourne, Berks., England) for radioactivity. Specific binding was defined as the [^{3}H]prazosin or [^{3}H]alprenolol binding that was inhibited by 10 μM phentolamine and 10 μM propranolol respectively.

Receptor densities (B_{max}) and dissociation constants (K_D) for the binding were calculated by fitting the data to the rectangular hyperbolic function

$$f(x) = \frac{B_{max} \cdot x}{K_D + x}$$

iterative curve fitting using the computer programme SigmaPlot Version 5.01 (Jandel Scientific Software, Erkrath, Germany).

2.9 Protein Content

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

2.10 Calculations And Statistics

Concentration-response curves were fitted to a four-parameter logistic function of the form:

$$f(x) = \frac{a - d}{1 + e^{-b(x-c)}} + d \qquad \text{Eqn 1.1}$$

where **a** = maximal response; **b** = slope coefficient; **c** = EC_{50}; **d** = minimum response; e = base of the natural logarithm, by using the computer software Inplot Version 3.14 (GraphPad Software, San Diego, CA, U.S.A.).

In some instances, the data were fitted to a modification (Rovati & Nicosia, 1994) of the logistic function expressed below due marked biphasic (bell-shaped) nature of the responses.

$$f(x) = \left[\frac{a_1 - d}{1 + (x/c_1)^{b_1}} + d\right] - \left[d - \left(\frac{a_2 - d}{1 + (x/c_2)^{b_2}} + d\right)\right] \qquad \text{Eqn 2.2}$$

where **a** = minimum response; **b** = slope parameter; **c** = EC_{50} and **d** = maximum response. Subscript 1 and 2 refer to R_s and R_i respectively. by using SigmaPlot Version 5.01 (Jandel Scientific Software, Erkrath, Germany).

Data were expressed as mean ± s.e. One-way analysis of variance was used to compare more than two means, followed by Bonferroni's multiple comparison if the overall ANOVA was significant (Wallenstein *et al.*, 1980; Ludbrook *et al.*, 1992). Unpaired Student's *t* test was used for comparison of two means. GraphPad InStat Version 2.02 (GraphPad Software, San Diego, CA, U.S.A.) was used for all statistical calculations.

2.11 Drugs And Chemicals

Sigma Chemicals, St. Louis, MO, U.S.A:

- α-isonitropropiophenone (INPP)
- 3,3',5,5'-tetramethylbenzidine (TMB)
- androst-4-ene-3,17-dione
- caffeine (anhydrous)
- calcium ionophore A23187
- dexamethasone
- dibutyryl cAMP
- DL-dithiotreitol
- DL-isoprenaline sulfate
- DMSO
- EGTA
- epinephrine bitartrate
- Folin-Ciocaltaeu's phenol reagent
- glucagon
- glucose 1 phosphate (dipotassium salt)
- heparin, sodium salt (bovine intestinal mucosa)
- HEPES
- insulin
- isocitric acid
- L-amino acids
- L-phenylephrine hydrochloride
- MOPS
- phenylmethyl sulfonyl fluoride
- PMA
- rabbit liver glycogen Type III
- sodium dodecyl sulfate
- Tris HCl
- Tween 20

Alexis Corp.,Nottingham, England

AIAP- [(2S)-(+)-2-Amino-5-iodoacetamidopentanoic acid]

E. Merck, Darmstadt, Germany

sodium fluoride,

disodium EDTA

Amersham, Buckinghamshire., England:

α-D[U^{14}C]Glucose-1 phosphate, potassium salt

[4-^{14}C]Androst-4-ene-3,17-dione

[*furanyl*-5-^{3}H]Prazosin

l-[*propyl*-2,3-^{3}H]Dihydroalprenolol

Ciba-Geigy, Basel, Switzerland

phentolamine mesylate

Boehringer, Mannheim, Germany:

collagenase Type A from *Clostridium histolyticum*

Isocitrate dehydrogenase from porcine heart (2 U mg^{-1})

Kyowa Medex, Tokyo, Japan

protein kinase inhibitors- K-252a; K-252b and KT5823

Fisons, Loughborough, England:

salts for physiological solutions and buffers

Wellcome

methoxamine

GIFTS

1. CYP1A1 antibody from Professor C. Roland Wolf and Dr. Colin J. Henderson, ICRF Molecular Pharmacology Unit, Biomedical Research Centre, Ninewells Hospital & Medical School, Dundee, Scotland.

2. HRP-labeled rabbit antibody from the Scottish Antibody Production Unit, Lanarkshire, Carluke, Scotland.

2.11.1 Vehicles for drugs

Phenylephrine and isoprenaline were prepared as 10^{-2} M stock solution in dH_20 containing 20 μM ascorbic acid. Further dilutions were made serially each day with the same vehicle.

Insulin and glucagon were prepared as stock solution in 0.1 M NaOH and were diluted before use with dH_20

Dexamethasone, ionophore A23187, forskolin, K252a, K252b, and KT5822 were prepared in DMSO. The drug were added to the cell at 100-1000 fold the final concentration to minimize the effect of the solvent. In all experiments in which DMSO was used as the solvent, DMSO was also added to the control cells at the same concentration as the test cells.

All other drugs were dissolved in dH_20 unless stated otherwise.

RESULTS

3.1 VALIDATION OF THE GLYCOGEN PHOSPHORYLASE ASSAY

The sections below present the results of preliminary studies undertaken to determine appropriate conditions for the glycogen phosphorylase *a* assay.

3.1.1 Kinetics of the glycogen phosphorylase a *assay*

Figure 3.1 shows the time course of the assay. Incorporation of ^{14}C into glycogen proceeded in a linear fashion for up to 50 min after which the reaction rate declines. Thus kinetics of the reaction deviates slightly from zero order. This is in contrast to the observation of Stalmans and Hers (1975).

A reaction time of 20 min (within the linear portion of the graph) was used in all subsequent assays.

3.1.2 Effect of protein concentration on the incorporation of ^{14}C into glycogen

Figure 3.2 shows the relationship between protein content of supernatant obtained from cell lysate (see Section 2.4.3 under *Materials and Methods*) and the amount of ^{14}C incorporated into glycogen. The amount of glycogen deposited was directly proportional to the protein concentration in the range of 0 to 6 mg ml^{-1}. The protein content of the supernatant was proportional to the amount of cells used and the upper limit of 6 mg ml^{-1} was the protein content in a supernatant obtained from ≈9-10 x 10^6 cell ml^{-1} suspension. However, in our studies, the density of cells used in hepatocyte suspensions were kept in the range from 2 to 5 million cells ml^{-1}. Activation of glycogen phosphorylase in response to drugs was minimal with cells densities above 5 x 10^6 cells ml^{-1}, probably due to rapid uptake and depletion of the drugs (Kunos, 1984). Furthermore, the cells rapidly deteriorate (loss of viability) when cultured at high densities.

3.1.3 Time course of drug action

The time courses for the effect of phenylephrine and isoprenaline are presented in Figure 3.3. Incubation of hepatocyte suspensions with phenylephrine or isoprenaline (final conc. 10 μM) caused a rapid activation of glycogen phosphorylase *a* activity from a basal level of 17.3 to 45.9 and 32.9 nmol min^{-1} mg^{-1} protein respectively for phenylephrine and isoprenaline. Maximum activation of the enzyme is achieved within 30-60 s which was persistent for at least 5 min. These results are consistent with observations made by other workers (Exton, 1988; García-Sáinz *et al.*, 1992; Keppens *et al.*, 1992).

Considering the results above, a contact time of 4 min was adopted in all subsequent experiments involving the activation of glycogen phosphorylase by drugs or hormones.

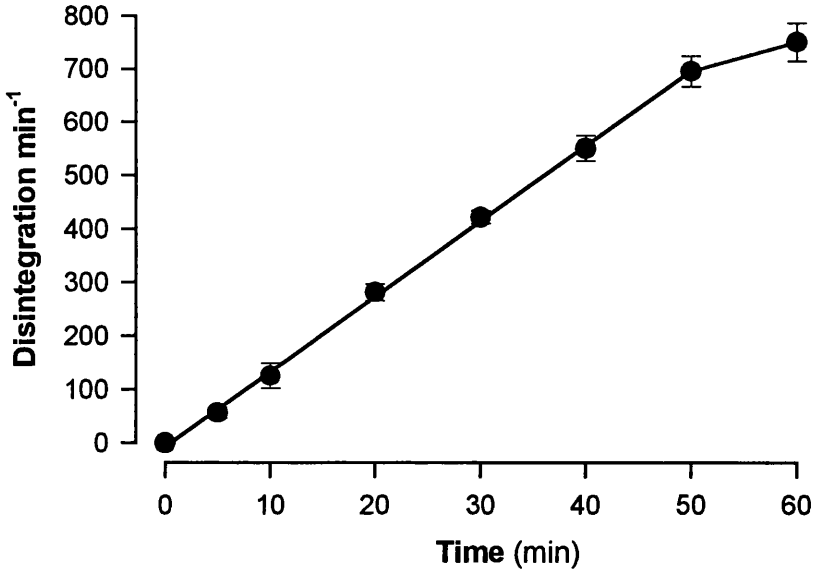


Figure 3.1 Time course of glycogen phosphorylase a assay. A 1:2 dilution of the supernatant obtained by lysing hepatocyte suspension (10^7 cells ml^{-1}) was used to establish several incubations. Reactions were terminated at the times indicated and the amount of radioactivity incorporated into glycogen measured. Each assay was done in duplicate and results are expressed as means ± s.e. (n=6).

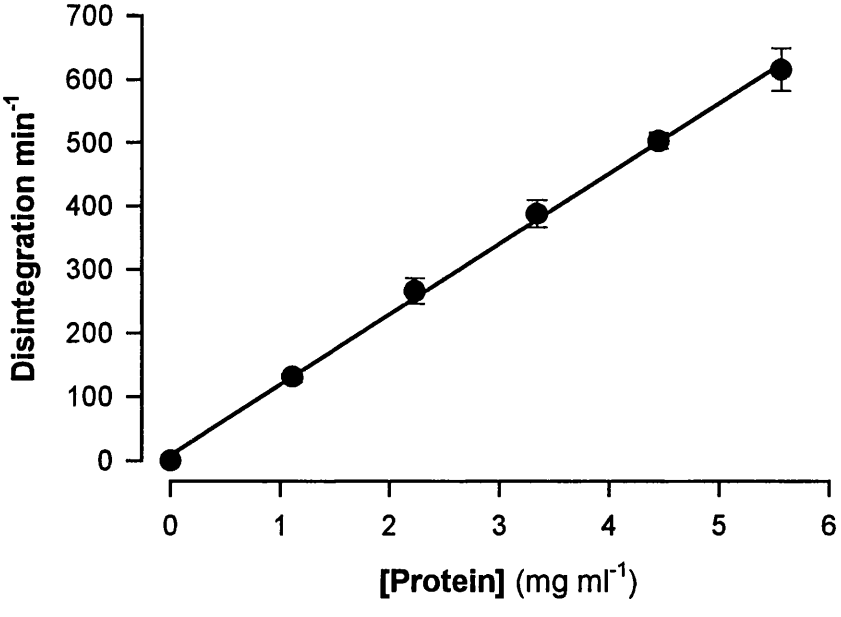


Figure 3.2 Amount of radioactivity incorporated into glycogen as a function of protein concentration. Various dilutions of the supernatant obtained from cell suspension (10^7 cells ml^{-1} ,equivalent to 6 mg ml^{-1} protein in the supernatant) were used to determine the amount of radioactive glycogen formed. Each assay was done in duplicate and results are expressed as means ± s.e. (n=6).

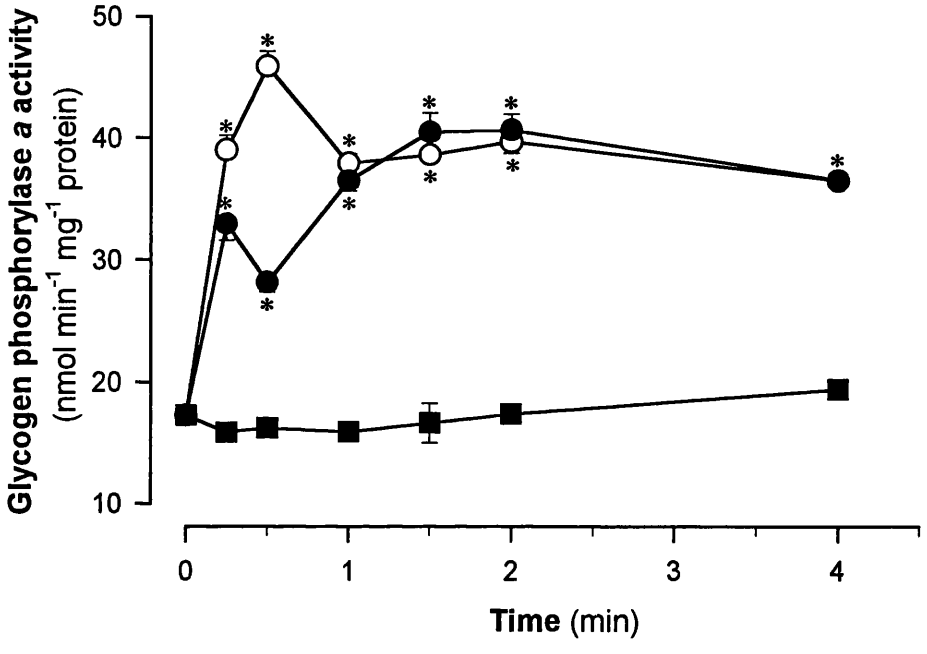


Figure 3.3 Time course of the effects of phenylephrine and isoprenaline on the activation of glycogen phosphorylase *a* in rat hepatocytes. Rat hepatocytes (3-5 x 10^6 cells ml^{-1}) were preincubated for 30 min in Williams' E medium. Aliquots (450 μl) were further incubated with 50 μl of 10 μM phenylephrine (●), 10 μM isoprenaline (O) or saline (■). Reactions were terminated at the times indicated and were assayed for glycogen phosphorylase *a* activity as described in *Materials and Methods*. Values are means ± s.e. (n=6). *$P < 0.05$ compared to basal activity at t=0 (17.3 ± 1.3 nmol min^{-1} mg^{-1} protein) by ANOVA followed by Student's t test with Bonferroni's correction.

3.1.4 Response of hepatocytes to hormones and other agents

Damage to hepatocyte membranes during isolation and thus loss of response to hormones has been reported by other workers (Zahlten & Stratman, 1974; Berry *et al.*, 1991). To confirm that our isolation procedure yielded cells with the receptors and signal transduction systems intact, responses to various hormones were examined.

Incubation of hepatocytes with increasing concentrations of hormones and other agents caused concentration-dependent increases in glycogen phosphorylase *a* activity (Figure 3.4). The order of potency of the agonists tested was glucagon > calcium ionophore A23187 = noradrenaline > dibutyryl cAMP >> isoprenaline (see Table 3.1 for EC_{50}s) Isoprenaline responses are expressed as a percentage of the maximum change produced by noradrenaline. The EC_{50} of isoprenaline could not be determined since it did not achieve its maximum. Although glucagon was the most potent of the agonist tested, A23187 was the most effective (high E_{max}) agent in the stimulation of glycogen phosphorylase *a* activity (see Table 3.1).. The basal phosphorylase a activity was not altered by the amount of ethanol added which was used as solvent for the calcium ionophore.

Insulin, on the other hand inhibited the activity of glycogen phosphorylase in a dose-dependent manner in the hepatocyte suspensions (Figure 3.5). The maximum change in phosphorylase *a* activity, 6.86 ± 0.93 nmol min^{-1} mg^{-1} protein, was not pronounced compared to the other hormones studied.

These results, therefore, indicate the presence of receptor/signal pathways in the hepatocytes used.

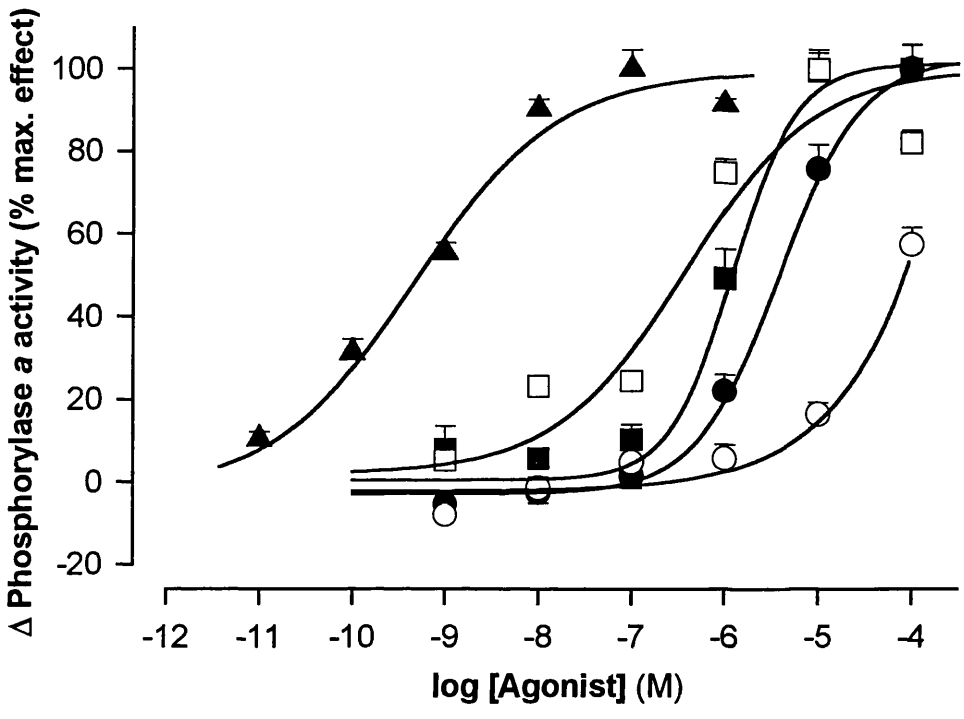


Figure 3.4 Activation of glycogen phosphorylase in response to hormones and other agents in isolated rat hepatocytes. Hepatocyte suspensions were preincubated for 30 min in Krebs-Henseleit buffer and then treated for 4 min with increasing concentrations of noradrenaline (●), isoprenaline (O), Bt_2cAMP(■), calcium ionophore A23187 (□), or glucagon (▲). Changes in glycogen phosphorylase *a* activity was determined as described in *Materials and Methods.* Assays were done in duplicate. Isoprenaline responses are presented as percentage of maximal noradrenaline response. Basal glycogen phosphorylase *a* activity was 26.3 ± 1.2 nmol min^{-1} mg^{-1} protein. Each data point represents mean ± s.e. (n=6). The curves are the least square fits of the data to the logistic function expressed in Eqn 2.1. Individual EC_{50} and E_{max} values are presented in Table 3.1.

Table 3.1 EC_{50} and E_{max} values for the activation of glycogen phosphorylase a in response to various agonists.

Hormone/effector	**EC_{50}** *(x 10^7 M)*	**E_{max}** (nmol min^{-1} mg^{-1} protein)
Noradrenaline	3.75 ± 1.17	16.1 ± 0.9
Isoprenaline	n.d.	9.2 ± 0.7
Glucagon	0.005 ± 0.001	58.2 ± 2.2
Bt_2 cAMP	12.10 ± 2.80	74.4 ± 4.4
A23187	3.71 ± 0.85	99.7 ± 4.6

EC_{50}s ± s.e were obtained by fitting the data points shown in Figure 3.4 to Eqn 2.1 (see *Materials and Methods*).
E_{max} values are means ± s.e.(n = 6).
n.d. - not determinable

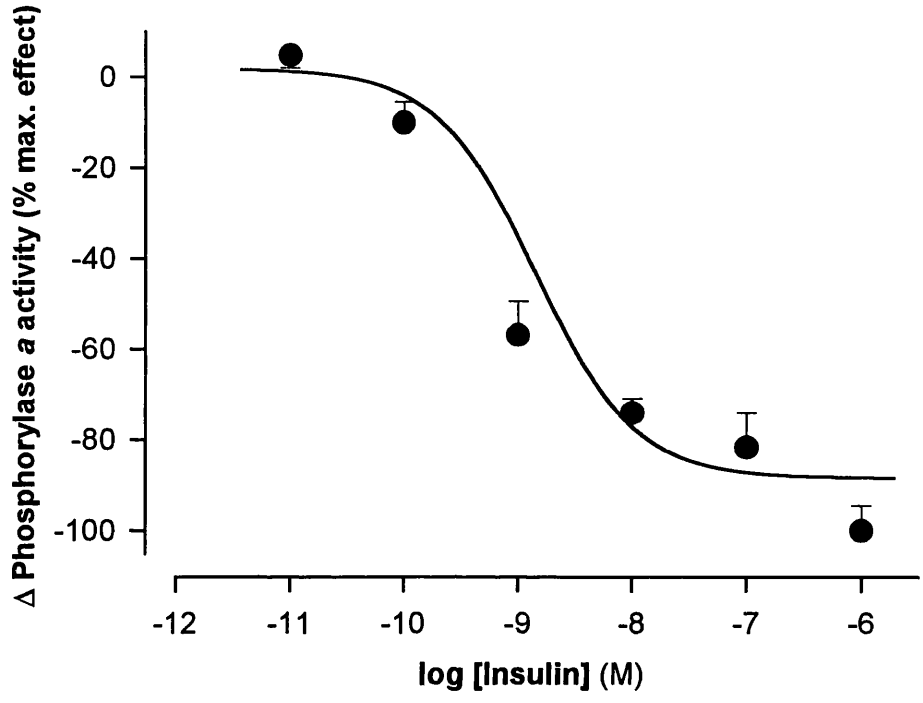


Figure 3.5 Inhibition of glycogen phosphorylase *a* activity by insulin in isolated rat hepatocytes. Hepatocyte suspensions were preincubated for 30 min in Krebs-Henseleit buffer and then treated for 4 min with increasing concentrations of insulin. Changes in glycogen phosphorylase *a* were determined as described in *Materials and Methods.* Assays were done in duplicate. Basal activity was 21.1 ± 0.7 nmol min^{-1} mg^{-1} protein. Each data point represents mean ± s.e. (n=6). The curve is the least square fit of the data to the logistic function expressed in Eqn 2.1.

3.2 Activation of Glycogen Phosphorylase *a* by Adrenergic Agonists in Isolated Rat Hepatocytes

To confirm that this was the case in our studies, we studied the effects of the α_1-blocker, prazosin and the β-blocker, propranolol on the activation of glycogen phosphorylase in response to phenylephrine (α-agonist) and isoprenaline (β-agonist).

The effects of the adrenergic agonists phenylephrine (α), isoprenaline (β) and methoxamine (α), are shown in Figure 3.6. Methoxamine was included in this study for comparison since phenylephrine is reported to have some amount of β-adrenergic property. All the agonists caused a concentration dependent activation of glycogen phosphorylase a with the maximum responses at 10^{-4} M. However, phenylephrine was ≈3.5-fold more potent than methoxamine (EC_{50}s- 0.65 ± 0.12 *vs.* 2.25 ± 0.45 μM; $P < 0.001$) and the maximal phenylephrine response was 3-fold greater that of methoxamine (17.1 ± 0.4 *vs.* 4.9 ± 0.4 nmol min^{-1} mg^{-1} protein; $P < 0.001$). The potency of methoxamine was comparable to isoprenaline (EC_{50},1.78 ± 0.82; $P > 0.05$). However, the E_{max} of isoprenaline (8.8 ± 1.2) was greater ($P < 0.001$) than the E_{max} of methoxamine. Decreased potency and efficacy of methoxamine in isolated hepatocytes have been reported by other workers (Aggerbeck *et al.*, 1980; Tsujimoto *et al.*, 1989).

3.2.1 Inhibition of responses to adrenergic agonists by prazosin and propranolol

Pretreatment of the cells with prazosin (1 μM) significantly decreased the basal glycogen phosphorylase a activity, whereas treatment with propranolol (10 μM) significantly increased the basal activity of glycogen phosphorylase *a* (see Table 3.2).

Both phenylephrine and isoprenaline caused concentration-dependent increases in glycogen phosphorylase activity (Figure 3.7). In the experiments,

isoprenaline was as potent ($P > 0.05$) as phenylephrine. See Table 3.2 for EC_{50}s. However, phenylephrine was twice as effective as isoprenaline

Activation of glycogen phosphorylase in response to adrenergic agonists is reported to be predominantly α in adult male (Ishac *et al.*, 1992). In contrast to this we found that the β-adrenergic response was significantly present when measured in Williams' E medium as shown in Figure 3.7. The responses were however, almost absent when the medium is changed to Krebs-Henseleit buffer. This 'phenomenon' is further investigated in a latter section (see *3.2.3 Effect of culture media on adrenergic responses*).

The presence of prazosin (1 μM) in the culture medium caused a dramatic displacement of the concentration-response curve of phenylephrine to the right by more than a 1000-fold (Figure 3.7a). Prazosin, also displaced the concentration-response-curve of isoprenaline slightly but significantly ($P > 0.05$) to the right by ≈2-fold. Thus responses to phenylephrine were much more sensitive to prazosin than responses to isoprenaline. The β-antagonist, propranolol displaced the curve of isoprenaline (β-agonist) dramatically to the right (Figure 3.7b) but in contrast potentiated the responses to phenylephrine ($P < 0.001$) - see Figure 3.7a.

Taken together, these results indicate that the use of phenylephrine as an α-agonist and propranolol as a β-agonist in this project was justifiable (see also 3.2.2 *Assay of [3H]prazosin and [3H]dihydroalprenolol binding).*

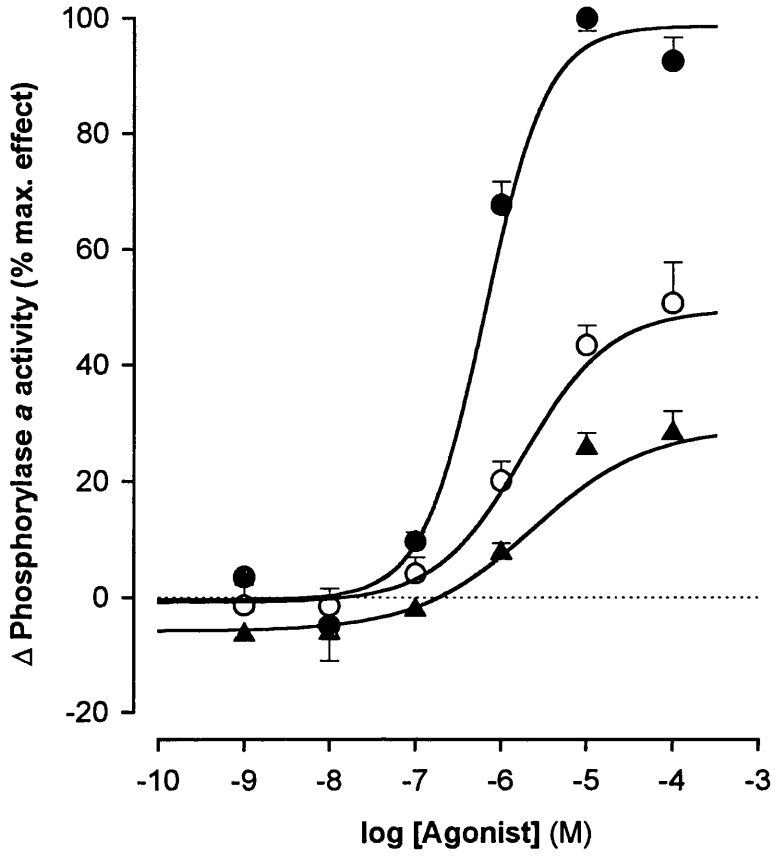


Figure 3.6 Activation of glycogen phosphorylase by adrenergic agonists in isolated rat hepatocyte suspensions. Hepatocyte suspensions were preincubated for 30 min in Krebs-Henseleit buffer and then treated for 4 min with increasing concentrations of phenylephrine (●), isoprenaline(O) and methoxamine (▲). Changes in glycogen phosphorylase *a* were determined as described in *Materials and Methods.* Assays were done in duplicate. Basal activity was 18.5 ± 0.8 nmol min^{-1} mg^{-1} protein. Each data point represents mean ± s.e. (n=6). For comparative purposes, responses to isoprenaline have been expressed as % of maximal phenylephrine response. The curves are the least square fits of the data to the logistic function expressed in Eqn 2.1.

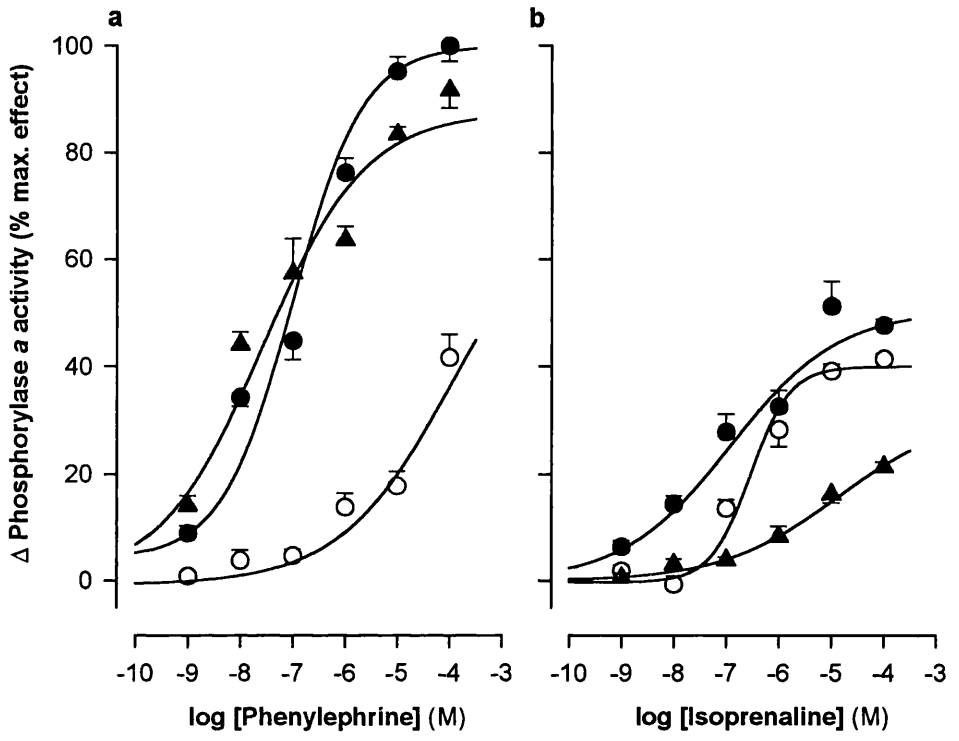


Figure 3.7 Effect of prazosin (1 μM: O) and propranolol (10 μM: ▲) or vehicle (●)on the activation of glycogen phosphorylase induced by phenylephrine (panel **a**) and isoprenaline (panel **b**). Hepatocytes were preincubated for 30 min Williams' E medium and the changes in glycogen phosphorylase *a* were determined as described in *Materials and Methods*. The cells were pretreated with the antagonists for 5 min before the exposure to the agonists. Basal glycogen phosphorylase *a* activity was 30.8 ± 0.4 nmol min^{-1} mg^{-1} protein. Each data point represents the mean ± s.e. (n=6). All responses are expressed as % of maximal phenylephrine response in vehicle-treated (control) cells. E_{max} in control cells was 42.2 ± 0.7 nmol min^{-1} mg^{-1} protein. The curves are the least square fits of the data to the logistic function expressed in Eqn 2.1. Individual EC_{50} and E_{max} values are presented in .Table 3.2

Table 3.2 Summary of inhibition of phenylephrine- and isoprenaline-induced activation of glycogen phosphorylase *a* (GPase *a*) by prazosin and propranolol in isolated rat hepatocytes.

Treatment	Basal GPase *a* activity[a]	EC_{50} *(x 10^7 M)*	
		Phenylephrine	*Isoprenaline*
Control	30.86 ± 0.41	1.00 ± 0.18	1.20 ± 0.49
Prazosin (1 μM)	28.31 ± 0.46	>1,000	2.88 ± 0.54
Propranolol (10 μM)	37.02 ± 1.14	0.26 ± 0.08	128.82 ± 26.38

[a]Values are expressed as nmol min^{-1} mg^{-1} protein and are means ± s.e. mean (n = 6). Values were obtained from the experiments shown in Figure 3.7.
EC_{50}s ± s.e. were obtained by least-square nonlinear regression as described in *Materials and Methods.*

3.2.2 Assay of [^{3}H]prazosin and [^{3}H]dihydroalprenolol binding

Preliminary studies were undertaken to determine appropriate conditions for the binding assays. Figure 3.8 shows the effect of altering the protein concentration of the membranes on the binding of [^{3}H]prazosin (Figure 3.8) and [^{3}H]dihydroalprenolol (Figure 3.9). The results demonstrate that under the conditions used in the studies, specific binding of both radioligands were directly proportional to the protein concentration. Specific binding represented about 80% of total [^{3}H]prazosin binding and more than 85% of total [^{3}H]DHA binding.

3.2.2.1 Kinetic studies

[^{3}H]Prazosin binding

Figure 3.10a describes the association and dissociation kinetics of [^{3}H]prazosin binding to purified membranes of rat hepatocytes. Binding to the membranes reached a steady state in about 30 min. Addition of a large excess of phentolamine (10 μM) rapidly displaced the radioligand from binding sites. Dissociation of bound [^{3}H]prazosin was also rapid and maximum dissociation was reached in 30 min.

Reaction kinetics were analysed as detailed in Materials and Methods section. The observed rate constant k_{ob}, calculated by linear regressional analysis from the association rate plot, was 0.103 ± 0.012 min^{-1} (Figure 3.10b).The first-order dissociation rate constant, calculated from the dissociation reaction after propranolol addition, was 0.035 ± 0.003 min^{-1} (Figure 3.10c). Thus, the pseudo-first-order rate constant k_1, was 0.339 nM min^{-1} and the K_D, 0.103 nM.

[^{3}H]Dihydroalprenolol

The binding kinetics of [^{3}H]DHA is illustrated in Figure 3.11a. Equilibrium was achieved with 30 min and dissociation was rapid after the

addition of excess (10 μM) propranolol. The rate constants, k_{ob} and k_2 , were 0.067 ± 0.010 and 0.038 ± 0.003 min-1 respectively (Figure 3.11b & c). The pseudo-first-order rate, k_1 was 0.029 and K_D calculated from the rate constants was 1.27 nM.

The K_Ds derived from the binding kinetics of both radioligands were close to K_Ds obtained from saturation experiments (see Table 3.3).

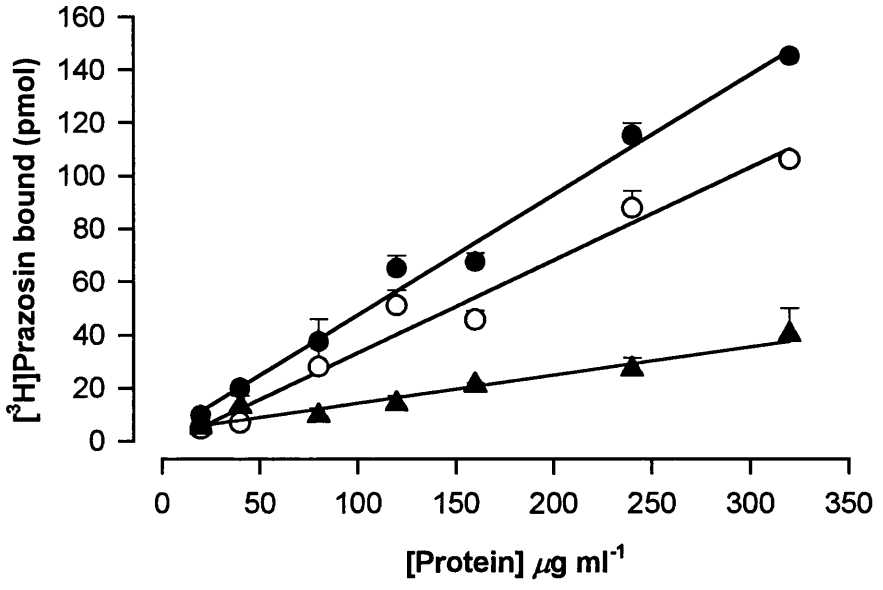


Figure 3.8 [^{3}H]Prazosin binding as a function of protein concentration. 200 pM concentrations of [^{3}H]prazosin were incubated with protein concentrations ranging from 20 to 320 mg ml^{-1} for 45 min in the absence (● total binding) and presence of 10 μM phentolamine (▲) for the determination of specific binding (○). Each data point represents means ± s.e. (n=4).

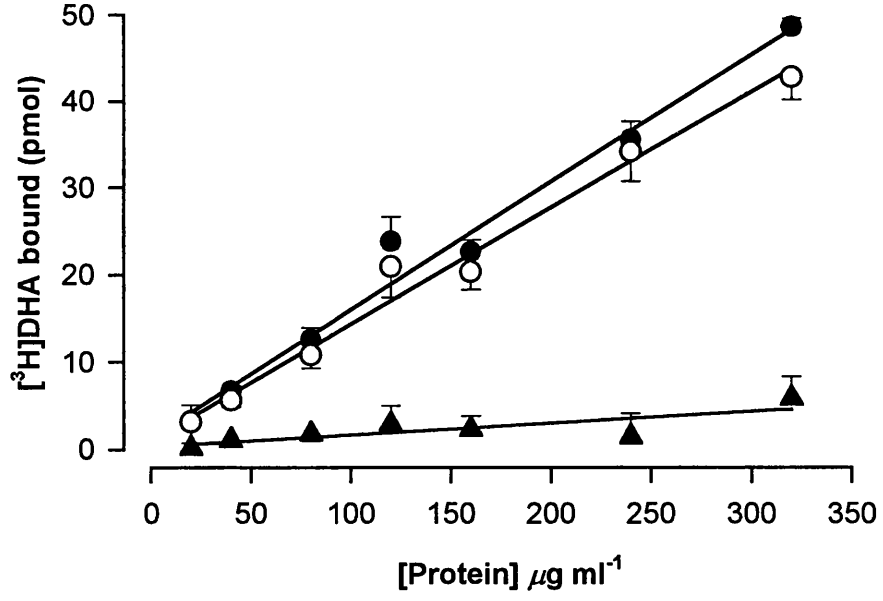


Figure 3.9 [^{3}H]Dihydroalprenolol binding as a function of protein concentration. 1 nM concentrations of [^{3}H]dihydroalprenolol were incubated with protein concentrations ranging from 20 to 320 mg ml^{-1} for 45 min in the absence (● total binding) and presence of 10 μM propranolol (▲) for the determination of specific binding (○). Each data point represents means ± s.e. (n=4).

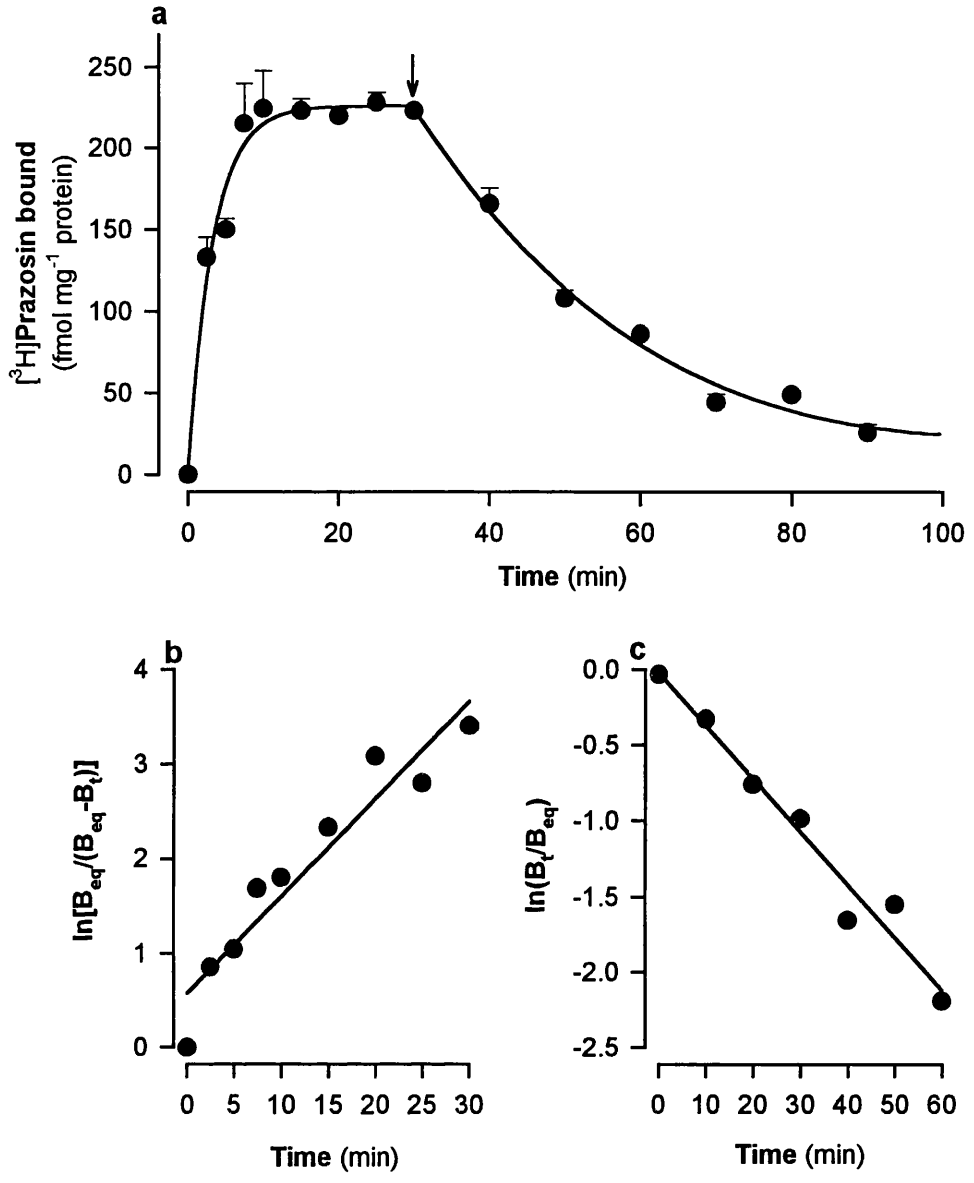


Figure 3.10 Association and dissociation kinetics of [^{3}H]prazosin binding to purified plasma membranes of isolated hepatocytes. **a**).Purified membranes (200 μg ml^{-1}) and [^{3}H]prazosin (100 pM) were incubated at 30 °C in a 250-ml Erlenmeyer flask; and at 30 min (indicated by arrow), phentolamine was added to a final concentration of 10 μM to initiate the dissociation reaction. At the times indicated 1-ml aliquots were removed in triplicate and assayed for bound [^{3}H]prazosin as described in *Materials and Methods.* Each point represents mean ± s.e. (n=3). Data plotted in panels **b** & **c** were taken from panel **a**. The association and dissociation rate constants were determined by regression analysis (see section *2.8.3*). r is 0.95 and 0.98 for plots **c** and **b** respectively.

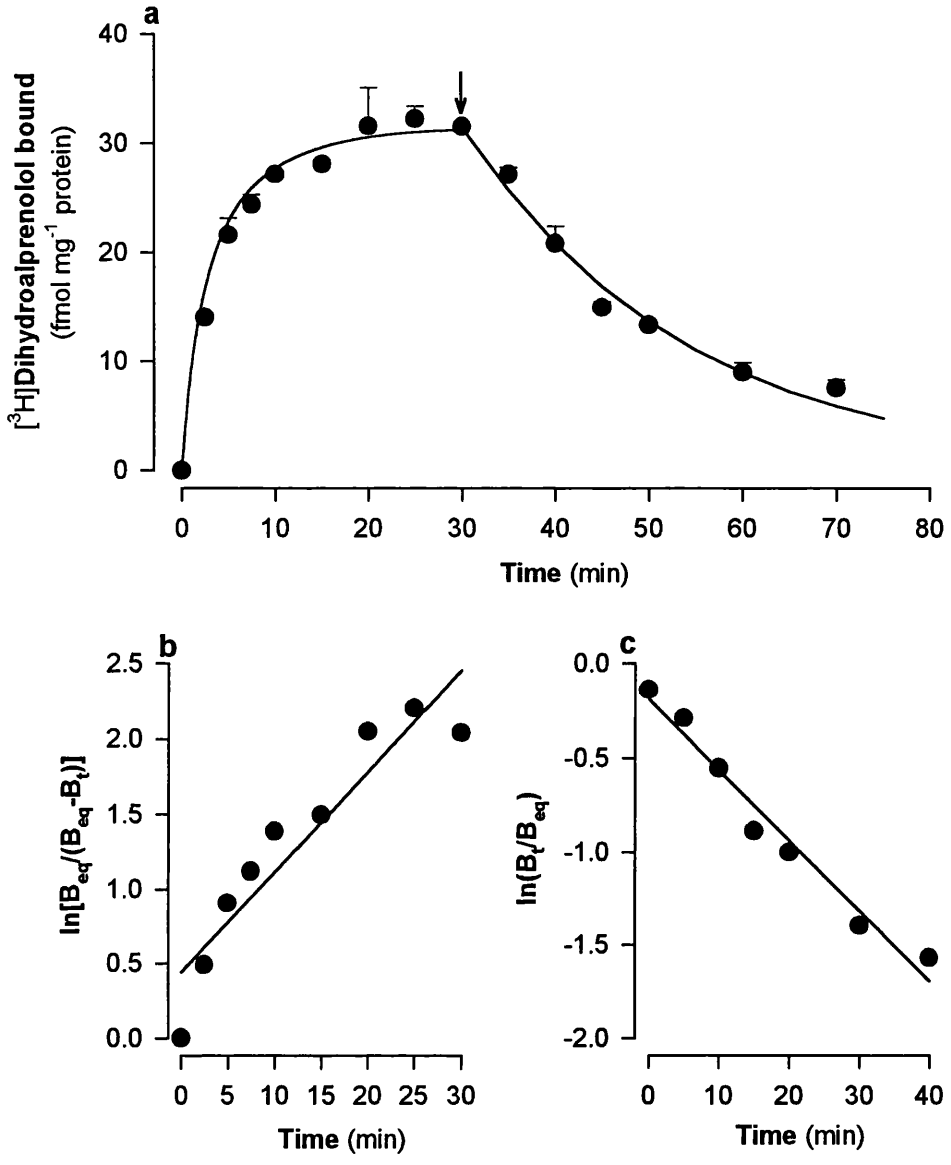


Figure 3.11 Association and dissociation kinetics of [^{3}H]dihydroalprenolol binding to purified plasma membranes of isolated hepatocytes. **a)**. Purified membranes (200 μg ml^{-1}) and [^{3}H]dihydroalprenolol (1 nM) were incubated at 30 °C in a 250-ml Erlenmeyer flask; and at 30 min (indicated by arrow), propranolol was added to a final concentration of 10 μM to initiate the dissociation reaction. At the times indicated 1-ml aliquots were removed in triplicate and assayed for bound [^{3}H] dihydroalprenolol as described in *Materials and Methods.* Each point represents mean ± s.e. (n=3). Data plotted in panels **b** & **c** were taken from panel **a**. The association and dissociation rate constants were determined by regression analysis (see section *2.8.3*). r is 0.93 and 0.98 for plots **c** and **b** respectively.

3.2.2.2 Saturation and competition studies

Saturation binding studies

Results of saturation binding studies are shown in Figure 3.12. The maximum number of binding sites (B_{max}) and the affinity constants K_D were determined by iterative curve fitting and are summarized in Table 3.3..

The value of K_D values for [^{3}H]prazosin obtained in our experiment were in agreement with values of 0.1-0.2 nM reported by other workers (Kunos et al., 1984). However, the number of binding sites was lower (30-50%) than reported values. There were some inconsistencies in values obtained for [^{3}H]DHA binding from previous studies. Differences in values may be attributed to the degree of purification of the membranes and the source of membranes; purified *versus* crude membranes and membranes from whole liver *versus* membranes from hepatocytes.

Competition studies

Figure 3.13 shows results of the displacement of the radioligands by phenylephrine and isoprenaline. IC_{50}s ± s.e. for the phenylephrine and isoprenaline *versus* [^{3}H]prazosin were 0.23 ± 0.02 μM and 73.2 ± 37.5 μM respectively and for phenylephrine and isoprenaline *versus* [^{3}H]DHA were 30.6 ± 17.3 μM and 0.508 ± 0.11 μM respectively.

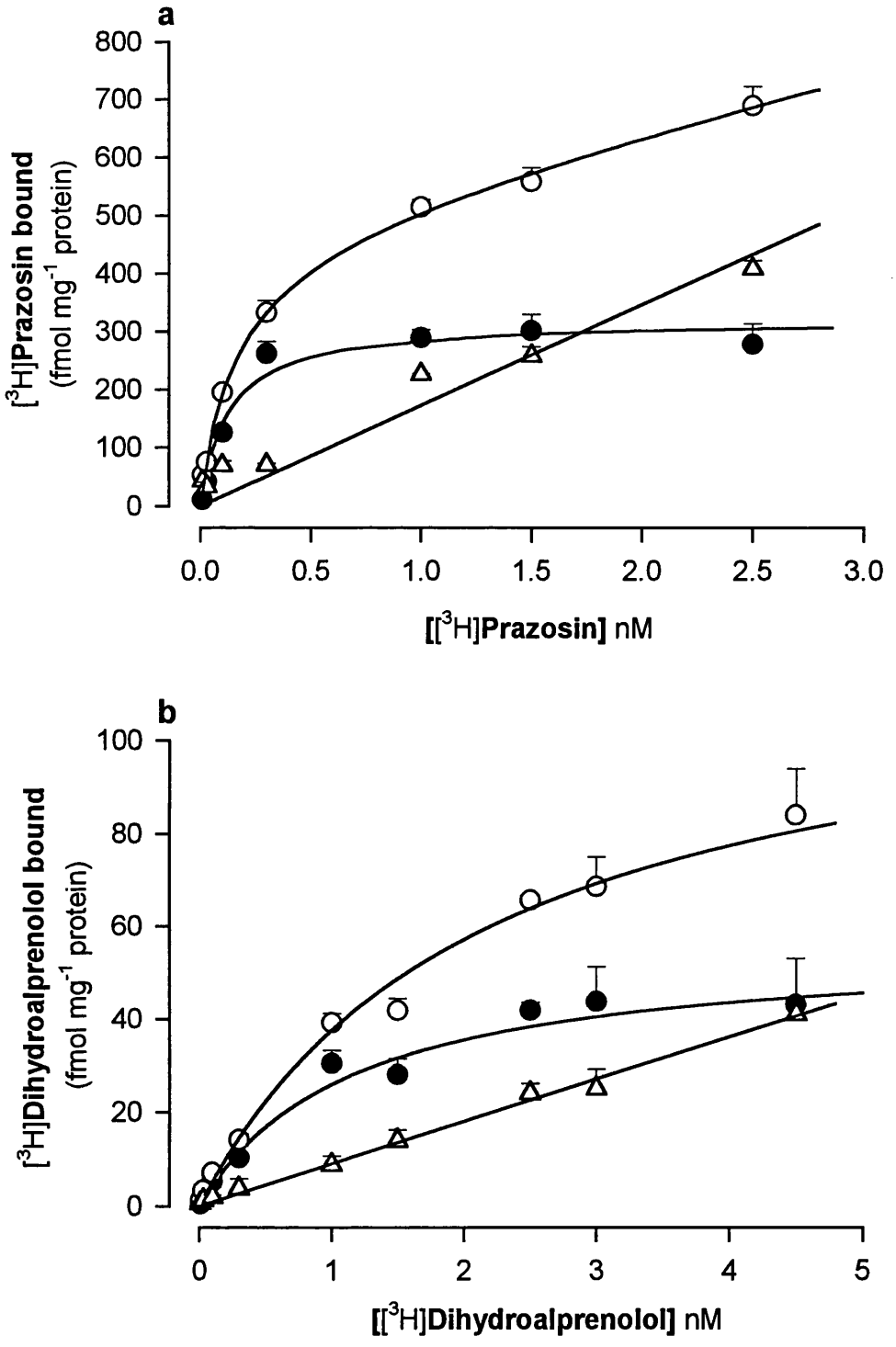


Figure 3.12 Saturation binding of [^{3}H]prazosin (**a**) and [^{3}H]dihydroalprenolol (**b**) to purified rat hepatocyte membranes. Specific radioligand binding (●) was determined by subtracting the binding (Δ nonspecific) in the presence of 10 μM phentolamine (**a**) and 10 μM propranolol (**b**) from total binding (○). Each data point represents mean ± s.d (n=4 and 3 for **a** and **b** respectively).

Table 3.3 Summary of saturation binding studies: affinities (K_D) and number of receptors (B_{max}).

Radioligand	K_D (*nM*)	B_{max} (*fmol* mg^{-1} *protein*)
[^{3}H]Prazosin	0.13 ± 0.03	319.8 ± 18.5
[^{3}H]Dihydroalprenolol	1.17 ± 0.22	56.2 ± 4.6

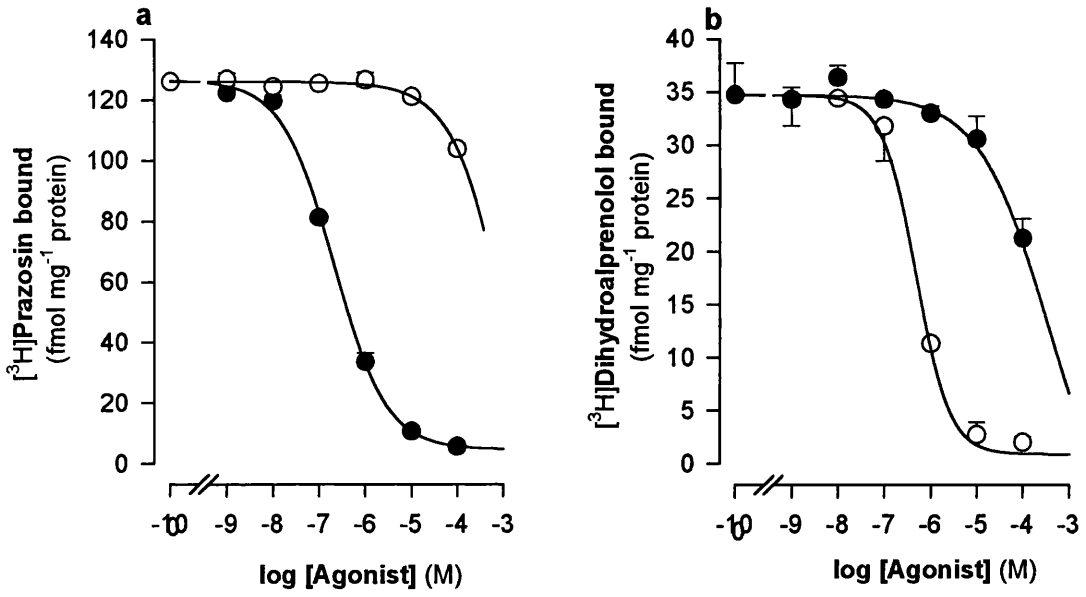


Figure 3.13 Displacement of [^{3}H]prazosin (panel **a**) and [^{3}H]dihydroalprenolol (panel **b**) from adrenoceptors by phenylephrine (●) and isoprenaline (○). Purified plasma membranes (200 μg ml^{-1}) were incubated for 30 °C for 30 min with either 100 pM [^{3}H]prazosin or 1 nM [^{3}H]dihydroalprenolol with increasing concentrations of phenylephrine or isoprenaline. Binding of the radioligands were determined as described in *Materials and Methods.* Each point is the mean of a triplicate determination. The curves are the least square fits of the data to the logistic function expressed in Eqn 2.1.

3.2.3 Effect of culture media on adrenergic responses

In the liver, activation of both α- and β-adrenoceptors leads to the same response. However in the adult male rat, the β-effect is barely detectable or absent and the size of the of the β-response is affected by several factors (see section *1.1 Reciprocal Expression of α- and β-Adrenoceptors in Isolated Hepatocytes*). Preliminary experiments with isolated rat hepatocytes in our laboratory indicated that the culture medium used may affect the functioning of the cells. The effect of culture medium on α- and β-response was therefore investigated and results are presented in Figure 3.14. The results indicate that the culture medium used may also affect the size of the β-response in isolated hepatocytes.

The basal glycogen phosphorylase *a* activity was greater ($P < 0.001$) in Williams' E than in Krebs-Henseleit (see Table 3.4). In Krebs-Henseleit (Figure 3.14**a**), phenylephrine (α-agonist) is ≈7-fold ($P < 0.01$) more potent than isoprenaline (β-agonist) — see Table 3.4 for EC_{50}s. Furthermore the maximal response of isoprenaline is 50% of maximal phenylephrine response. In Williams' E, however, the agonists are equipotent ($P > 0.05$) and the E_{max} of isoprenaline is 84% of the phenylephrine response (see Figure 3.14**b**). Thus Williams' E appears to 'unmask' the β-adrenergic response.

The major differences between the two media, in terms of composition, are the amino acids and the vitamins present in Williams' E but not in Krebs-Henseleit (see Appendix B for compositions). We therefore examined the effects of amino acids or vitamins supplement to Krebs-Henseleit. The amino acids added were identical in composition and concentrations to Williams' E. Stock amino acid solution was prepared from the standard compounds and was added to the culture medium at 100-fold the final concentrations (identical to the amino acid fraction of Williams' E). RPMI 1640 vitamin solution (100X) was used as the source of vitamins.

The addition of amino acids to Krebs' buffer significantly increased the basal glycogen phosphorylase *a* activity whereas the addition of vitamins had no effect on the basal activity (see Table 3.4).

Figure 3.14c & d show the results of the effect of amino acids and vitamins on adrenergic responses (see also Table 3.4 for EC_{50} and E_{max} values). Addition of vitamins (RPMI-1640) to Krebs-Henseleit did not cause an increase in the β-response but rather a decrease compared with the β-response in Krebs-Henseleit—E_{max} of isoprenaline was 20% of the maximal phenylephrine response. There was however no significant ($P > 0.05$) change in the EC_{50}. Furthermore, the presence of vitamins in the medium significantly ($P < 0.05$) increased the potency of phenylephrine.

In contrast to the effects of vitamins, the addition of L-amino acids to Krebs-Henseleit caused a dramatic increase in the β-response. This increase was similar to that observed in Williams' E. However, the maximal isoprenaline response was greater (by ≈30%) than the maximal response to phenylephrine. Thus the amino acids fraction plays an important role in 'unmasking' of the β-response.

3.2.3.1 Effect of amino acids

A series of experiments were carried out to identify the amino acid(s) responsible for the increased β-adrenergic response. Due to time limitation, the effects of the individual amino acids in Williams' E were not investigated; Williams' E contains 21 amino acids (see *Appendix B*).

Figure 3.15 shows the effects of the individual amino acids on basal glycogen phosphorylase *a* activity. Arginine, glutamic acid, proline and valine increased the basal activity. In contrast, lysine and methionine decrease the activity whilst glycine and cysteine had no effect.

Results of the effects of individual amino acids on adrenergic responses are presented in Figure 3.14 (arginine, glutamic acid, and proline); Figure 3.15

(glycine, cysteine, and methionine); Figure 3.16 and summarized in Table 3.5. From these results, the amino acids may be placed in of one of three groups. In the first group are glycine, cysteine, and valine; these have little or no effect on α-responses. Furthermore cysteine and lysine appear to wipe out the β-responses (see Table 3.5). The second group of amino acids, arginine, glutamic acid and methionine caused dramatic increases in the affinity (EC_{50}) of isoprenaline without an increase in the efficacy (E_{max}) — see Figure 3.16**a** & **b** for the effects of arginine and glutamic acid. In addition to this effect glutamic acid also decreased the affinity of phenylephrine. Lastly, proline causes an increase in both affinity and efficacy of phenylephrine (Figure 3.16**c**). In the presence of proline, the EC_{50} of isoprenaline was comparable ($P = 0.07$) to that of phenylephrine and the E_{max} was ≈86% of the maximal phenylephrine response. Thus proline can unmask the β-response in hepatocytes though arginine and glutamic acid may contribute to the increased affinity of isoprenaline.

3.2.3.2 α- and β-response in female rat hepatocytes

Earlier reports showed that the size of the β- and α-adrenergic response are almost equal in female rat hepatocytes (Studer & Borle, 1982; Studer & Ganas, 1988). In the present experiments we have examined the effects of phenylephrine and the isoprenaline in isolated hepatocytes from female rats to determine the role of gender in the α- and β-response

Figure 3.19 confirms the earlier observations mentioned above. In Krebs-Henseleit, the E_{max} of phenylephrine was greater (by 46%; $P < 0.001$) than that of isoprenaline; however there was no significant difference between the potencies (EC_{50}) - see Table 3.6. The agonist were equipotent and equiactive in Williams' E. Furthermore, the potencies of the agonist were lower in Williams' E compared to potencies in Krebs-Henseleit. The EC_{50}s of phenylephrine and isoprenaline were not significantly different from values obtained from male

hepatocytes (see Table 3.5 and Table 3.6), although the phenylephrine responses were considerably smaller in the female rat hepatocytes.

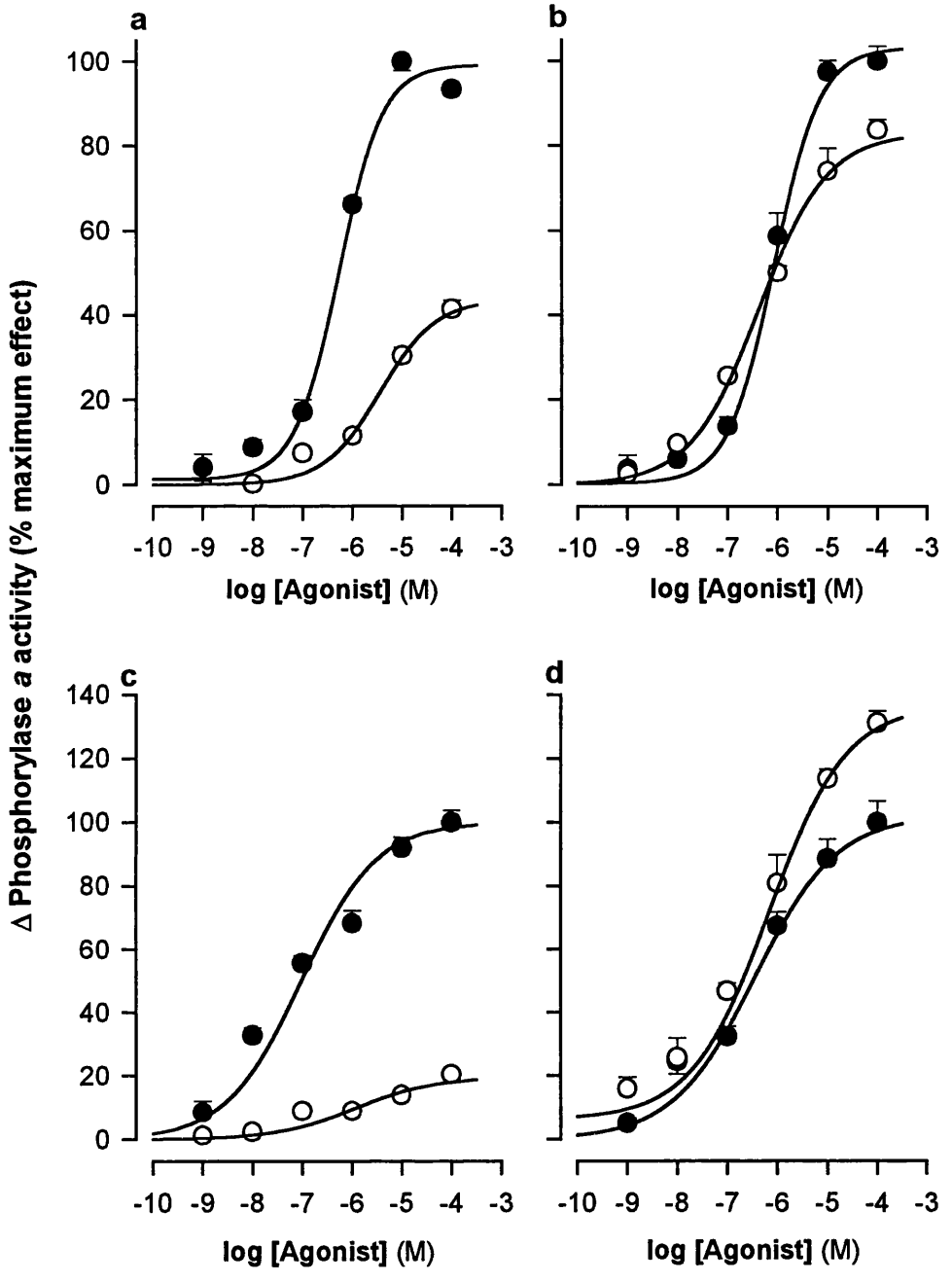


Figure 3.14 Effect of culture medium on adrenergic responses. Hepatocyte suspensions were preincubated for 30 min in Krebs-Henseleit (panel **a**), Williams' E (panel **b**) or in Krebs-Henseleit supplemented with either RPMI-1640 vitamins (panel **c**) or L-amino acids (panel **d**) and then treated for 4 min with increasing concentrations of phenylephrine (●) or isoprenaline (○). Phosphorylase *a* activity was as described in *Materials and Methods*. For comparative purposes, isoprenaline responses have been expressed as % of respective maximal phenylephrine responses. Assays were done in duplicate and each data point represents mean ± s.e. (n=6). The curves are least square fits of the data to the logistic function expressed in Eqn 2.1. Basal activities and individual EC_{50} and E_{max} values are presented in Table 3.4.

Table 3.4 Effect of culture medium on activation of glycogen phosphorylase *a* (GPase *a*) in response to adrenergic agonists.

Medium	Basal GPase *a* activity[a]	EC_{50} (x 10^7 M)		E_{max}[a]	
		Phenylephrine	*Isoprenaline*	*Phenylephrine*	*Isoprenaline*
Krebs-Henseleit	24.43 ± 0.73	5.30 ± 1.17	36.26 ± 7.82†††	42.17 ± 0.88	17.47 ± 0.85†††
+ L-Amino acids	41.19 ± 0.58***	3.55 ± 1.35$^{n.s.}$	6.76 ± 3.08**,‡	16.23 ± 1.08***	21.27 ± 0.59**,††
+ RPMI-1640 vitamins	20.24 ± 1.25$^{n.s.}$	0.95 ± 0.22**	11.63 ± 0.71*,†††	21.98 ± 0.80***	4.50 ± 0.34***,†††
Williams' E	32.98 ± 0.79**	8.42 ± 1.94$^{n.s.}$	6.61 ± 1.77**,‡	40.87 ± 1.42$^{n.s.}$	34.22 ± 0.95***,††

[a]Values are expressed as nmol min^{-1} mg^{-1} protein are means ± s.e. (n = 6). The values were obtained from the experiments shown in Figure 3.14.

EC_{50}s ± s.e. were obtained by least-square nonlinear regression as described in *Materials and Methods.*

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; $^{n.s.}P \geq 0.05$ represent significant differences from respective controls (Student's t test with Bonferroni's correction) and †$P < 0.05$; ††$P < 0.01$; †††$P < 0.001$; ‡$P \geq 0.05$ represent significant differences from corresponding values for phenylephrine.

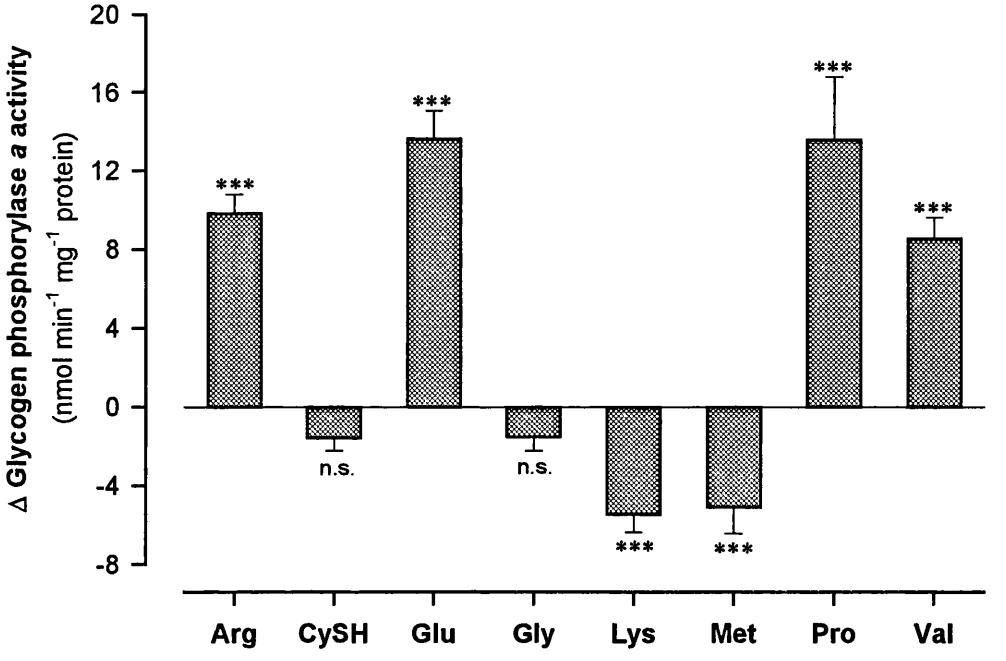


Figure 3.15 Effect of amino acids on glycogen phosphorylase *a* activity in isolated hepatocytes. Values are means ± s.e. (n = 6). ***$P < 0.001$; $^{n.s.}P \geq 0.05$ represent significant differences from respective controls (Student's *t* test with Bonferroni's correction) See Table 3.5 for individual values.

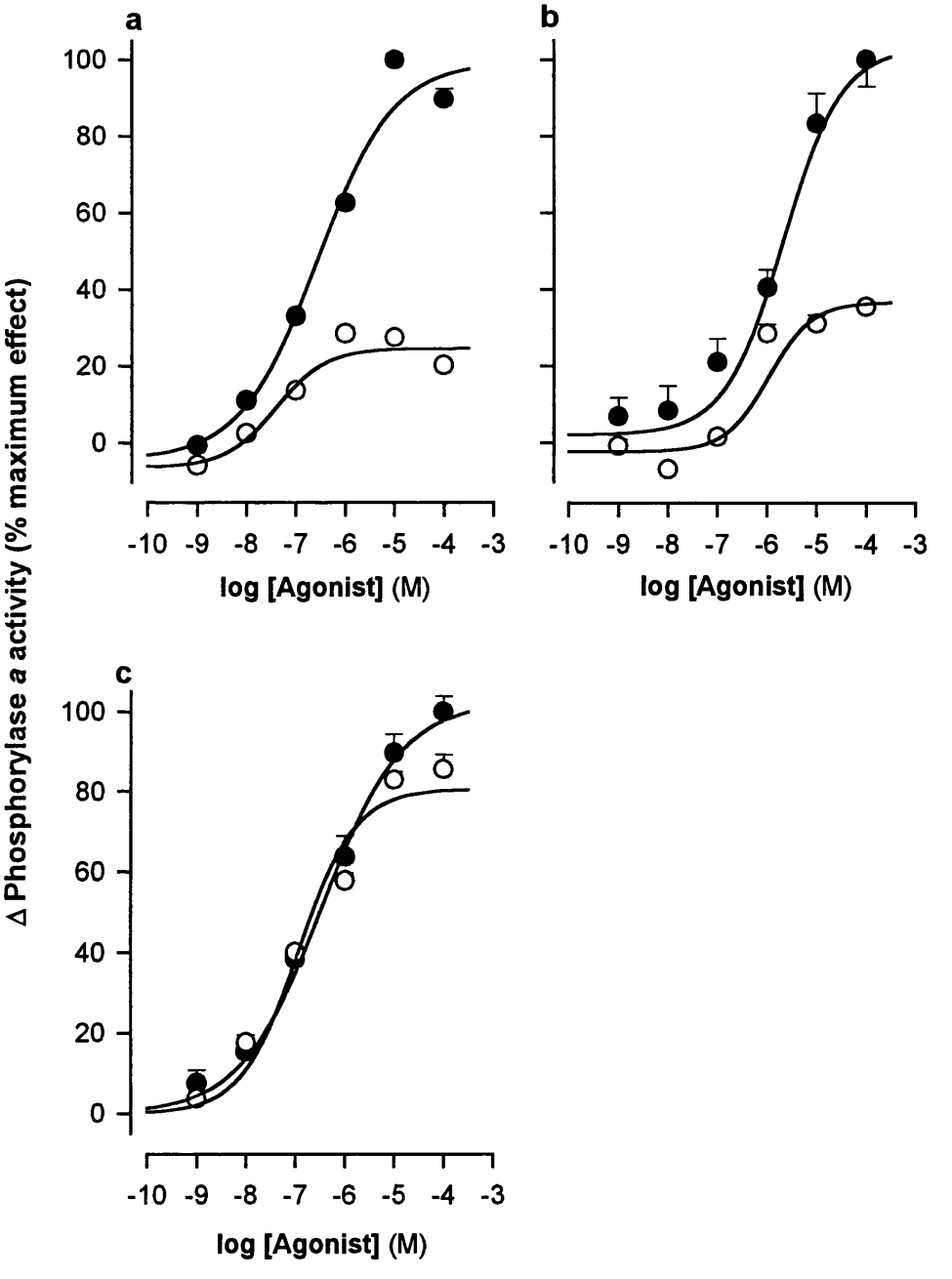


Figure 3.16 Effect of arginine, glutamic acid and proline on adrenergic responses. Hepatocyte suspensions were preincubated for 30 min in Krebs-Henseleit supplemented with L-arginine (50 mg ml^{-1}; panel **a**); L-glutamic acid (45 mg ml^{-1}; panel **b**) or L-proline (30 mg ml^{-1}; panel **c**) and then treated for 4 min with increasing concentrations of phenylephrine (●) or isoprenaline (○). Phosphorylase *a* activity was assayed as described in *Materials and Methods.* For comparative purposes, isoprenaline responses have been expressed as % of respective maximal phenylephrine responses. Assays were done in duplicate and each data point represents the mean ± s.e. (n=6). The curves are least square fits of the data to the logistic function expressed in Eqn 2.1. Basal activities and individual EC_{50} and E_{max} values are presented in Table 3.5.

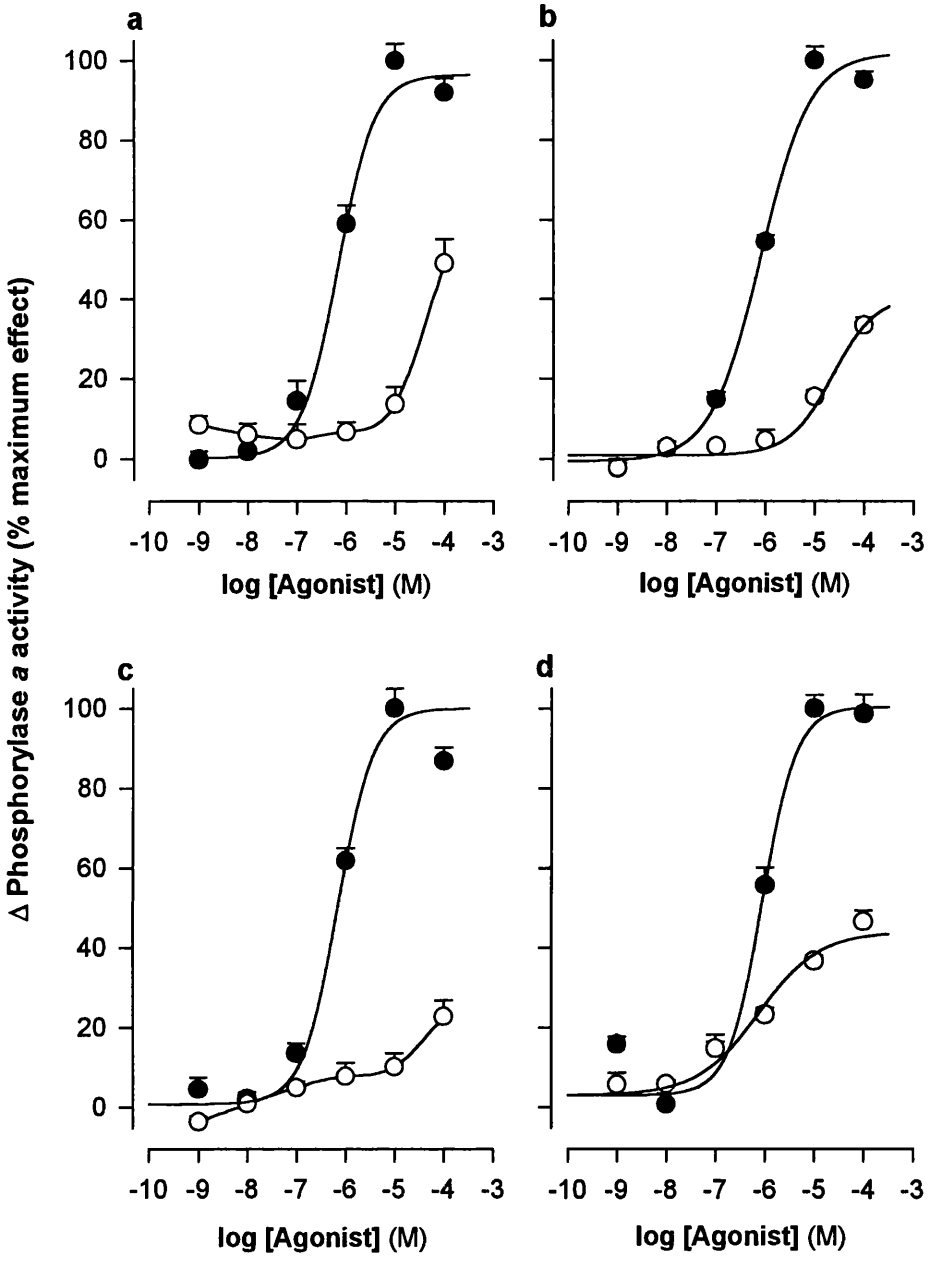


Figure 3.17 Effect of glycine, cysteine and methionine on adrenergic responses. Hepatocyte suspensions were preincubated for 30 min in Krebs-Henseleit (panel **a**) or in Krebs-Henseleit buffer supplemented with glycine (50 mg ml^{-1}; panel **b**), L-cysteine (40 mg ml^{-1}; panel **c**), or L-methionine (30 mg ml^{-1}; panel **d**) and then treated for 4 min with increasing concentrations of phenylephrine (●) or isoprenaline (○). Phosphorylase *a* activity was then assayed as described in Materials and Methods. For comparative purposes, isoprenaline responses have been expressed as % of respective maximal phenylephrine responses. Assays were done in duplicate and each data point represents the mean ± s.e (n=6). The curves are least square fits of the data to the logistic function expressed in Eqn 2.1. Basal activities and individual EC_{50} and E_{max} values are presented in Table 3.5.

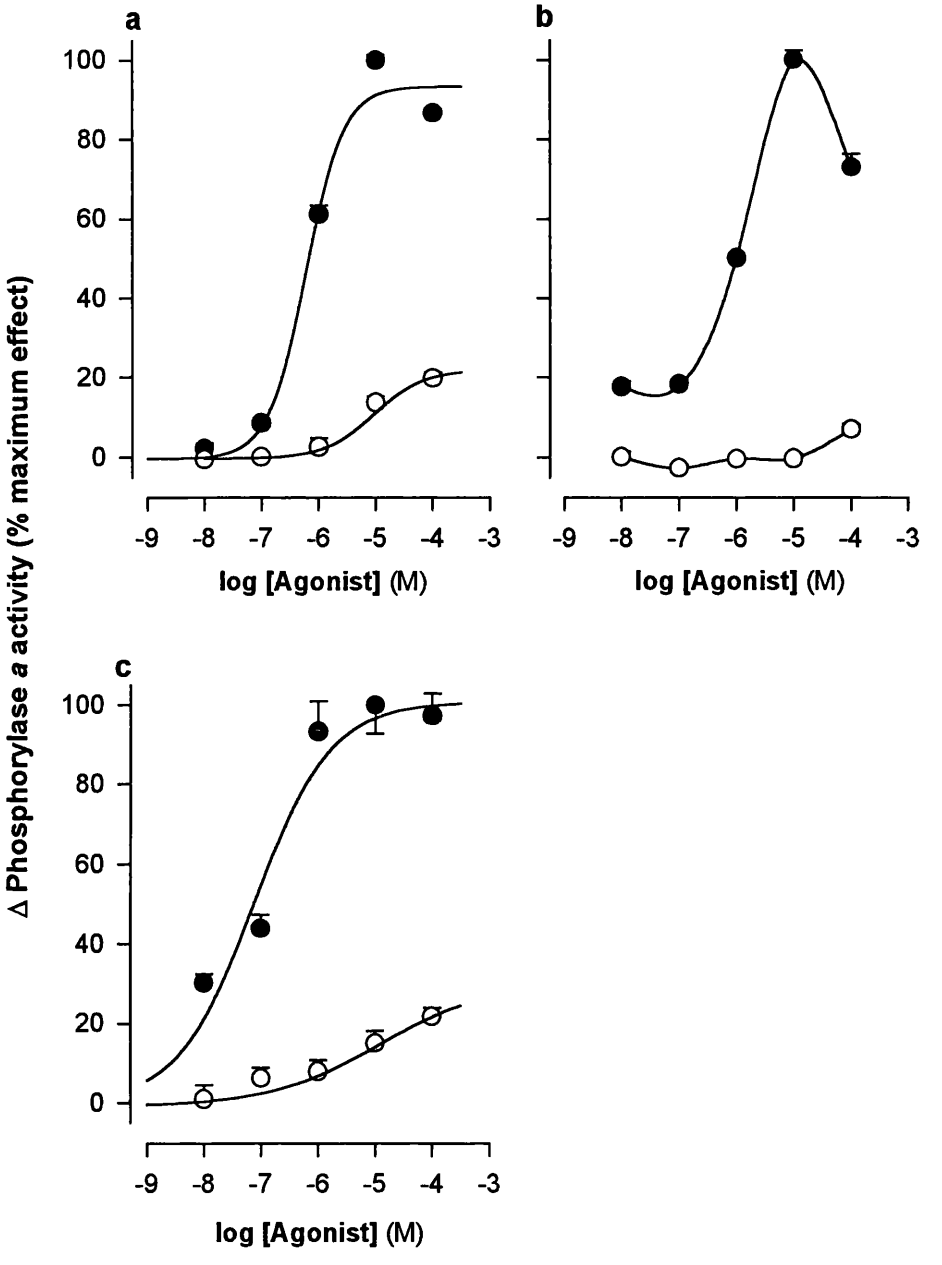


Figure 3.18 Effect of lysine and valine on adrenergic responses. Hepatocyte suspensions were preincubated for 30 min in Krebs-Henseleit buffer or in Krebs-Henseleit supplemented with L-lysine (88 mg ml^{-1}; panel **b**) or L-valine (50 mg ml^{-1}; panel **c**) and then treated for 4 min with increasing concentrations of phenylephrine (●) or isoprenaline (○). Phosphorylase *a* activity was then assayed as described in *Materials and Methods.* For comparative purposes, isoprenaline responses have been expressed as % of respective maximal phenylephrine responses. Assays were done in duplicate and each data point represents the mean ± s.e. (n=6). The curves, except for phenylephrine response in panel **b,** are least square fits of the data to the logistic function expressed in Eqn 2.1. The curve for phenylephrine in panel **b** is the least square fit of the data to Eqn 2.2. Basal activities and individual EC_{50} and E_{max} values are presented in Table 3.5.

Table 3.5 Summary of the effects of amino acids on the activation of glycogen phosphorylase *a* (GPase *a*) induced by adrenergic agonists in isolated rat hepatocytes.

Amino acid	Basal GPase *a* activity[a]	EC_{50} ($\times 10^7$ M)		E_{max}[a]	
		Phenylephrine	*Isoprenaline*	*Phenylephrine*	*Isoprenaline*
Glycine (50 mg l^{-1})[b]	$14.60 \pm 0.43^{n.s.}$	$7.94 \pm 1.35^{n.s.}$	$227.23 \pm 88.38^{***}\dagger$	$38.52 \pm 1.29^{**}$	$12.92 \pm 0.74^{n.s.}\dagger\dagger\dagger$
L-Arginine (50 mg l^{-1})[c]	$34.24 \pm 0.68^{***}$	$2.66 \pm 0.56^{n.s.}$	$0.40 \pm 0.08^{***}\dagger\dagger\dagger$	$59.53 \pm 0.94^{***}$	$16.37 \pm 0.57^{n.s.}\dagger\dagger\dagger$
L-Cysteine (40 mg l^{-1})[b]	$14.53 \pm 0.28^{n.s.}$	$6.95 \pm 1.10^{n.s.}$	n.d.	$35.78 \pm 1.76^{*}$	$8.22 \pm 1.44^{**}\dagger\dagger\dagger$
L-Glutamic acid (45 mg l^{-1})[c]	$38.05 \pm 1.24^{***}$	$21.38 \pm 8.51^{***}$	$10.71 \pm 2.95^{*}\dagger\dagger\dagger$	$53.41 \pm 3.77^{***}$	$18.94 \pm 0.30^{n.s.}\dagger\dagger\dagger$
L-Lysine (88 mg l^{-1})[d]	$11.55 \pm 0.70^{***}$	$14.12 \pm 2.91^{n.s.}$	n.d.	$58.65 \pm 1.33^{n.s.}$	$4.10 \pm 0.75^{***}\dagger\dagger\dagger$
L-Methionine (15 mg l^{-1})[b]	$11.02 \pm 1.21^{***}$	$8.91 \pm 2.02^{n.s.}$	$7.41 \pm 2.51^{***}\ddagger$	$30.46 \pm 1.47^{n.s.}$	$14.38 \pm 0.82^{n.s.}\dagger\dagger\dagger$
L-Proline (30 mg l^{-1})[c]	$37.99 \pm 3.16^{***}$	$3.47 \pm 1.22^{n.s.}$	$1.17 \pm 0.32^{***}\ddagger$	$34.09 \pm 1.27^{*}$	$29.15 \pm 1.25^{***}\dagger\dagger\dagger$
L-Valine (50 mg l^{-1})[d]	$25.55 \pm 0.93^{***}$	$1.07 \pm 0.58^{***}$	>1,000	$43.77 \pm 1.30^{***}$	$9.57 \pm 0.37^{*}\dagger\dagger\dagger$

[a]Values are expressed as nmol min^{-1} mg^{-1} protein and means ± s.e. (n = 6). The values were obtained from the experiments shown in $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{n.s.}P \geq 0.05$ represent significant differences from respective controls (Student's t test with Bonferroni's correction) and $\dagger P < 0.05$; $\dagger\dagger\dagger P < 0.001$; $\ddagger P \geq 0.05$ represent significant differences from corresponding values for phenylephrine.

n.d. denotes not determinable

Values in control cells i.e. Krebs' with no amino acids were as follows:

[b]Basal activity, 16.10 + 0.59. EC_{50} and E_{max} - $6.76 \pm 1.51 \times 10^{-7}$ M & 29.94 ± 1.51 for PHE; > 1000×10^{-7} M & 14.92 ± 1.79 for ISO.

[c]Same as Krebs-Henseleit in Table

[d]Basal activity, 17.01 ± 0.56. EC_{50} and E_{max} $-9.55 \pm 1.04 \times 10^{-7}$ M & 62.23 ± 0.87 for PHE; > 100×10^{-7} M & 12.37 ± 0.95 for ISO.

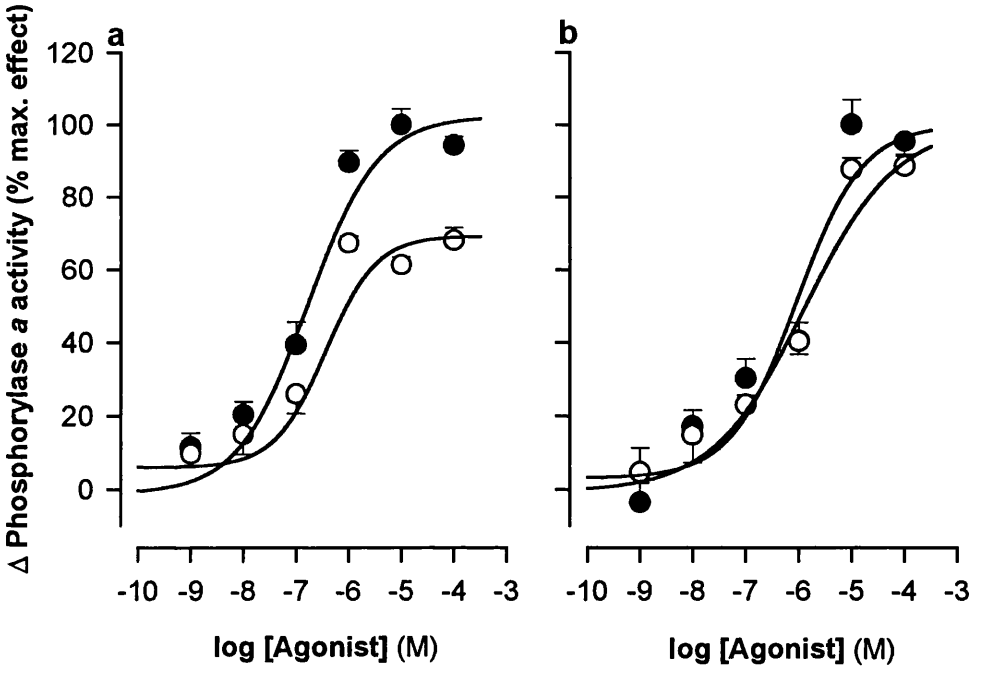


Figure 3.19 Activation of glycogen phosphorylase *a* in response to adrenergic agonists in female rat hepatocytes. Hepatocyte suspensions were preincubated for 30 min in Krebs-Henseleit (panel **a**) or in Williams' E (panel **b**) and then treated for 4 min with increasing concentrations of phenylephrine (●) or isoprenaline (○). Phosphorylase *a* activity was assayed as described in *Materials and Methods*. Assays were done in duplicate and each data point represents are mean ± s.d. (n=6). The curves are least-square fits of the data to the logistic function expressed in Eqn 2.1. Basal glycogen phosphorylase *a* activity was 23.9 ± 0.8 & 34.8 ± 2.5 nmol min^{-1} mg^{-1} protein in Krebs-Henseleit and Williams' E respectively. Individual EC_{50} and E_{max} values are presented in Table 3.6.

Table 3.6 EC_{50} and E_{max} values for the effect of adrenergic agonists in female rat hepatocytes.

Medium	Basal GPase a activity[a]	EC_{50} (x 10^7 M)		E_{max}[a]	
		Phenylephrine	*Isoprenaline*	*Phenylephrine*	*Isoprenaline*
Krebs-Henseleit	23.87 ± 0.31	1.65 ± 0.56	3.63 ± 1.28‡	16.83 ± 0.74	11.47 ± 0.57†††
Williams' E	34.79 ± 1.02***	9.33 ± 2.87***	12.88 ± 4.37*,‡	17.16 ± 1.20[n.s.]	15.21 ± 0.50***,‡

[a]Values are expressed as nmol min^{-1} mg^{-1} protein are means ± s.e. (n = 6). The values were obtained from the experiments shown in Figure 3.19.

EC_{50}s ± s.e. were obtained by least-square nonlinear regression as described in *Materials and Methods.*

*$P < 0.05$; ***$P < 0.001$; [n.s.]$P \geq 0.05$ (not significant) *vs* respective values in Williams' E medium (*t*-test).

†††$P < 0.001$ and ‡$P \geq 0.05$ (not significant) *vs* corresponding values for phenylephrine.

3.3 Time-Dependent Changes in α- and β-Adrenergic Responses

Since the main aim of this project was to investigate the change from a predominantly α to β-adrenergic response in hepatocytes, a phenomenon well documented by several workers (Okajima & Ui, 1982; Refsnes *et al.*, 1983; Christoffersen *et al.*, 1984; Ishac *et al.*, 1992), we set up some experiments to confirm the change under the conditions used in our laboratory.

In the present study, adrenergic responses were measured as: a) activation of glycogen phosphorylase in response to phenylephrine (α-agonist) and isoprenaline (β-agonist) and b) accumulation of cyclic AMP in response to isoprenaline.

3.3.1 Activation of glycogen phosphorylase a

The studies on glycogen phosphorylase *a* were carried out in Krebs-Henseleit buffer (Figure 3.20) and in Williams' E medium (Figure 3.21a & b) and Dulbecco's modified Eagle's medium (Figure 3.21c & d).

Basal glycogen phosphorylase *a* activity in the media was in the order Krebs' << Williams' E < DMEM(see Table 3.7). Furthermore the basal activity in Krebs' significantly ($P < 0.001$) increased with time in contrast to the significant ($P < 0.01$) decreases in Williams' E and DMEM. The higher values of enzyme activity in Williams' E and DMEM may probably be due to the presence of amino acids in the two culture media (see also section *3.2.3.1* ***Effect of amino acids***).

The potency of phenylephrine was the same in the different media at 0 h (see Table 3.7) but the order of potency of isoprenaline in the media at 0 h was Williams' E << DMEM = Krebs'. Also, phenylephrine was ≈4 to 5 fold as potent as isoprenaline in cells kept in Krebs' buffer and DMEM but equipotent with isoprenaline in Williams' E. Although there was no significant ($P > 0.05$) difference between the EC_{50} of isoprenaline in Krebs' buffer and in DMEM, the

maximal isoprenaline response was greater by 4-fold in DMEM. Furthermore, the E_{max} of isoprenaline was comparable ($P > 0.05$) to that of phenylephrine in hepatocytes kept in DMEM.

After 6 h, the concentration-response curve of phenylephrine was shifted significantly ($P < 0.05$) to the right (decreased potency) in all the culture media. However, the rightward shift was greatest in DMEM- a 40-fold shift in comparison to a 2- and 4-fold shift in Krebs' buffer and Williams' E respectively. A decrease in the potency of phenylephrine was accompanied by dramatic increases in the potency of isoprenaline in the Krebs' buffer (60 fold) and DMEM (12 fold). There was a slight but not significant ($P = 0.46$) increase in the EC_{50} of isoprenaline in the cells maintained in Williams' E. Similarly, a decrease in the intrinsic activity (E_{max}) of phenylephrine was accompanied by significant increases in the intrinsic activity of isoprenaline. However, there was no significant difference between the maximal responses of phenylephrine and isoprenaline in DMEM.

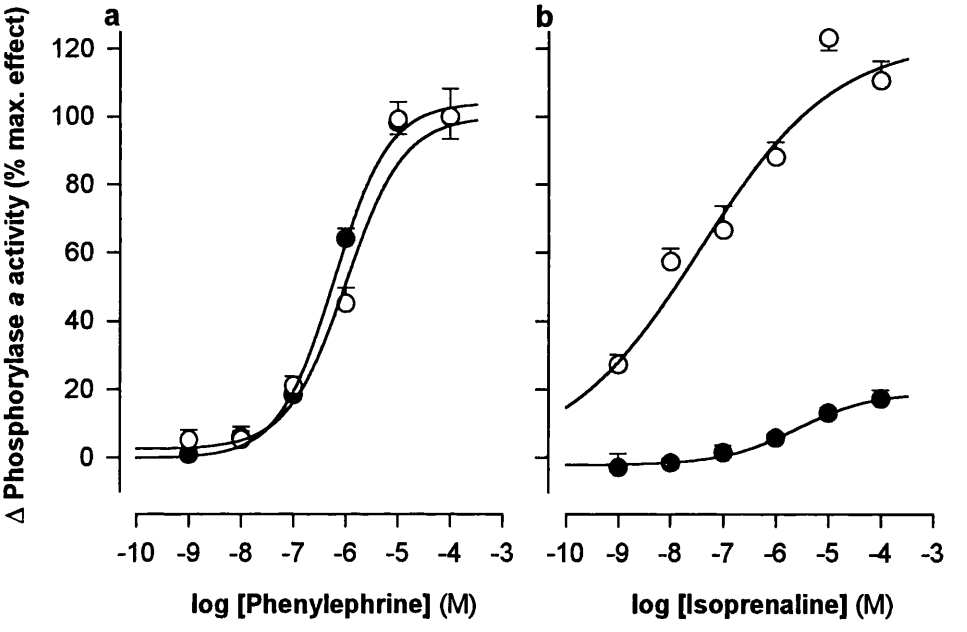


Figure 3.20 Effect of incubation (6 h) of isolated rat hepatocytes on the activation of glycogen phosphorylase in response to phenylephrine (panel **a**) and isoprenaline (panels **b**). Hepatocyte suspensions were preincubated for 30 min in Krebs-Henseleit and then treated for 4 min with increasing concentrations of phenylephrine (panel **a**) or isoprenaline (panel **b**) at 0 h (●) and 6 h (○). Phosphorylase *a* activity was assayed as described in *Materials and Methods*. Assays were done in duplicate and each data point represents the mean ± s.e. (n=6). The curves are least square fits of the data to the logistic function expressed in Eqn 2.1. For comparative purposes, isoprenaline responses have been expressed as % of respective maximal phenylephrine responses. Basal activities, EC_{50} and E_{max} values are presented in Table 3.7.

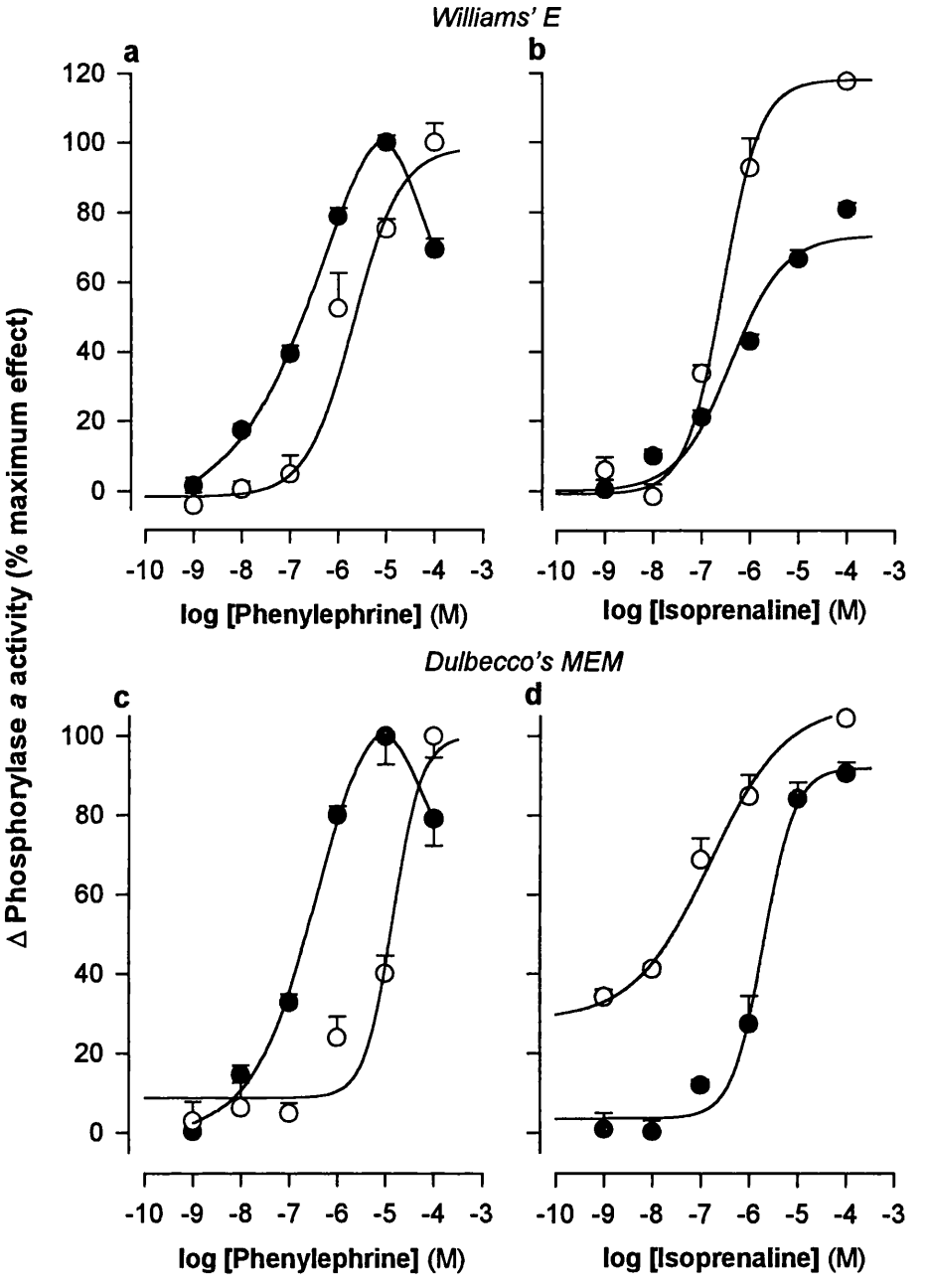


Figure 3.21 Effect of incubation (6 h) of isolated rat hepatocytes on the activation of glycogen phosphorylase in response to phenylephrine (panels **a** & **c**) and isoprenaline (panels **b** & **d**). Hepatocyte suspensions were preincubated for 30 min in either Williams' E (panels **a** & **b**) or Dulbecco's modified Eagle's medium (panels **c** & **d**) and then treated for 4 min with increasing concentrations of phenylephrine or isoprenaline at 0 h (●) and 6 h (○). Phosphorylase *a* activity was assayed as described in *Materials and Methods*. Assays were done in duplicate and each data point represents the mean ± s.e. (n=6). The curves, except for phenylephrine at *0 h* in panels **a** & **c**, are least square fits of the data to the logistic function expressed in Eqn 2.1. The curves for phenylephrine in panel **a** & **c** are the least-square fits of the data to Eqn 2.2. For comparative purposes, isoprenaline responses have been expressed as % of respective maximal phenylephrine responses. Basal activities, EC_{50} and E_{max} values are presented in Table 3.7.

Table 3.7 Summary of time-dependent changes in phenylephrine- and isoprenaline-induced activation of glycogen phosphorylase *a* (GPase *a*) in isolated hepatocytes.

Medium	Basal GPase *a* activity[a]	EC_{50} x 10^7 (M)		E_{max}[a]	
		Phenylephrine	*Isoprenaline*	*Phenylephrine*	*Isoprenaline*
Krebs-Henseleit					
0 h	25.84 ± 0.84	5.88 ± 0.88	25.12 ± 10.81††	38.36 ± 1.27	6.64 ± 0.40†††
6 h	30.72 ± 0.35***	10.00 ± 1.29*	0.42 ± 0.16***†††	18.56 ± 0.51***	20.53 ± 0.44***†
Williams' E					
0 h	37.94 ± 1.14	5.01 ± 1.50	3.99 ± 1.27‡	18.55 ± 0.52	14.98 ± 0.38†††
6 h	23.37 ± 0.76***	20.72 ± 11.09*	2.80 ± 1.03***†††	17.24 ± 0.99[n.s.]	22.20 ± 1.25***†
DMEM					
0 h	45.86 ± 2.06	3.63 ± 0.87	20.00 ± 5.56††	30.13 ± 2.21	27.31 ± 0.88‡
6 h	34.90 ± 1.74**	143.96 ± 28.64***	1.57 ± 0.87***†††	28.42 ± 1.58[n.s.]	30.11 ± 0.60*‡

[a]Values are expressed as nmol min^{-1} mg^{-1} protein are means ± s.e. (n = 6). The values were obtained from the experiments shown in Figure 3.20 and Figure 3.21

EC_{50}s ± s.e. were obtained by least-square nonlinear regression as described in *Materials and Methods.*

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; [n.s.]$P \geq 0.05$ represent significant differences from respective controls (Student's t test with Bonferroni's correction) and †$P < 0.05$; ††$P < 0.01$; †††$P < 0.001$; ‡$P \geq 0.05$ represent significant differences from corresponding values for phenylephrine.

DMEM - Dulbecco's modified Eagle's medium

3.3.2 Cyclic AMP accumulation

In cyclic AMP studies, activation of glycogen phosphorylase was also studied in order to determine the possible link between the two.

Figure 3.22 shows the effect of isoprenaline on cyclic AMP levels in isolated rat hepatocytes. At 0 h, isoprenaline had no effect on cyclic AMP levels in the hepatocytes (Figure 3.22**a**). In contrast, isoprenaline caused significant concentration-dependent increases in cyclic AMP levels in hepatocytes that had been kept in culture for 6 hours (Figure 3.22**b**). Thus the effect of isoprenaline on glycogen phosphorylase at 0 h did not correlate with its actions on cyclic AMP-it caused a concentration-dependent increase in glycogen phosphorylase *a* activity. The effect on glycogen phosphorylase *a* was not expected since isoprenaline is known to have this effect *via* the accumulation of cyclic AMP. At 6 h, the potency of isoprenaline with respect to glycogen phosphorylase activation was increased by ≈40 fold (see Table 3.8).

For comparison, the effects of glucagon (Figure 2.22) and forskolin (Figure 2.23) on cyclic AMP levels and glycogen phosphorylase were investigated with the same batch of cells. Glucagon caused concentration-dependent increases in the level of cAMP paralleled by corresponding increases in glycogen phosphorylase *a* activity. The effects of glucagon were consistent with its reported mechanism of action. Similar results were obtained with forskolin. Forskolin, a diterpene from the plant, *Coleus forskholii*, acts by increasing intracellular cAMP by direct activation of adenylate cyclase in the plasma membrane, thus mimicking the actions of cAMP-induced stimuli (Laurenza *et al.*, 1989; Huang *et al.*, 1982).

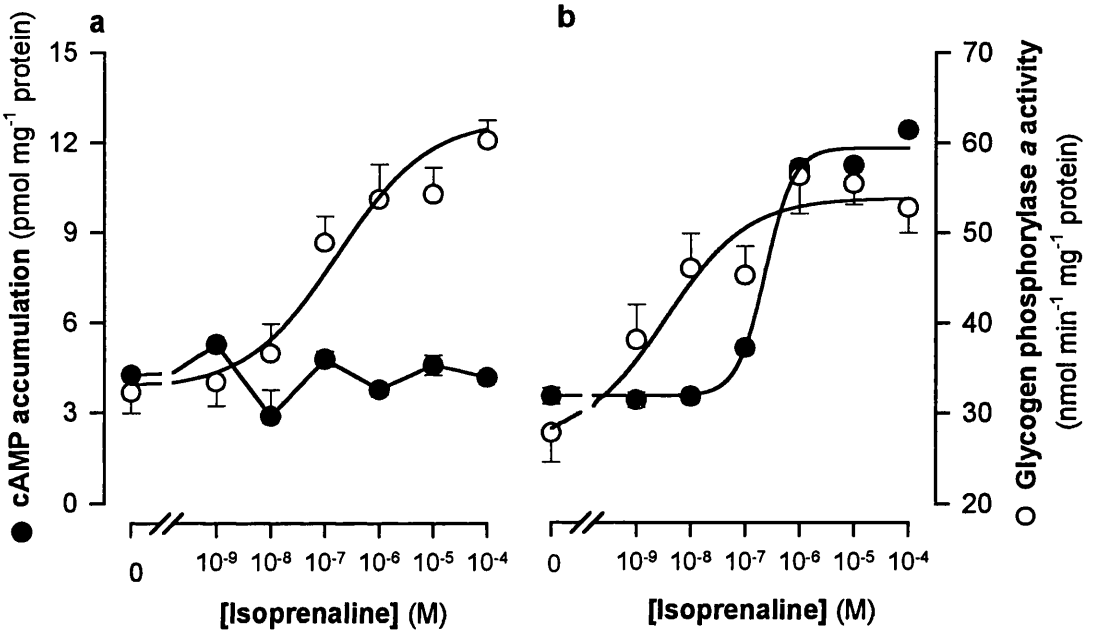


Figure 3.22 Cyclic AMP accumulation and activation of glycogen phosphorylase *a* in response to isoprenaline in freshly isolated and 6-h cultured rat hepatocyte suspensions. Hepatocyte suspensions were preincubated for 30 min in Krebs-Henseleit and then treated for 4 min with increasing concentrations of isoprenaline at 0 h (panel **a**) and 6 h (panel **b**) for the assay of cAMP accumulation (●) or glycogen phosphorylase *a* activity (○) as described in *Materials and Methods*. The curves are least square fits of the data to the logistic function expressed in Eqn 2.1. Each data point represents the mean ± s.e. (n = 3 and 6 for the cAMP and glycogen phosphorylase *a* assays respectively). Basal activities, EC_{50} and E_{max} values are presented in Table 3.8.

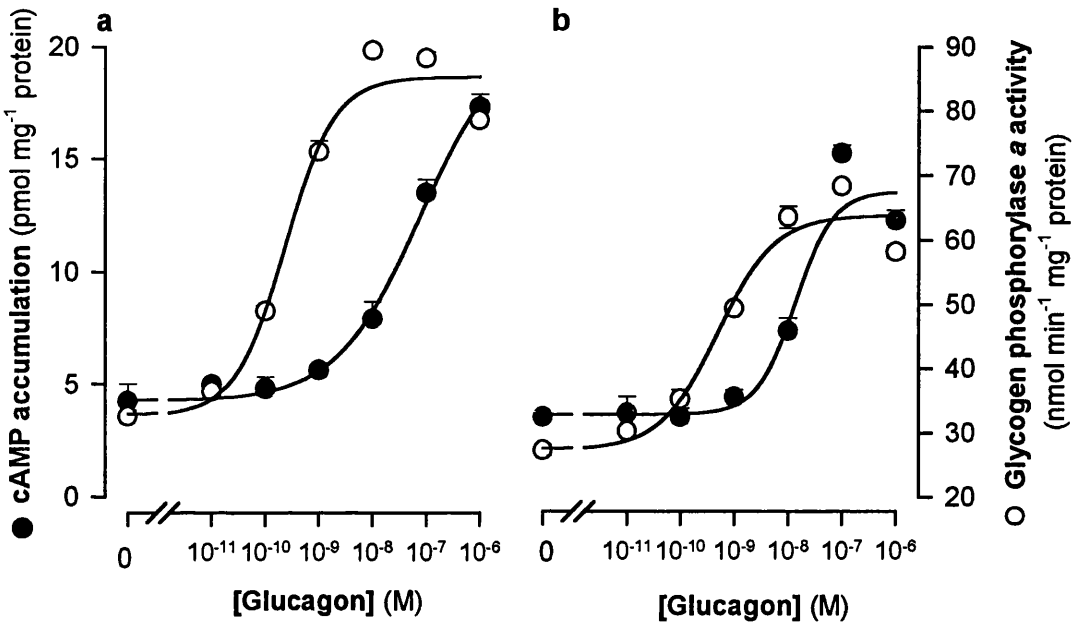


Figure 3.23 Cyclic AMP accumulation and activation of glycogen phosphorylase *a* in response to glucagon in freshly isolated and 6-h cultured rat hepatocyte suspensions. Hepatocyte suspensions were preincubated for 30 min in Krebs-Henseleit and then treated for 4 min with increasing concentrations of glucagon at 0 h (panel **a**) and 6 h (panel **b**) for the assay of cAMP accumulation (●) or glycogen phosphorylase *a* activity (○) as described in *Materials and Methods*. The curves are least square fits of the data to the logistic function expressed in Eqn 2.1. Each data point represents the mean ± s.e. (n = 3 and 6 for the cAMP and glycogen phosphorylase *a* assays respectively). Basal activities, EC_{50} and E_{max} values are presented in Table 3.8.

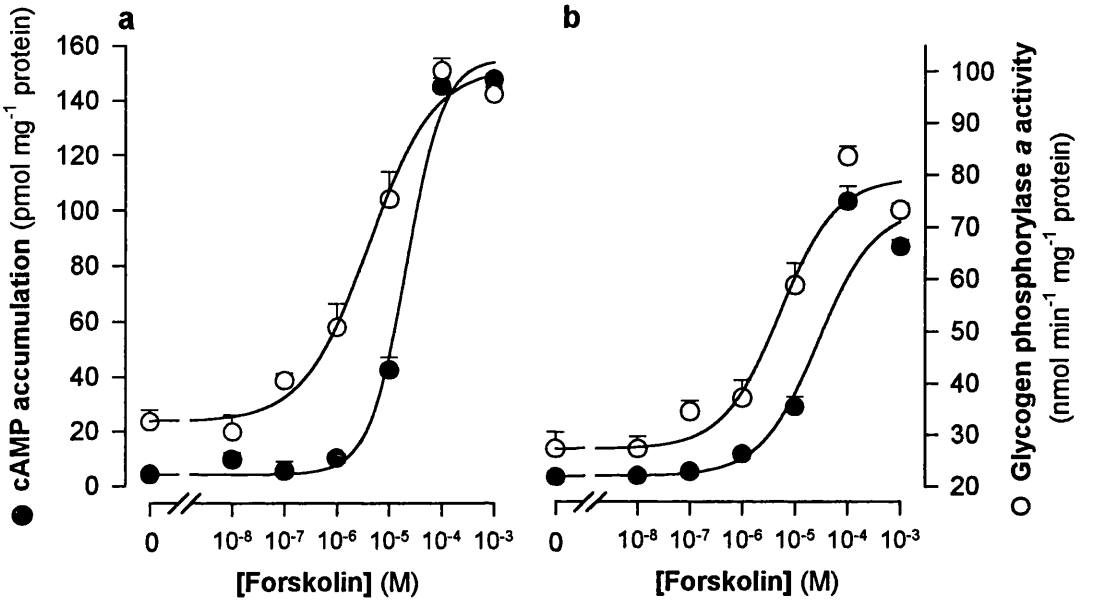


Figure 3.24 Cyclic AMP accumulation and activation of glycogen phosphorylase *a* in response to forskolin in freshly isolated and 6-h cultured rat hepatocyte suspensions. Hepatocyte suspensions were preincubated for 30 min in Krebs-Henseleit and then treated for 4 min with increasing concentrations of forskolin at 0 h (panel **a**) and 6 h (panel **b**) for the assay of cAMP accumulation (●) or glycogen phosphorylase *a* activity (○) as described in *Materials and Methods*. The curves are least square fits of the data to the logistic function expressed in Eqn 2.1. Each data point represents the mean ± s.e. (n = 3 and 6 for the cAMP and glycogen phosphorylase *a* assays respectively). Basal activities, EC_{50} and E_{max} values are presented in Table 3.8.

Table 3.8 Summary of the effects of isoprenaline, glucagon, forskolin, and phenylephrine on the levels of cyclic AMP and glycogen phosphorylase *a* activity in isolated rat hepatocytes.

	Cyclic AMP accumulation		*Phosphorylase* a *activity*	
Agonist	EC_{50} x 10^7*(M)*	E_{max} [a]	EC_{50} x 10^7*(M)*	E_{max} [b]
Isoprenaline				
0 h	n.d.	n.d.	1.69 ± 0.38	62.53 ± 2.10
6 h	2.36 ± 0.45	11.82 ± 0.13	0.04 ± 0.02**	53.85 ± 1.17**
Glugacon				
0 h	0.13 ± 0.08	14.58 ± 1.46	0.002 ± 0.001	85.26 ± 3.04
6 h	0.66 ± 0.08**	19.82 ± 2.65[n.s.]	0.005 ± 0.001*	70.30 ± 1.65**
Forskolin				
0 h	200.31 ± 57.19	155.32 ± 13.59	39.95 ± 1.41	100.72 ± 2.22
6 h	266.77 ± 88.50[n.s.]	100.99 ± 8.23**	49.80 ± 2.25**	79.33 ± 2.52***
Phenylephrine				
0 h	N.A.	N.A.	0.74 ± 0.36	69.77 ± 2.37
6 h	N.A.	N.A.	8.57 ± 1.58***	20.54 ± 1.12***

[a]Values are expressed as pmol mg^{-1} protein are means ± s.e. (n = 3). The values were obtained from the experiments shown in Figure 3.22, Figure 3.23 and Figure 3.24

[b]Values are expressed as nmol min^{-1} mg^{-1} protein are means ± s.e. (n = 6).

n.d. = not determinable; N.A. = not assayed.

EC_{50}s ± s.e. were obtained by least-square nonlinear regression as described in *Materials and Methods.*

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; [n.s.]$P \geq 0.05$ represent significant differences from respective values at 0 h (Student's *t* test).

3.3.3 Effect of dimethyl sulfoxide and sodium butyrate on the time-dependent changes in α- and β-adrenergic responses

In these experiments, DMSO and sodium butyrate were added to the culture in a bid to prevent the reciprocal changes in α- and β-adrenergic activation of glycogen phosphorylase in the adult male hepatocytes.

Dimethyl sulfoxide and the short-chain fatty acid, *n*-butyrate, were chosen because they have been used with some success as differentiating agents in hepatocytes and some cell lines (Kim *et al.*, 1980; Souleimani & Asselin, 1986; Staecker *et al.*, 1988; Padgham *et al.*, 1992).

3.3.3.1 Effect of DMSO and sodium butyrate in hepatocyte suspensions

Basal glycogen phosphorylase a activities

Figure 3.25 shows the effect of DMSO and sodium butyrate on the basal activities of glycogen phosphorylase *a*. These agents decreased glycogen phosphorylase *a* activity in freshly isolated cells, but in contrast, increased the activity in 6-h old cultures. This finding again emphasizes the difference between the response of isolated rat hepatocytes at the two time points.

Figure 3.26 shows the effect of 2% DMSO and 2 mM sodium butyrate on the activity of glycogen phosphorylase *a* in response to phenylephrine and isoprenaline in isolated hepatocyte suspension.

0 hour

At 0 hour (Figure 3.26**a** & **b**), both agents displaced the concentration-response curve of phenylephrine significantly to the right. EC_{50} for phenylephrine was decreased by 15-fold and 5-fold by DMSO and butyrate respectively (see Table 3.9). In contrast, the isoprenaline curves were shifted to the left by the addition of DMSO and sodium butyrate to the medium. Furthermore, the potency of the isoprenaline was increased 500-fold by DMSO

($P < 0.001$) with no significant increase in the maximal response. Butyrate, however had no effect on the concentration-response curve of isoprenaline.

6 hour

Control: After six hours, the EC_{50} of phenylephrine significantly decreased by 50%, while the EC_{50} of isoprenaline was dramatically increased by 100-fold. See Figure 3.26**c** & **d** and Table 3.9. Furthermore, the maximal isoprenaline effect increased by 3-fold ($P < 0.001$) whilst the E_{max} for phenylephrine decreased by a third, thus making isoprenaline a more effective agonist than phenylephrine in the six-hour hepatocyte suspension.

DMSO: Addition of 2% DMSO to the culture medium did not prevent the increase in E_{max} of isoprenaline with time. Isoprenaline and phenylephrine were equipotent (similar EC_{50} values), though isoprenaline was more effective (greater E_{max}) in the 6-hour old hepatocyte suspensions. The maximum responses to both agonists were increased significantly ($P < 0.001$ *vs.* control values) by DMS0 after six hours in culture.

Sodium butyrate: As with DMSO, sodium butyrate did not arrest the increased efficacy of isoprenaline in the 6-hour old cultures. However the E_{max} of isoprenaline in the presence of butyrate was similar ($P < 0.001$) to that of phenylephrine.

Similar results were obtained for DMSO in a preliminary experiment (Woode & Skett, 1994). Experiments were carried out in Krebs-Henseleit, Williams' E and DMEM.

Summary

The addition of the differentiating agents, DMSO and sodium butyrate to culture medium does not prevent the emergence and predominance of the β-adrenoceptor response in the isolated hepatocytes from the adult male rat but rather enhance the process.

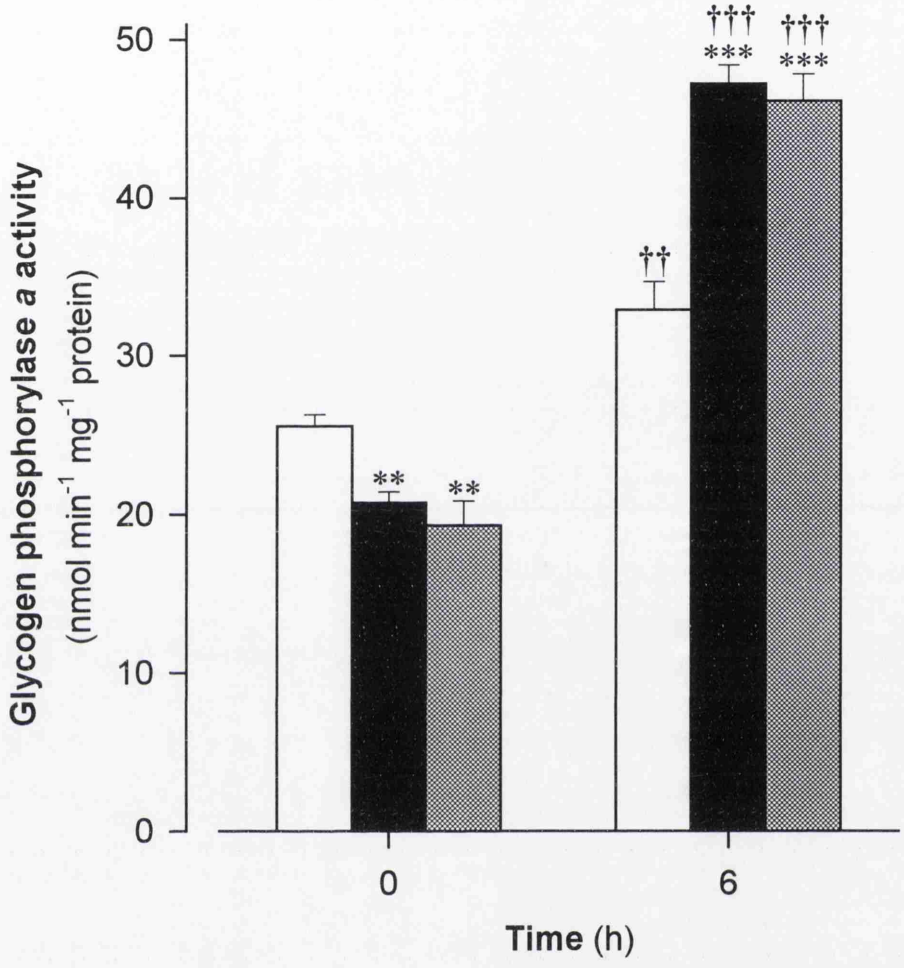


Figure 3.25 Effect of DMSO (solid columns) and sodium butyrate(cross-hatched columns) on glycogen phosphorylase *a* in hepatocyte suspensions. Results represent means ± s.e. (n=6). $^{**}P < 0.01$; $^{***}P < 0.001$ compared to control values (open columns) of the group (determined by ANOVA followed by Student's t test with Bonferroni's adjustment). $\dagger\dagger P < 0.01$ and $\dagger\dagger\dagger P < 0.001$ compared to control value at 0 h.

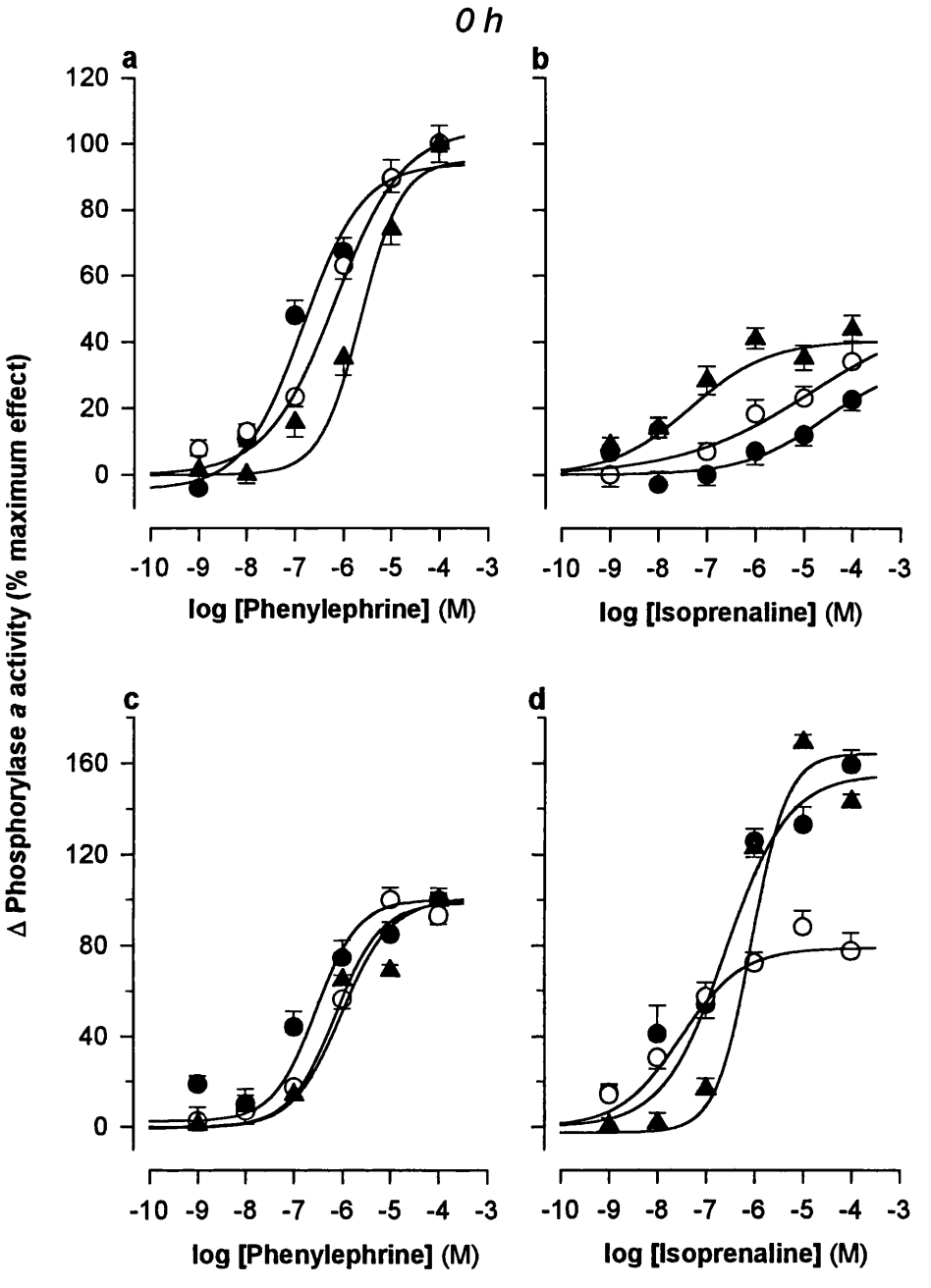


Figure 3.26 Effect of dimethyl sulfoxide (DMSO) and sodium butyrate on the time-dependent changes in phenylephrine- and isoprenaline-induced increases in glycogen phosphorylase *a* activity in isolated hepatocyte suspensions. Hepatocyte suspensions were preincubated in Williams' E medium without (●) and with 2% DMSO (▲) or 2 mM sodium butyrate (○) for 30 min and then treated for 4 min with increasing concentrations of phenylephrine or isoprenaline at 0 h (panel **a** & **b**) and at 6 h (panel **c** & **d**). Phosphorylase *a* activity was assayed as described in *Materials and Methods*. Assays were done in duplicate and each data point represents the mean ± s.e. (n=6). The curves are least square fits of the data to the logistic function expressed in Eqn 2.1. For comparative purposes, isoprenaline responses have been expressed as % of respective maximal phenylephrine responses. Basal activities, EC_{50} and E_{max} values are presented in Table 3.9.

Table 3.9 Effect of dimethyl sulfoxide (DMSO) and sodium butyrate on phenylephrine- and isoprenaline-induced activation of glycogen phosphorylase *a* (GPase *a*) in isolated rat hepatocytes.

Additions	Basal GPase *a* activity[a]	EC_{50} x 10^7 *(M)*		E_{max}[a]	
		Phenylephrine	*Isoprenaline*	*Phenylephrine*	*Isoprenaline*
0 h					
None	25.55 ± 0.72	1.44 ± 0.42	263.03 ± 150.15 n.s.,‡	31.01 ± 1.77	7.01 ± 1.07†††
DMSO (2%)	20.72 ± 0.70**	22.91 ± 8.12***	0.46 ± 0.22*,†††	23.38 ± 0.58**	10.22 ± 0.98 n.s.,†††
Butyrate (2 mM)	19.32 ± 1.55**	6.61 ± 2.34*	107.15 ± 100.24 n.s.,‡	25.86 ± 1.43 n.s.	8.8 ± 1.57 n.s.,†††
6 h					
None	32.90 ± 1.78	2.82 ± 0.82	2.51 ± 0.99 n.s.,‡	14.31 ± 0.44	22.81 ± 0.91†††
DMSO (2%)	47.22 ± 1.20***	9.33 ± 2.25*	8.71 ± 1.63 n.s.,‡	19.84 ± 0.97***	28.45 ± 0.63***,†††
Butyrate (2 mM)	46.13 ± 1.69***	7.08 ± 2.29 n.s.	0.30 ± 0.11**,†††	13.62 ± 0.56 n.s.	11.3 ± 1.16***,‡

[a]Values are expressed as nmol min^{-1} mg^{-1} protein are means ± s.e. (n = 6). The values were obtained from the experiments shown in Figure 3.26.

EC_{50}s ± s.e. were obtained by least-square nonlinear regression as described in *Materials and Methods.*

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; n.s. $P \geq 0.05$ represent significant differences from respective controls (Student's t test with Bonferroni's correction) and †$P < 0.01$; ††$P < 0.001$; †††$P < 0.001$; ‡$P \geq 0.05$ represent significant differences from corresponding values for phenylephrine.

3.3.3.2 Effect of DMSO and sodium butyrate in monolayer cultures

Basal phosphorylase activity of glycogen phosphorylase *a* activity decreased dramatically to 28% of initial values for the control cells and 16% for the butyrate-treated cells after 48 hours (see Figure 3.27 and Table 3.10). Dimethyl sulfoxide-treatment, however seemed to prevent the loss of enzyme activity -values fall to 50% at 24 h and increase significantly ($P < 0.001$) to 75% at 48 h. Decreases in glycogen phosphorylase *a* was accompanied by corresponding decreases in the total glycogen phosphorylase activity- from 108.5 ± 2.3 nmol min^{-1} mg^{-1} protein at 0 h to 75.2 ± 1.5 and 33.6 ± 2.1 nmol min^{-1} mg^{-1} protein at 24 h and 48 h respectively. Similar reports of decrease in phosphorylase activity have been reported by other workers (Christoffersen, 1984; Kauffman et al., 1990). The decline in glycogen phosphorylase *a* activity is similar to changes in activities of other enzymes, notably the cytochrome P450 system, in hepatocytes maintained in primary culture (Bequé *et al.;* 1984; Isom *et al.,* 1985; Kuri-Harcuch & Mendoza-Figueroa, 1989; Saad *et al.,* 1993).

Figure 3.28 and Table 3.10 show the results of the effect of dimethyl sulfoxide and sodium butyrate on the activation of glycogen phosphorylase in short-term monolayer cultures of isolated hepatocytes.

Isoprenaline was as potent as phenylephrine (Table 3.10; $P < 0.05$) at 0 h. This was consistent with the effect of isoprenaline in Williams' E. The E_{max} was however, ≈50% of the maximal phenylephrine response. The effects of DMSO and butyrate were not measured at 0 h -they were assumed to be similar to the effects in hepatocyte suspensions (see previous section).

The addition of 2% DMSO and 2 mM sodium butyrate caused significant ($P < 0.001$) decreases in the potency of isoprenaline to undetectable levels at 48 h. In contrast, the EC_{50} of isoprenaline increased significantly ($P < 0.001$) with time in the control cultures, with isoprenaline being most potent in 48-hour old cultures ($P < 0.05$; 24 h *vs.* 48 h).

In DMSO-treated cultures, isoprenaline had inhibitory effects on glycogen phosphorylase *a* activity. This effect was not anticipated since there are no reports of isoprenaline having this effect (Refsnes *et al.*, 1983; Kunos & Ishac, 1985; Exton, 1985; Ruffolo *et al.*, 1991).

The EC_{50} of phenylephrine decreased significantly ($P < 0.001$) to 25% of initial value within 24 hours in the control cells and remained constant over the next 24 h. Similar values were obtained for DMSO-treated cells. In the butyrate-treated cells, however, the EC_{50} was decreased by a much smaller but significant ($P < 0.001$) amount (30%) during the first 24 hour. EC_{50} values in the latter cells were comparable to the other treatment groups at 48 h.

In control cells, there was a progressive decrease in the maximal phenylephrine response. Responses to isoprenaline, however, did not change ($P < 0.05$) at 24 h and was greater than the phenylephrine responses (Table 3.10). DMSO-treatment maintained the maximal responses to both agonist at 24 h and the E_{max} of phenylephrine was significantly ($P < 0.05$) greater than for isoprenaline responses at all times. The maximal responses to both agonists progressively decreased in butyrate-treated cells.

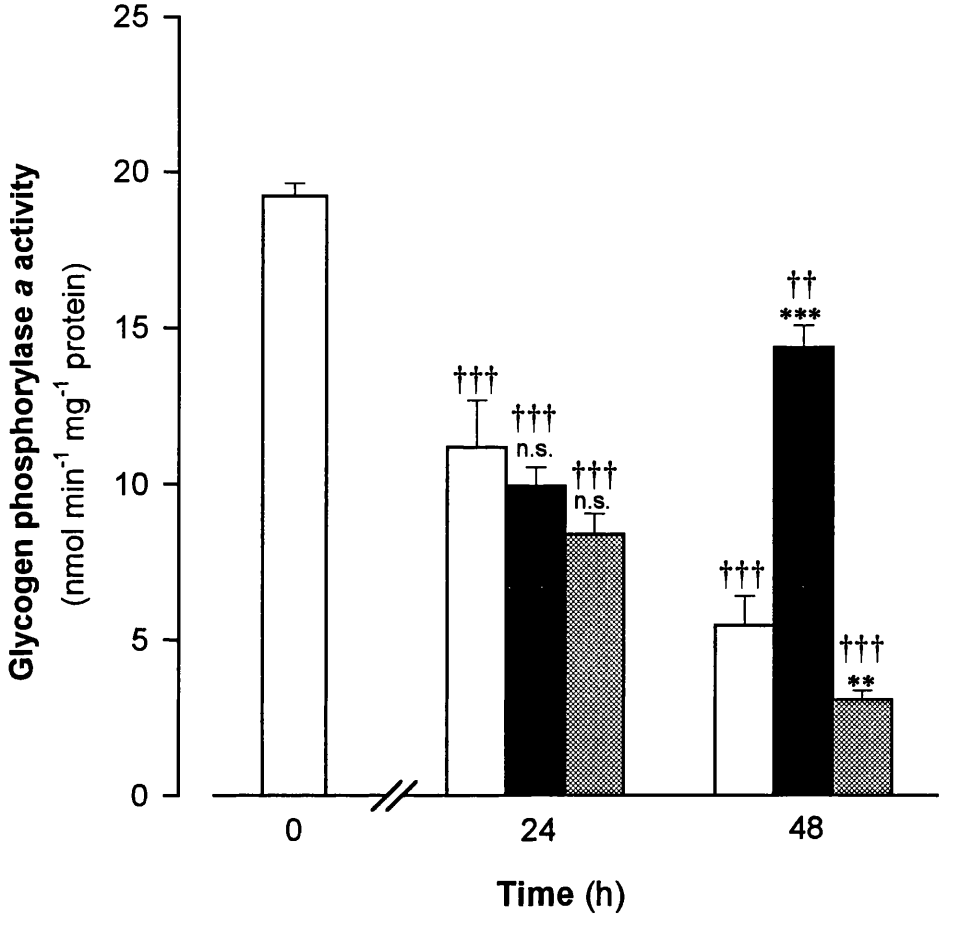


Figure 3.27 Effect of DMSO (solid columns) and sodium butyrate (cross-hatched columns) on glycogen phosphorylase *a* activity in short-term monolayer cultures of isolated hepatocytes. Results represent means ± s.e. (n=6). $^{n.s.}P > 0.05$ (not significant);$^{**}P < 0.01$; $^{***}P < 0.001$ compared to control value of the group and $\dagger\dagger P < 0.01$; $\dagger\dagger\dagger P < 0.001$ compared to control activity at 0 h (determined by ANOVA followed by Students' *t* test with Bonferroni's correction).

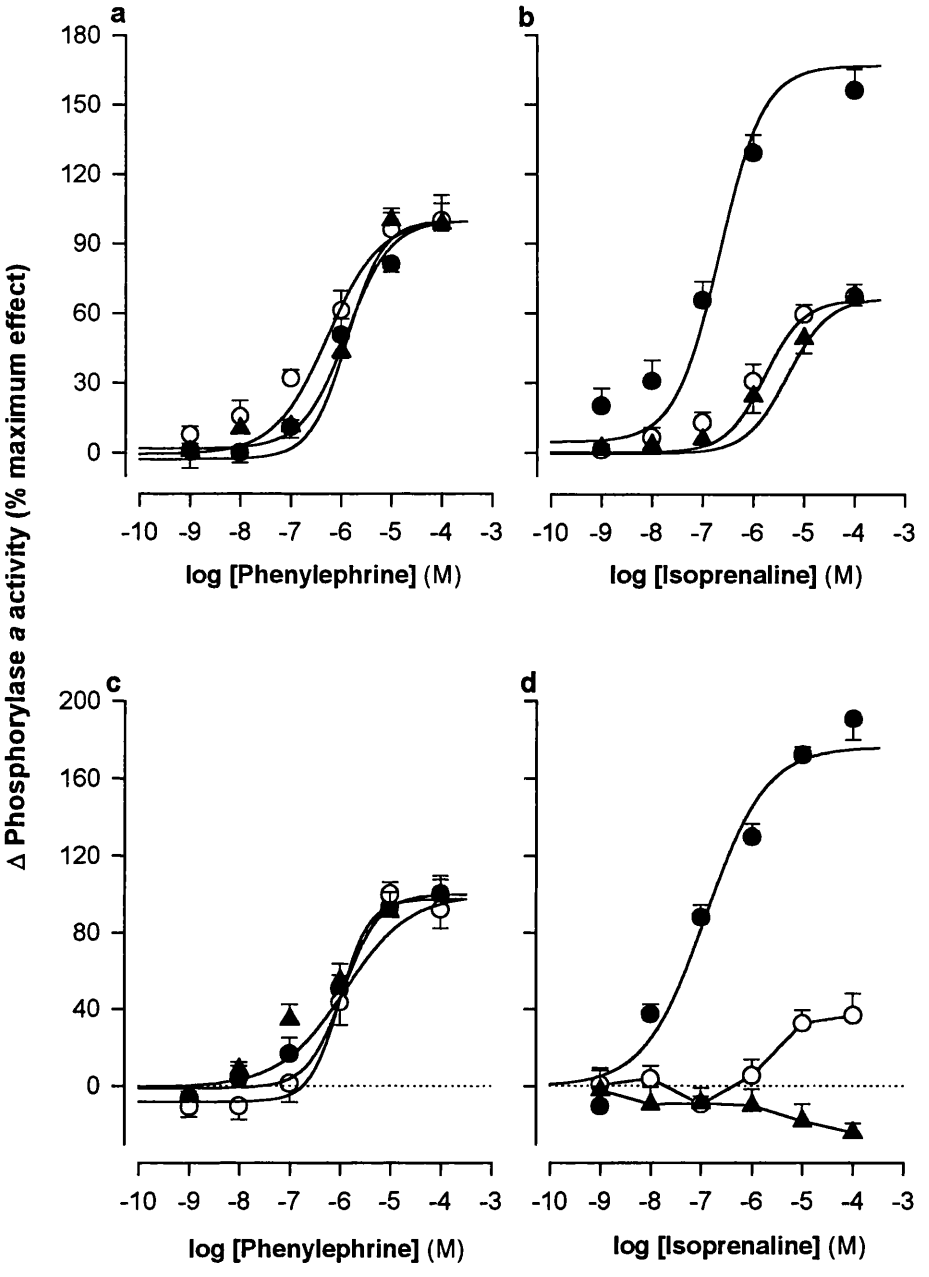


Figure 3.28 Effect of dimethyl sulfoxide (DMSO) and sodium butyrate on the time-dependent changes in phenylephrine- and isoprenaline-induced increases in glycogen phosphorylase *a* activity in monolayer cultures of rat hepatocytes. Hepatocyte monolayers were cultured in Williams' E medium without (●) and with 2% DMSO (▲) or 2 mM sodium butyrate (○). After 24 h (panels **a** & **b**) and 48 h (panels **c** & **d**) in culture, the cells were treated for 4 min with increasing concentrations of phenylephrine (panels **a** and **c**) or isoprenaline (panels **b** and **d**). Phosphorylase *a* activity was assayed as described in *Materials and Methods*. Assays were done in duplicate and each data point represents the mean ± s.e. (n=6). The curves are least square fits of the data to the logistic function expressed in Eqn 2.1. For comparative purposes, isoprenaline responses have been expressed as % of respective maximal phenylephrine responses. Basal activities, EC_{50} and E_{max} values are presented in Table 3.10.

Table 3.10 Effect of DMSO and sodium butyrate on phenylephrine- and isoprenaline-induced activation of glycogen phosphorylase *a* in primary monolayer cultures of isolated rat hepatocytes.

Addition	Basal GPase *a* activity[a]	EC_{50} x 10^7 *(M)*		E_{max}[a]	
		Phenylephrine	*Isoprenaline*	*Phenylephrine*	*Isoprenaline*
0 h					
None	19.22 ± 0.40	3.02 ± 0.73	6.03 ± 1.75‡	20.54 ± 0.83	11.23 ± 1.04†††
24 h					
None	11.17± 1.50***	13.18 ± 2.95***	2.13 ± 0.69*,†††	5.30 ± 0.58***,‡	10.07 ± 0.17$^{n.s.}$,†††
DMSO (2%)	9.93 ± 0.61***	12.88 ± 2.41***	47.86 ± 20.32***,†	18.90 ± 0.97$^{n.s.}$	12.81 ± 0.88$^{n.s.}$,†††
Butyrate (2 mM)	8.39 ± 0.67***	5.37 ± 1.57$^{n.s.}$	16.98 ± 6.27 $^{n.s.}$,†	3.75 ± 0.27***,‡	2.52 ± 0.19***,††
48 h					
None	5.48 ± 0.95***	11.74 ± 2.83**	1.20 ± 0.37***,†††	3.98 ± 0.36***,‡	7.59 ± 0.44*,†††
DMSO (2%)	14.39 ± 0.70**	12.59 ± 4.27*	n.d.	3.61 ± 0.26***,‡	-0.86 ± 0.16***,†††
Butyrate (2 mM)	3.09 ± 0.29***	10.00 ± 2.23**	n.d.	3.80 ± 0.23***,‡	1.39 ± 0.43***,†††

[a]Values are expressed as nmol min^{-1} mg^{-1} protein are means ± s.e. (n = 6). The values were obtained from the experiments shown in Figure 3.28..

EC_{50}s ± s.e. were obtained by least-square nonlinear regression as described in *Materials and Methods.*

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; $^{n.s.}P \geq 0.05$ represent significant differences from respective controls (Student's t test with Bonferroni's correction) and †$P < 0.01$; ††$P < 0.001$; †††$P < 0.001$; ‡$P \geq 0.05$ represent significant differences from corresponding values for phenylephrine.

3.3.4 Effect of dexamethasone on the time-dependent changes in adrenergic responses in hepatocytes

Glucocorticoid insufficiency is one of several physiological conditions leading to a switch from a predominantly α- to a β-adrenergic response in the liver of adult male rats (Brønstad & Christoffersen, 1980; Goodhardt *et al.*, 1982; Christoffersen *et al.*, 1984; Borle & Studer, 1990). Reciprocal changes in the adrenergic responses are reversible by glucocorticoid replacement (Goodhardt *et al.*, 1982). However, dexamethasone supplementation to culture medium has been reported to be ineffective in arresting the reciprocal change in hepatocyte cultures (Christoffersen *et al.*, 1984; Kunos & Ishac, 1985). Results presented in this section are from experiments we performed to reexamine the effect of dexamethasone, a glucocorticoid which has been used successfully to maintain differentiated functions of hepatocytes in our lab (Khan *et al.*, 1992) and elsewhere (Laishes & Williams, 1976; Saad *et al.*, 1993).

3.3.4.1 Effect of dexamethasone on adrenergic responses in hepatocyte suspensions

The presence of dexamethasone in the culture medium increased, dose-dependently, the basal glycogen phosphorylase *a* activity (Table 3.11). The enzyme activity was decreased significantly ($P < 0.001$) and maintained over the six hour period in control cultures and in cultures with the higher concentration (1.0 μM) of dexamethasone. In contrast, the basal activity remained the same in cultures containing 0.1 μM dexamethasone.

At 0 h, the presence of 0.1 μM dexamethasone in the culture medium caused a significant ($P < 0.01$) displacement of the concentration-response curve of phenylephrine to the left (i.e. increased potency) of the control curve and significantly increased ($P < 0.001$) the maximal phenylephrine response (see Figure 3.29a and Table 3.11). The presence of 1 μM dexamethasone had no effect on the curve of phenylephrine but decreased the maximal response to

phenylephrine. In contrast to these effects on phenylephrine, dexamethasone decreased the maximal isoprenaline response in a concentration-dependent manner (Figure 3.29**b** and Table 3.11). Thus, the presence of 0.1 μM dexamethasone in the culture medium increased both the potency and efficacy of phenylephrine at the expense of isoprenaline.

After six hours in culture, the potency of phenylephrine was decreased by $\approx$2 fold ($P < 0.05$) in control cells but increased by $\approx$5 fold in cultures supplemented with 1 μM dexamethasone and remained unchanged in cultures with 0.1 μM dexamethasone (see Figure 3.29**c** and Table 3.11). However, the maximal responses were decreased by dexamethasone (compared to control cultures). In contrast there was a dramatic increases in the potency of isoprenaline with time in both control and dexamethasone-treated cells (see Figure 3.29**d** and Table 3.11). Furthermore, dexamethasone caused dramatic increases in maximal responses to isoprenaline at 6 h in comparison to a decrease in control cultures. Also at 6 h, The maximal response to isoprenaline was significantly lower (by 3 fold) than the maximal response to phenylephrine, though the potency was greater than phenylephrine..

In some of the cultures, dexamethasone was added at 5½ h and then the activation of glycogen phosphorylase a determined at 6 h (Figure 3.30). In these cells, dexamethasone (0.1 μM) had no effect on potency of phenylephrine in comparison to the effect of phenylephrine in control cells at 6 h (Figure 3.29**d** and Figure 3.30**a**), while the higher concentration of 1 μM dexamethasone increased (decreased EC_{50}) the potency of phenylephrine (see Table 3.11). Furthermore, the presence of dexamethasone failed to increase the maximal response of isoprenaline above values for phenylephrine as was observed in cultures which had dexamethasone throughout the incubation (Figure 3.29**d**).

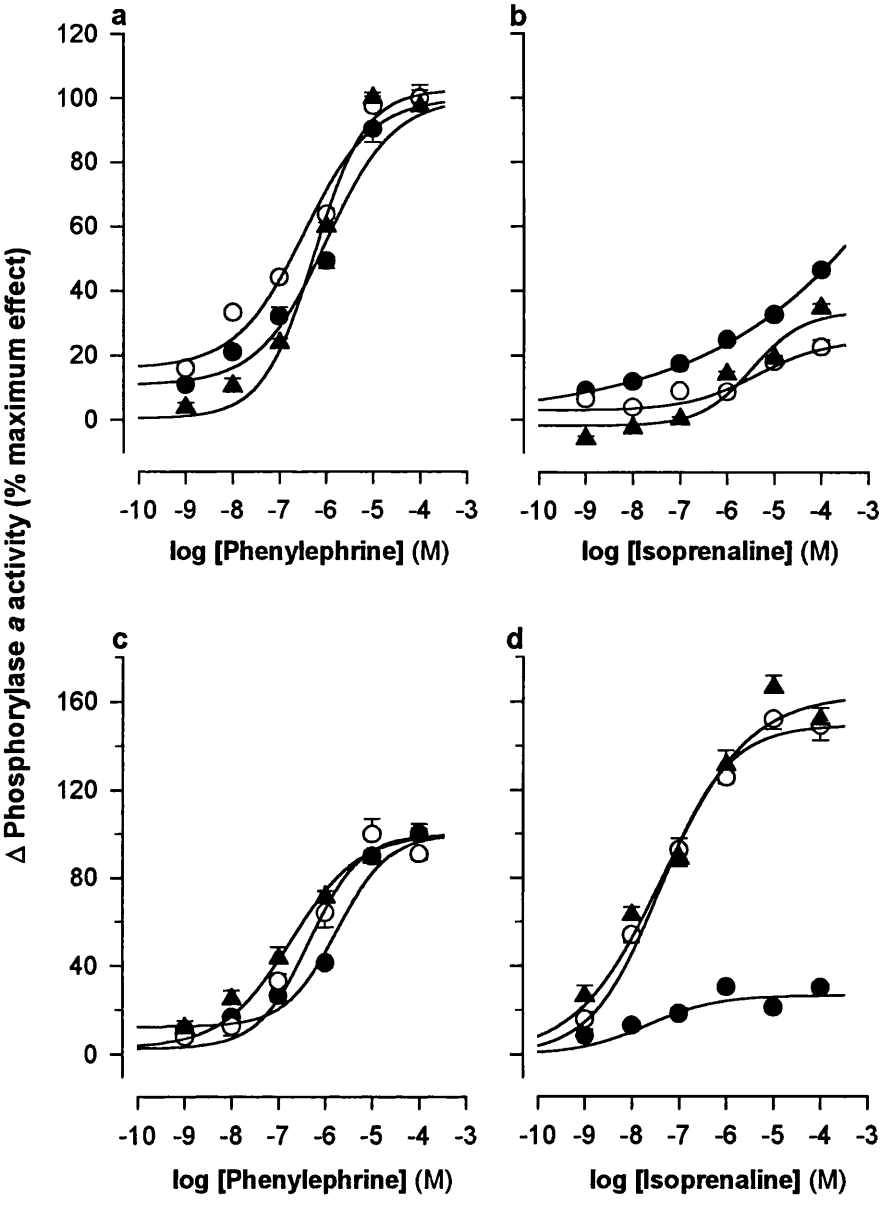


Figure 3.29 Effect of dexamethasone on time-dependent changes in the activation of glycogen phosphorylase induced by isoprenaline and phenylephrine in isolated rat hepatocyte suspensions. Hepatocyte suspensions in Williams' E without (●) and with dexamethasone (final conc., 0.1 μM ○ and 1 μM ▲) were preincubated for 30 min and then treated for 4 min with increasing concentrations of phenylephrine (panels **a** and **b**) or isoprenaline (panels **b** and **d**) at 0 h (upper panels) and at 6 h (lower panels). Phosphorylase *a* activity was assayed as described in *Materials and Methods*. Assays were done in duplicate and each data point represents the mean ± s.e. (n=6). The curves are least square fits of the data to the logistic function expressed in Eqn 2.1. For comparative purposes, isoprenaline responses have been expressed as % of respective maximal phenylephrine responses. Basal activities, EC_{50} and E_{max} values are presented in Table 3.11

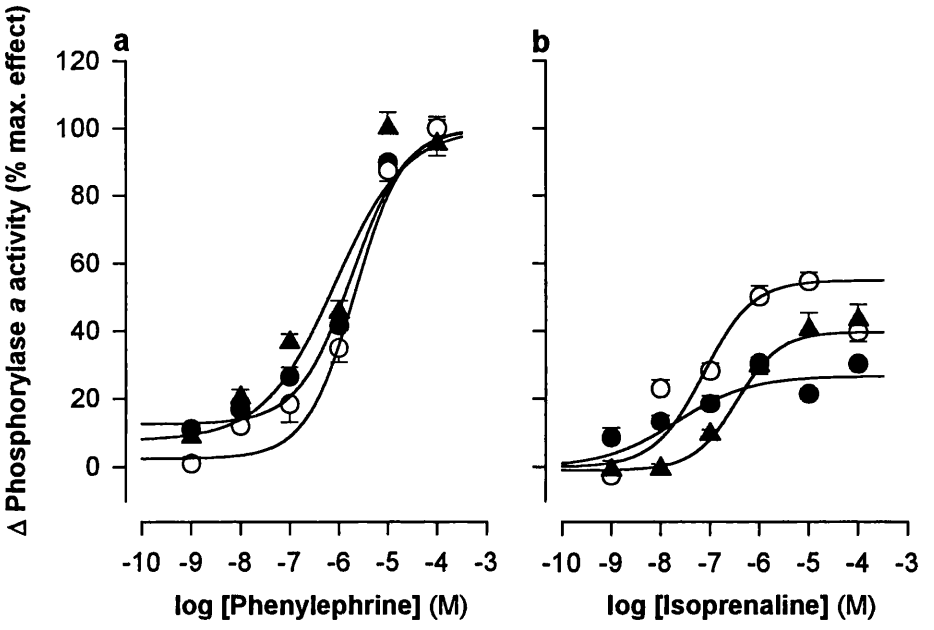


Figure 3.30 Effect of dexamethasone on the activation of glycogen phosphorylase induced by phenylephrine and isoprenaline. Hepatocyte suspensions in Williams' E were incubated for 5½ h. Dexamethasone was then added to the cell suspensions to give final concentrations of 0.1 μM (○) and 1 μM (▲). The cell were incubated further for 30 min. and then treated for 4 min with increasing concentrations of phenylephrine (panel **a)** or isoprenaline (panels **b).** Control responses (without dexamethasone, ●) have been reproduced from Figure 3.9 for comparison Phosphorylase *a* activity was assayed as described in *Materials and Methods.* Assays were done in duplicate and each data point represents the mean ± s.e. (n=6). The curves are least square fits of the data to the logistic function expressed in Eqn 2.1. For comparative purposes, isoprenaline responses have been expressed as % of respective maximal phenylephrine responses. Basal activities, EC_{50} and E_{max} values are presented in Table 3.11

Table 3.11 Effect of dexamethasone (DEX) on phenylephrine- and isoprenaline-induced activation of glycogen phosphorylase *a* (GPase *a*)in isolated rat hepatocyte suspensions.

Addition	Basal GPase *a* activity[a]	EC_{50} x 10^7 *(M)*		E_{max}[a]	
		Phenylephrine	*Isoprenaline*	*Phenylephrine*	*Isoprenaline*
0 h					
None	32.70 ± 0.89	9.77 ± 1.83	n.d.	46.90 ± 1.86	21.76 + 0.63†††
DEX (0.1 μM)	38.87 ± 1.14**	3.39 ± 0.63**	n.d.	56.08 ± 0.91***	19.37± 0.71***,†††
DEX (1.0 μM)	46.95 ± 2.05***	5.50 ± 1.22[n.s.]	33.88 ± 15.26‡	39.89 ± 0.92**	8.98 ± 0.84***,†††
6 h					
None	26.27 ± 0.12	17.78 ± 2.64	0.22 ± 0.09†††	36.13 ± 0.86	11.0 3± 0.57*,†††
DEX (0.1 μM)	37.79 ± 2.58**	4.57 ± 1.02***	0.39 ± 0.08[n.s.],†††	16.77 ± 1.13***	25.54 ± 0.80***,†††
DEX (1.0 μM)	29.11 ± 1.91[n.s.]	1.86 ± 0.42***	0.48 ± 0.15[n.s.],††	22.21 ± 0.98***	36.98 ± 1.13***,†††
DEX (0.1 μM)[b]	19.85 ± 0.55***	20.89 ± 4.67[n.s.]	0.74 ± 0.20[n.s.],†††	14.12 ± 0.46***	7.71 ± 0.38**,†††
DEX (1.0 μM)[b]	21.44 ± 1.18*	8.12 ± 1.96*	3.54 ± 1.15***,‡	6.70 ± 0.83***	3.14 ± 1.43***,‡

[a]Values are expressed as nmol min^{-1} mg^{-1} protein are means ± s.e. (n = 6). The values were obtained from the experiments shown in Figure 3.29 and Figure 3.30

EC_{50}s ± s.e. were obtained by least-square nonlinear regression as described in *Materials and Methods.*

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; [n.s.]$P \geq 0.05$ represent significant differences from respective controls (Student's t test with Bonferroni's correction) and †$P < 0.05$; ††$P < 0.01$; †††$P < 0.001$; ‡$P \geq 0.05$ represent significant differences from corresponding values for phenylephrine.

[b]dexamathasone was added at $t = 5.5$ h and the assay carried out at $t = 6$ h.

3.3.4.2 Effect of cycloheximide on the actions of dexamethasone

Cycloheximide was used to examine the effect of protein synthesis on the increased potency and efficacy of isoprenaline in the presence of dexamethasone.

The effect of dexamethasone on basal glycogen phosphorylase *a* activity was similar to results shown in the previous section (section *3.3.4.1*). The presence of 0.1 μM dexamethasone in the culture medium increased significantly ($P < 0.001$) the basal phosphorylase *a* activity in the hepatocytes (see Table 3.12). After 6 h, however, the enzyme activity in dexamethasone-treated cells decreased to values comparable to basal activities in control cells. Treatment of the cells with cycloheximide (2 μM) increased the basal glycogen phosphorylase a activity with time. Basal enzyme activity was also increased in cells treated with both cycloheximide and dexamethasone.

Although both agonists had similar EC_{50} ($P > 0.05$) at 0 h, the maximal response to phenylephrine was greater than to isoprenaline in control cells (Table 3.12). Dexamethasone (0.1 μM, final concentration) had no effect on the concentration-response curve of phenylephrine (Figure 3.31**a**) but shifted significantly ($P < 0.001$) the curve of isoprenaline to the right *i.e.* decreased activity (Figure 3.31**b**). Furthermore, the presence of dexamethasone increased the maximal response to isoprenaline without any significant effect on the maximal response to phenylephrine (see Table 3.12).

After six hours, the agonists still had similar potencies (EC_{50}s) with respect to control cells, though the potencies were increased by ≈150 fold in comparison to control cells at 0 h (Table 3.12). The maximal response to phenylephrine was unchanged while the response to isoprenaline significantly increased ($P < 0.001$, *vs.* control cells at 0 h). Thus the reciprocal change in the α- and β-adrenergic response with time was not obvious in these cultures. However the presence of 0.1 μM dexamethasone in the medium 'enhanced' the

reciprocal changes in the responses (see Figure 3.31**c** & **d**). The potency of phenylephrine in the presence of dexamethasone at 6 h was 6 fold and 280 fold lesser compared to values obtained in control cells at 0 h and 6 h respectively. In contrast, the potency increased by 15 fold compared to control cells at 0 h but there was no significant difference ($P > 0.05$) when compared to control cells at 6 h. Furthermore, the maximal response to isoprenaline was significantly ($P < 0.001$) greater than the response to phenylephrine.

The addition of cycloheximide (2 μM) to the culture medium effectively blocked the emergence of the β-response with time, even in the presence of dexamethasone (see Figure 3.32**b**). There was also no significant ($P > 0.05$) change in the EC_{50} of phenylephrine with time (see Figure 3.32 and Table 3.12) in cultures treated with cycloheximide. EC_{50} values in cells treated with cycloheximide alone and with cycloheximide/dexamethasone were similar ($P = 0.057$). Furthermore, the presence of cycloheximide led to decreases in the maximal responses to both phenylephrine and isoprenaline; more so with isoprenaline responses. The E_{max} of isoprenaline was decreased to values 3 to 9 fold less than values obtained from cultures without cycloheximide while responses to phenylephrine are decreased by 1.2 to 1.6 fold (see Table 3.12).

These results suggest that protein synthesis may be involved in the emergence of the β-adrenergic response in isolated hepatocytes with time.

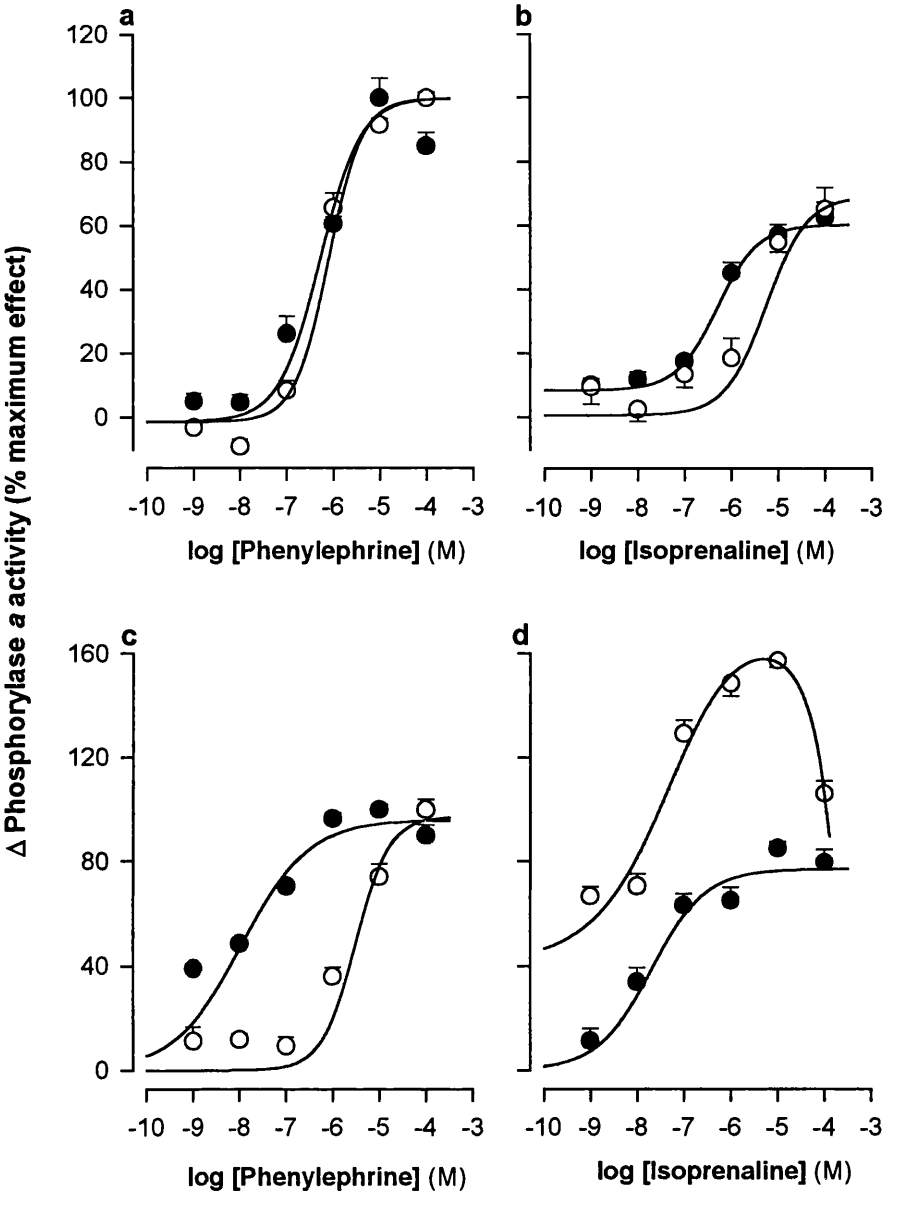


Figure 3.31 Effect of dexamethasone (0.1 μM) on time-dependent changes in the activation of glycogen phosphorylase induced isoprenaline and phenylephrine in isolated rat hepatocyte suspensions. Hepatocyte suspensions in Williams' E without (●) and with dexamethasone (○; final conc., 0.1 μM) were preincubated for 30 min and then treated for 4 min with increasing concentrations of phenylephrine (panels **a** and **b**) or isoprenaline (panels **b** and **d**) at 0 h (upper panels) and at 6 h (lower panels). Phosphorylase *a* activity was assayed as described in *Materials and Methods*. Assays were done in duplicate and each data point represents the mean ± s.e. (n=6). The curves, except for the bell-shaped, are least square fits of the data to the logistic function expressed in Eqn 2.1. The bell-shaped curve is least-square fit of the data to Eqn 2.2. For comparative purposes, isoprenaline responses have been expressed as % of respective maximal phenylephrine responses. Basal activities, EC_{50} and E_{max} values are presented in Table 3.12.

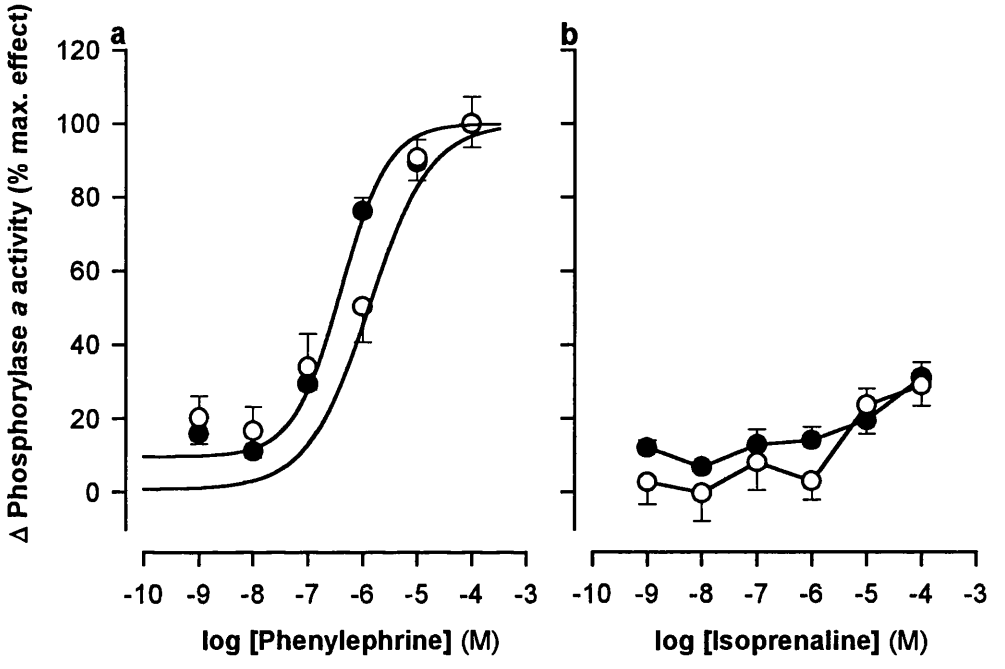


Figure 3.32 Inhibition of the effects of dexamethasone on adrenergic responses by cycloheximide in rat hepatocytes. Hepatocyte suspensions in Williams' E without (●) and with cycloheximide (○; final conc., 2 μM) were incubated for 6 h and then treated for 4 min with increasing concentrations of phenylephrine (panels **a**) or isoprenaline (panels **b**). Phosphorylase *a* activity was assayed as described in *Materials and Methods*. Assays were done in duplicate and each data point represents the mean ± s.e. (n=6). The curves are least square fits of the data to the logistic function expressed in Eqn 2.1. For comparative purposes, isoprenaline responses have been expressed as % of respective maximal phenylephrine responses. Basal activities, EC_{50} and E_{max} values are presented in Table 3.12.

Table 3.12 Summary of the effect of cycloheximide on dexamethasone actions in hepatocyte suspensions.

Addition	Basal GPase *a* activity[a]	EC_{50} x 10^7 *(M)*		E_{max}[a]	
		Phenylephrine	*Isoprenaline*	*Phenylephrine*	*Isoprenaline*
0 h					
None	27.81 ± 0.6	5.25 ± 1.08	5.01 ± 1.12‡	45.50 ± 2.82	27.05 ± 1.09†††
DEX (0.1 μM)	38.95 ± 0.99***	7.76 ± 3.64[n.s.]	54.95 ± 20.28***,‡	50.87 ± 0.86[n.s.]	36.49 ± 3.67*,††
6 h					
None	29.14 ± 0.71	0.11 ± 0.03	0.20 ± 0.06‡	40.83 ± 0.71	33.54 ± 0.91†††
DEX (0.1 μM)	26.14 ± 1.28[n.s.]	31.38 ± 6.97***	0.33 ± 0.02[n.s.],†††	39.40 ± 1.51[n.s.]	55.94 ± 2.65***,†††
CHX (2 μM)[b]	41.64 ± 1.64***	3.98 ± 0.82***	n.d.	31.63 ± 2.29**	9.88 ± 1.29***,†††
DEX + CHX[b]	32.26 ± 0.49**	13.18 ± 4.67***	n.d.	21.84 ± 1.42***	6.37 ± 1.25***,†††

[a]Values are expressed as nmol min^{-1} mg^{-1} protein are means ± s.e. (n = 6). The values were obtained from the experiments shown in Figure 3.31 and Figure 3.32.

EC_{50}s ± s.e. were obtained by least-square nonlinear regression as described in *Materials and Methods.*

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; [n.s.]$P \geq 0.05$ represent significant differences from respective controls (Student's t test with Bonferroni's correction) and †$P < 0.05$; ††$P < 0.01$; †††$P < 0.001$; ‡$P \geq 0.05$ represent significant differences from corresponding values for phenylephrine.

3.3.4.3 Effects of dexamethasone on adrenergic responses in short-term monolayer cultures of hepatocytes

In addition to studies in hepatocyte suspensions, which is over a shorter period of time, the effect of dexamethasone was also studied in monolayer cultures over a 24 hour period (see Figure 3.33 and Table 3.13).

Basal glycogen phosphorylase *a* activity increased significantly ($P < 0.01$) over the 24-hour incubation period (Table 3.13). The increase was not expected as previous findings indicated dramatic decreases in basal phosphorylase activity with time. Dexamethasone increased significantly ($P < 0.01$) the basal glycogen phosphorylase *a* activity and the increase was maintained over the incubation period.

At 0 h, 0.1 μM dexamethasone caused a 10-fold and 44-fold rightward displacement of the concentration-response curves to phenylephrine and isoprenaline respectively (see Figure 3.33**a** & **b** and Table 3.13) without any effect on the maximal responses.

After 24 h, there is a 280-fold increase in the EC_{50} (decreased potency) of phenylephrine compared to 12-fold for isoprenaline in control cells. Thus at 24 h isoprenaline is $\approx$8 times more potent than phenylephrine. In dexamethasone-treated cells the decrease in potency of phenylephrine was much smaller (20-fold) than in control cells. However, due a further decrease in the EC_{50} of isoprenaline in the dexamethasone-treated cells, the potency ratio of isoprenaline to phenylephrine is increased from 8 to 32. Thus the presence of dexamethasone enhances the emergence of the β-adrenergic response. Although the addition of dexamethasone to the medium caused an increase in the E_{max} of isoprenaline at 24 h, this value was considerably lower than E_{max} of phenylephrine.

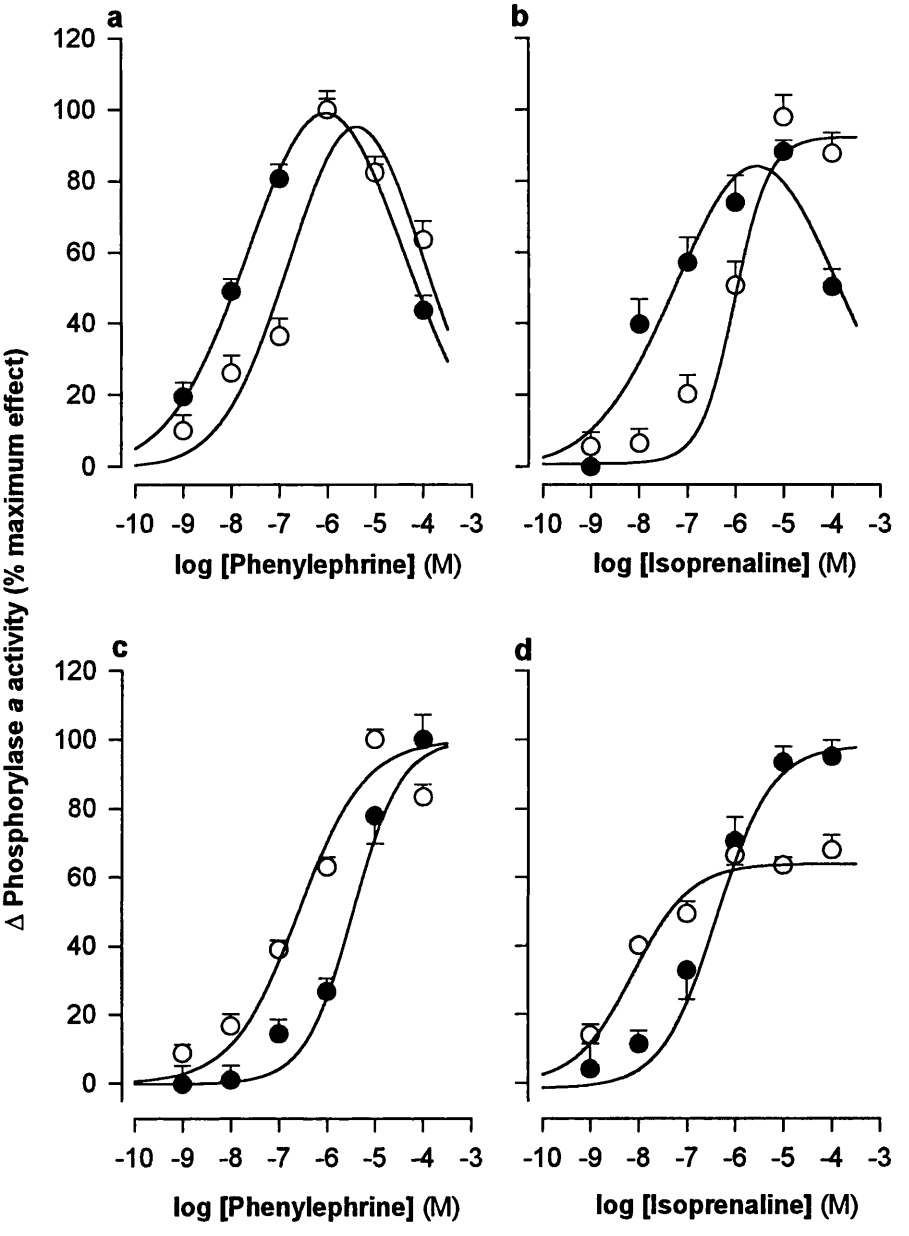


Figure 3.33 Effect of dexamethasone (0.1 μM) on time-dependent changes in the activation of glycogen phosphorylase induced isoprenaline and phenylephrine in short-term primary cultures of rat hepatocytes. Hepatocyte suspensions in Williams' E without (●) and with dexamethasone (○; final conc., 0.1 μM) were preincubated for 30 min and then treated for 4 min with increasing concentrations of phenylephrine (panels **a** and **b**) or isoprenaline (panels **b** and **d**) at 0 h (upper panels) and at 24 h (lower panels). Phosphorylase *a* activity was assayed as described in *Materials and Methods*. Assays were done in duplicate and each data point represents the mean ± s.e. (n=6). For comparative purposes, isoprenaline responses have been expressed as % of respective maximal phenylephrine responses. Basal activities, EC_{50} and E_{max} values are presented in Table 3.13.

Table 3.13 Effect of dexamethasone (DEX) on the activation of glycogen phosphorylase *a* (GPase *a*) in response to adrenergic agonist in primary monolayer cultures of hepatocyte.

Addition	Basal GPase *a* activity[a]	EC_{50} x 10^7 *(M)*		E_{max}[a]	
		Phenylephrine	*Isoprenaline*	*Phenylephrine*	*Isoprenaline*
0 h					
None	32.03 ± 1.05	0.12 ± 0.01	0.34 ± 0.03††	17.00 ± 0.89	14.98 ± 0.52‡
DEX (0.1 μM)	38.02 ± 0.93**	1.07 ± 0.07**	15.14 ± 4.64***,†††	16.77 ± 0.50$^{n.s.}$	16.40 ± 1.01$^{n.s.}$,‡
24 h					
None	36.50 ± 0.54	34.67 ± 8.96	4.17 ± 1.66†††	13.56 ± 0.96	12.10 ± 0.30‡
DEX (0.1 μM)	38.58 ± 1.84$^{n.s.}$	2.57 ± 0.11***	0.08 ± 0.01***,†††	32.44 ± 0.96***	22.04 ± 1.43***,†††

[a]Values are expressed as nmol min^{-1} mg^{-1} protein are means ± s.e. (n = 6). The values were obtained from the experiments shown in Figure 3.33.

EC_{50}s ± s.e. were obtained by least-square nonlinear regression as described in *Materials and Methods.*

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; $^{n.s.}P \geq 0.05$ represent significant differences from respective controls (Student's t test with Bonferroni's correction) and †$P < 0.05$; ††$P < 0.01$; †††$P < 0.001$; ‡$P \geq 0.05$ represent significant differences from corresponding values for phenylephrine.

3.3.5 Effect of polyamines on the reciprocal changes of adrenergic responses

The purpose of these experiments was to incubate hepatocytes in the presence of known inhibitors of polyamine synthesis in an attempt to arrest the switch in the adrenergic subtypes controlling glycogenolysis.

3.3.6 Effect of (+)-S-2-amino-5-iodoacetamidopentanoic acid (AIAP) and putrescine on adrenergic responses

At 0 h, the presence of 100 μM AIAP and 100 μM putrescine caused a dramatic shift ($P < 0.001$ and $P < 0.001$ respectively) of the concentration-response curve of phenylephrine to the right (Figure 3.34). Furthermore, AIAP had no effect on the maximal phenylephrine responses and the basal glycogen phosphorylase *a* activity (Table 3.14) . Putrescine however, caused a significant ($P < 0.001$) increase in the basal activity (i.e. activated glycogen phosphorylase) and a significant decrease in the E_{max} of phenylephrine. In comparison, isoprenaline response was not affected by AIAP but was completely inhibited by putrescine (Figure 3.35). Activation of glycogen phosphorylase by putrescine is probably due to its effects on Ca^{2+} homeostasis and phosphoinositide metabolism, thought to be mediated by the ability of polyamines to stimulate GTPase activity in G-proteins (Schuber, 1989; Bueb, 1992). Polyamines are known regulate Ca^{2+} fluxes across mitochondrial membranes (Rottenberg & Marbach, 1990; Lenzen *et al.*, 1992), and exposure to polyamines can increase $[Ca^{2+}]_i$ (Morgan et al., 1990; Groblewski *et al.*, 1992).

After 2 h, the affinity of phenylephrine decreases ($P < 0.001$) accompanied by a dramatic increase in the affinity of isoprenaline in the control cultures (Figure 3.34**b** & Figure 3.35**b**). The maximal responses to both agonists were significantly ($P < 0.001$) decreased; however the maximal responses to both were equal. Thus a switch in the adrenergic subtypes had already occurred by this time- the EC_{50} of isoprenaline is greater (≈10-fold) than that of

phenylephrine. Maintenance of the cells in culture for the next 4 h (i.e. at 6 h), led to further decrease in the EC_{50} of phenyleprine and about a 2.4-fold increase in the E_{max} of phenylephrine. There was no further increase in the EC_{50} of isoprenaline as expected, but rather a decrease. The ratio of isoprenaline to phenylephrine activity was ≈14 at 6 h.

AIAP blocked the increase in the isoprenaline response with time; by 6 h isoprenaline had lost its ability to stimulate glycogen phosphorylase activity and was instead inhibitory. Addition of putrescine to the medium, in contrast, led to an initial increase in the β-response at 2 h and then a dramatic 'inversion' of the response (see Figure 3.35**c**) After the initial decrease, the phenylephrine response increased with time in both the AIAP- and putrescine-treated cultures but not to the levels in the control cells at 0 h.

At the concentrations tested, AIAP was better than putrescine at preserving the α-response- the E_{max} and EC_{50} were greater in the AIAP-treated cultures at 6 h ($P < 0.05$; EC_{50} in AIAP-treated *vs.* EC_{50} in putrescine-treated cells)

Measurements were made at 2 h, because ornithine decarboxylase (ODC), the first and key enzyme in polyamine biosynthesis has a rapid and dramatic turnover rate- its half-life is usually from several minutes to one hour (Wagner *et al.*, 1994; Hayashi and Murakami, 1995).

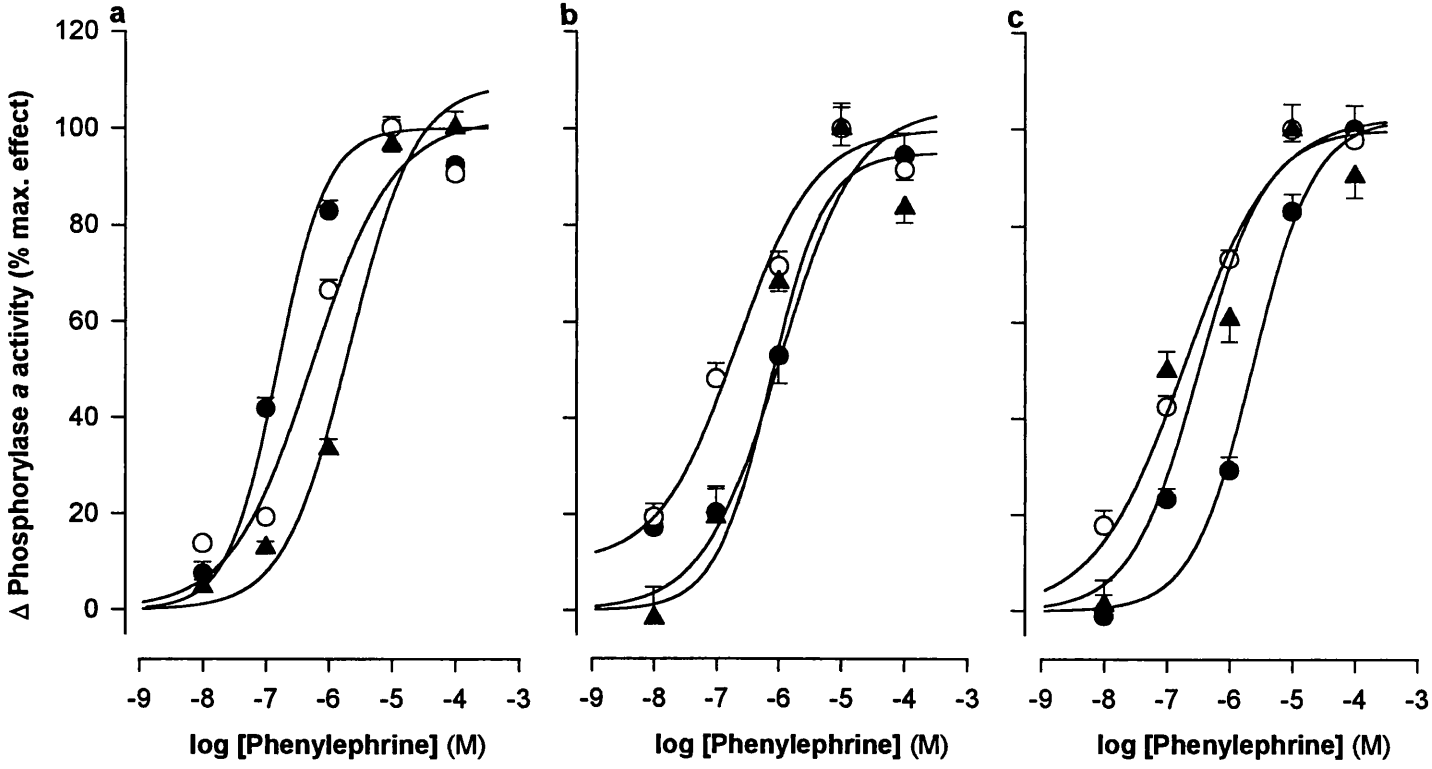


Figure 3.34 Effects of AIAP and putrescine on phenylephrine-induced activation of glycogen phosphorylase *a* in rat hepatocytes suspensions. Hepatocyte suspensions in Williams' E without (●) and with 100 μM AIAP (○) or 100 μM putrescine (▲) were preincubated for 30 min. Then the hepatocytes were treated for 4 min with increasing concentrations of phenylephrine at 0 h (panel **a**), 4 h (panel **b**) and 6 h (panel **c**). Phosphorylase *a* activity was assayed as described in *Materials and Methods*. Assays were done in duplicate and each data point represents the mean ± s.e. (n=6). The curves are least square fits of the data to the logistic function expressed in Eqn 2.1. Basal activities and individual EC_{50} and E_{max} values in Table 3.14.

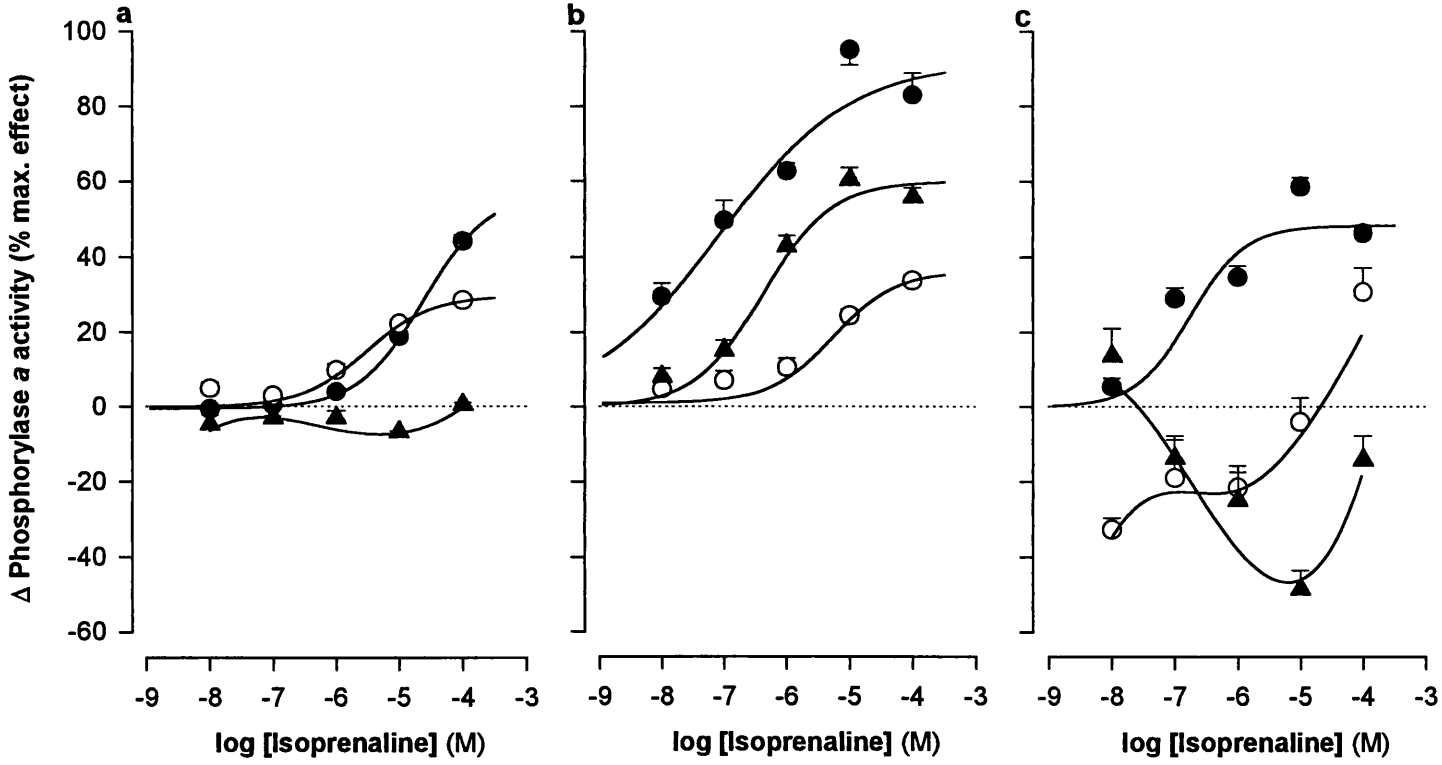


Figure 3.35 Effects of AIAP and putrescine on isoprenaline-induced activation of glycogen phosphorylase *a* in rat hepatocytes suspensions. Hepatocyte suspensions in Williams' E without (●) and with 100 μM AIAP (○) or 100 μM putrescine (▲) were preincubated for 30 min. Then the hepatocytes were treated for 4 min with increasing concentrations of isoprenaline at 0 h (panel **a**), 4 h (panel **b**) and 6 h (panel **c**). Phosphorylase *a* activity was assayed as described in *Materials and Methods*. Assays were done in duplicate and each data point represents the mean ± s.e. (n=6). The sigmoidal curves are least square fits of the data to the logistic function expressed in Eqn 2.1. For comparative purposes, the responses have been expressed as % of respective maximal phenylephrine responses in Figure 3.34. Basal activities and individual EC_{50} and E_{max} values in Table 3.14.

Table 3.14 Effect of AIAP and putrescine on phenylephrine- and isoprenaline induced activation of glycogen phosphorylase *a* in hepatocytes suspensions.

Addition	Basal GPase *a* activity[a]	EC_{50} x 10^7 *(M)*		E_{max}[a]	
		Phenylephrine	*Isoprenaline*	*Phenylephrine*	*Isoprenaline*
0 h					
None	32.10 ± 0.59	1.51 ± 0.16	239.88 ± 91.06†††	33.17 ± 0.53	14.68 ± 0.51†††
AIAP (100 μM)	30.24 ± 0.58 $^{n.s.}$	5.37 ± 1.48***	30.20 ± 12.822***,††	42.23± 0.93***	12.04 ± 0.62*,†††
Putrescine (100 μM)	40.72 ± 1.14***	21.38 ± 5.16***	n.d.	25.74 ± 0.85***	0.17 ± 0.11***,†††
2 h					
None	24.42± 0.28	10.2 ± 4.34	0.85 ± 0.40††	5.11 ± 0.27	4.86 ± 0.21‡
AIAP (100 μM)	24.50 ± 0.29$^{n.s.}$	2.09 ± 0.43***	53.7 ± 29.71***,†††	10.42 ± 0.46***	3.52 ± 0.16**,†††
Putrescine (100 μM)	23.75 ± 0.29$^{n.s.}$	7.24 ± 1.74$^{n.s.}$	4.07 ± 1.18$^{n.s.}$,‡	6.70 ± 0.23**	4.06 ± 0.21*,†††
6 h					
None	35.80 ±0.39	23.44 ± 6.06	1.62 ± 0.42†††	12.30 ± 0.38	7.21 ± 0.28†††
AIAP (100 μM)	40.52 ± 0.58***	1.91 ± 0.43*	n.d.	9.73 ± 0.48**	3.10 ± 0.62***,†††
Putrescine (100 μM)	31.17 ± 0.72***	3.16 ± 0.87***	n.d.	4.49 ± 0.23***	-2.18 ± 0.22***,†††

[a]Values are expressed as nmol min^{-1} mg^{-1} protein are means ± s.e. (n = 6). The values were obtained from the experiments shown in Figure 3.34 and Figure 3.35

EC_{50}s ± s.e. were obtained by least-square nonlinear regression as described in *Materials and Methods.*

$^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{n.s.}P \geq 0.05$ represent significant differences from respective controls (Student's t test with Bonferroni's correction) and †$P < 0.05$; ††$P < 0.01$; †††$P < 0.001$; ‡$P \geq 0.05$ represent significant differences from corresponding values for phenylephrine.

‡inhibition of glycogen phosphorylase *a* activity.

3.3.7 Effect of AIAP on arginase activity in isolated rat hepatocytes

Figure 3.36 shows the effect of AIAP on arginase activity in isolated hepatocytes and the accumulation of urea in the culture medium.

At 0 h urea levels in the media of control and treated cells were the same and increased with time (cumulative effect). However, after 2 hours in culture, AIAP caused concentration-dependent increases in the urea concentration. Thereafter, the urea levels remained fairly constant in cultures treated with 100 μM AIAP - 1.74 ± 0.17 at 0h and 2.01 ± 0.28 at 4 h (see Figure 3.36).

Similarly, AIAP-induced increases in arginase activity peaked at 2 h. There was, however, no significant increase in activity with 50 mM AIAP. Basal arginase activity (0.32 ± 0.04 mM of urea produced min^{-1} mg^{-1} protein) did not change appreciably during culture of the hepatocytes. To confirm the activation of arginase by AIAP, cell lysate was incubated with various concentrations of AIAP (Figure 3.37). As shown, the activation of arginase was concentration-dependent.

These results are in contrast to results obtained with arginase in the uterus (Mendez *et al.*, 1986).

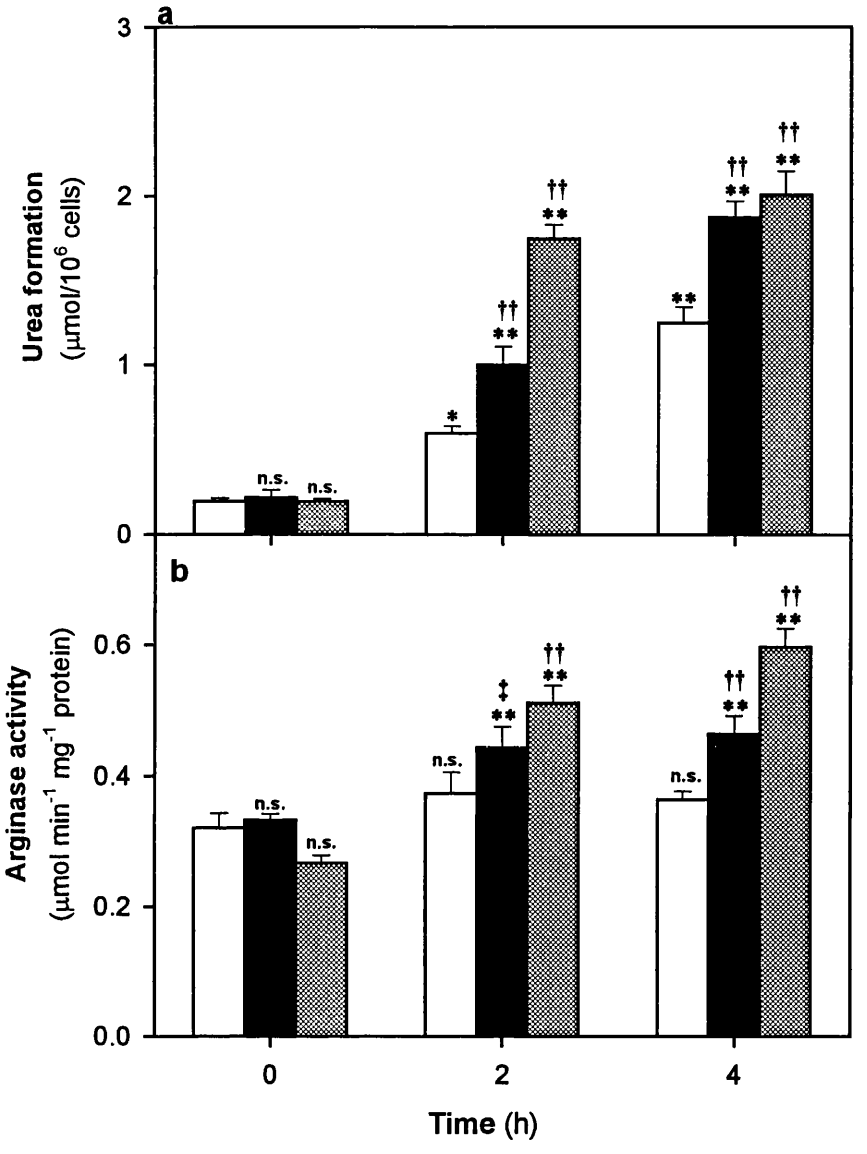


Figure 3.36 Effect of AIAP on urea production and arginase activity in isolated rat hepatocyte suspensions. Hepatocyte suspensions (3-5 x 10^6 cells ml^{-1}) were incubated in Williams' E medium in the absence (open columns) and presence of 50 μM AIAP (solid columns) or 100 μM AIAP (cross-hatched columns). **a** At the indicated times, the urea concentration was determined in the supernatant obtained by centrifuging, maximum speed in a microcentrifuge, 1-ml aliquot of the suspensions. **b** Arginase activity was determined as described in *Materials and Methods.* The times indicated exclude the preincubation time (30 min). Results are presented as means ± s.e. (n=6). $^{**}P < 0.01$; $^{n.s.}P \geq 0.05$ represent significant differences from control (no AIAP) value at 0 h and respective controls and $‡P \geq 0.05$; $††P < 0.01$; represent significant differences from corresponding values for phenylephrine .. (ANOVA followed Student's *t* test with Bonferroni's correction)

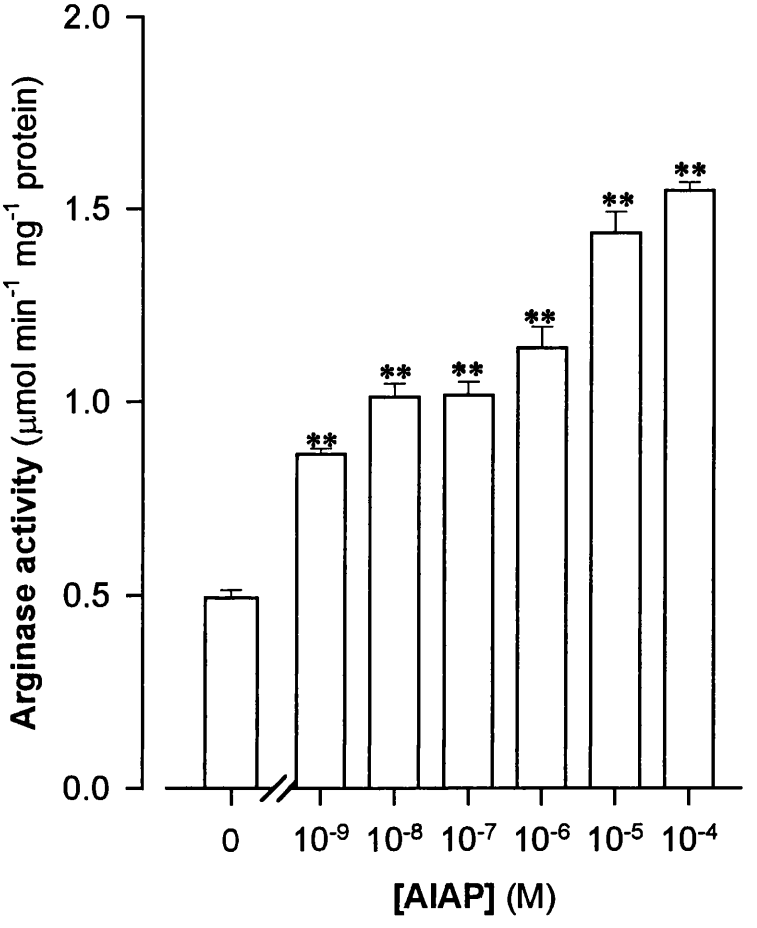


Figure 3.37 Activation of arginase activity by AIAP in isolated hepatocytes. Several incubations were established with cell lysate (0.1-0.2 mg protein ml^{-1}) with various concentrations of AIAP and arginase activity assayed as described in *Materials and Methods*. Arginase activity is expressed as the amount of urea formed min^{-1} mg^{-1} protein. Values are means ± s.d. (n=6). $^{**}P < 0.01$ compared to control values. (ANOVA followed by Bonferroni's multiple comparison test)

3.4 Cytochrome P450 And Adrenergic Responses In Rat Hepatocytes

There is considerable evidence that suggests the involvement of the cytochrome P-450 mono-oxygenase system in signal transduction and the control of ion fluxes across cell membranes in several cell types and tissues (Alvarez *et al.*, 1991, 1992; Escalante *et al.*, 1994; Oyekan *et al.*, 1994). These findings together with the well-established changes in cytochrome P-450 levels (Bequé et al., 1984; Paine, 1990; Padgham et al., 1993;) prompted us to investigate the possible link between the inventory of cytochromes P-450 and the adrenergic stimulation of glycogen phosphorylase activity in isolated and cultured hepatocytes.

3.4.1 Effect of cytochrome P450 inhibitors

As a first step, the effect of the cytochrome P-450 inhibitors, metyrapone and SKF-525A, on the activation of glycogen phosphorylase in response to α- and β-agonists was investigated.

Effect of metyrapone

In the presence of metyrapone (100-200 μM) the concentration-response curve of phenylephrine was shifted significantly to the right ($P < 0.001$) in a concentration-dependent manner (Figure 3.38**a**; see Table 3.15 for EC_{50} values). The difference between shifts caused by both concentrations was slight but significant. Furthermore, the maximal phenylephrine response was depressed by metyrapone in a concentration-dependent manner, (see Table 3.15). Concentration-response curves to isoprenaline stimulation were also shifted to the right, though less dramatically, by metyrapone (Figure 3.38**b**). However, the maximal isoprenaline response remained virtually unchanged.

Effect of SKF-525A

The presence of SKF-525A (100-200 μM) also shifted the concentration response curve of phenylephrine to the right (Figure 3.39**a**). The shift in the

curve was parallel i.e. identical slopes In contrast to metyrapone, SKF-525A shifted the concentration response to the left without depressing the maximum response (Figure 3.39b; see Figure 3.39 for individual values). Thus SKF-525A causes a reciprocal change in the response to α- and β-agonists.

These findings suggest that cytochrome P-450 plays a role in the activation of glycogen phosphorylase in response to adrenergic agonists.

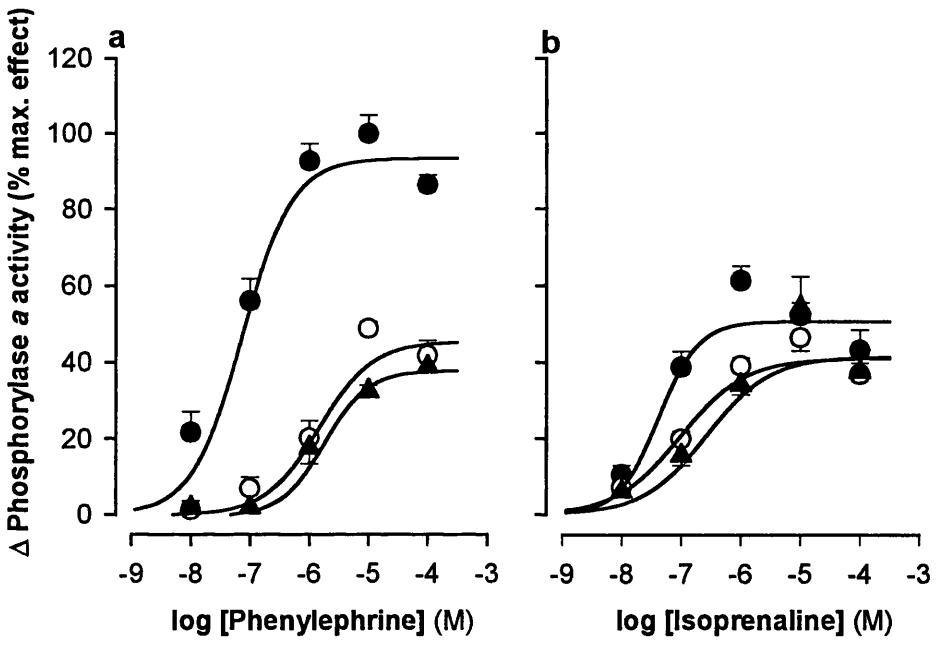


Figure 3.38 Inhibition of adrenergic-induced activation of glycogen phosphorylase *a* by metyrapone in isolated hepatocytes. Hepatocyte suspensions were preincubated in Williams' E medium for 30 min and then treated for 4 min with increasing concentrations of phenylephrine (panel **a**) and isoprenaline (panel **b**) in the absence (●) and presence of metyrapone (100 μM, ○ or 200 μM, ▲). Metyrapone was added to the 2 ml hepatocytes suspensions (see section 2.3 'Culture of Hepatocytes') in a total volume of 200 μl 5 min before the effects of the agonists were measured. Samples were processed for phosphorylase assay as described in *Materials and Methods*. Assays were done in duplicate and each data point represents the mean ± s.e. (n=6). The curves are least square fits of the data to the logistic function expressed in Eqn 2.1. For comparative purposes, isoprenaline responses have been expressed as % of respective maximal phenylephrine responses. Basal activities and individual EC_{50} and E_{max} values are presented in Table 3.15.

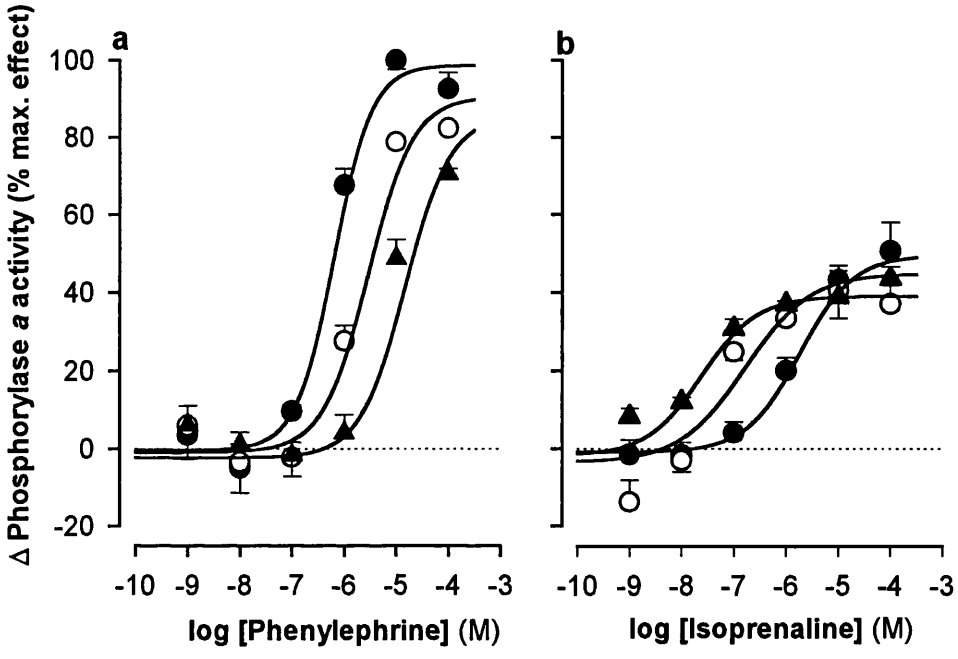


Figure 3.39 Effect of SKF-525A on adrenergic-induced activation of glycogen phosphorylase a in isolated hepatocytes. Hepatocyte suspensions were preincubated in Williams' E medium for 30 min and then treated for 4 min with increasing concentrations of phenylephrine (panel **a**) and isoprenaline (panel **b**) in the absence (●) and presence of SKF-525A (100 μM, ○ or 200 μM, ▲). SKF-525A was added to the 2 ml hepatocytes suspensions (see section 2.3 'Culture of Hepatocytes') in a total volume of 200 μl 5 min before the effects of the agonists were measured. Samples were processed for phosphorylase assay as described in *Materials and Methods.* Assays were done in duplicate and each data point represents the mean ± s.e. (n=6). The curves are least square fits of the data to the logistic function expressed in Eqn 2.1. For comparative purposes, isoprenaline responses have been expressed as % of respective maximal phenylephrine responses. Basal activities and individual EC_{50} and E_{max} values are presented in Table 3.15.

Table 3.15 Basal activities, EC_{50} and E_{max} values for the effects of metyrapone and SKF-525A on the activation of glycogen phosphorylase *a* induced by phenylephrine and isoprenaline in isolated rat hepatocytes.

Cytochrome P450 inhibitor	Basal GPase *a* activity[a]	EC_{50} x 10^7 (M)		E_{max}[a]	
		Phenylephrine	*Isoprenaline*	*Phenylephrine*	*Isoprenaline*
Metyrapone					
Control	32.25 ± 1.42	0.72 ± 0.16	0.42 ± 0.12‡	21.85 ± 1.07	11.39 ± 0.75†††
100 μM	29.98 ± 0.46$^{n.s.}$	14.12 ± 5.41***	0.95 ± 0.23$^{n.s.}$,†††	10.67 ± 0.0.4***	10.16 ± 0.76$^{n.s.}$,‡
200 μM	25.44 ± 1.02***	17.78 ± 7.06***	2.63 ± 1.11*,‡	8.50 ± 0.36***	11.84 ± 1.76$^{n.s.}$,‡
SKF 525A					
Control	18.53 ± 0.91	6.45 ± 1.21	17.78 ± 8.23‡	17.09 ± 0.40	8.67 ± 1.25†††
100 μM	23.84 ± 0.75***	28.84 ± 7.95**	1.62 + 0.64*,†††	14.07 ± 0.18***	6.95 ± 0.85$^{n.s.}$,†††
200 μM	21.2 ± 0.90$^{n.s.}$	151.36 ± 51.35***	0.24 ± 0.08***,††	12.08 ± 0.22***	7.48 ± 0.49$^{n.s.}$,††

[a]Values are expressed as nmol min^{-1} mg^{-1} protein are means ± s.e. (n = 6). The values were obtained from the experiments shown in Figure 3.38 and Figure 3.39.

EC_{50}s ± s.e. were obtained by least-square nonlinear regression as described in *Materials and Methods.*

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; $^{n.s.}P \geq 0.05$ represent significant differences from respective controls (Student's t test with Bonferroni's correction) and †$P < 0.05$; ††$P < 0.01$; †††$P < 0.001$; ‡$P \geq 0.05$ represent significant differences from corresponding values for phenylephrine.

3.4.2 Effect of β-naphthoflavone-inducible cytochromes P450 on adrenergic responses in rat hepatocytes

To further investigate the effect of cytochrome P450 on adrenergic responses in hepatocytes, experiments were performed with hepatocytes from adult male rats treated with the cytochrome P450 inducer, β-naphthoflavone (β-NF).

Treatment of the rats with β-NF led to the deposition of the latter between the Glisson's capsule and the liver. This however, did not have any effect on the isolation or the viability of the hepatocytes.

3.4.2.1 Cytochrome P450 and CYP1A1 content

Pretreatment of the rats with β-NF (80 mg kg^{-1}, i.p., daily for 3 days) caused a 2-fold increase (from 0.37 ± 0.05 to 0.78 ± 0.12 nmol mg^{-1} protein) in the total cytochrome P450 (Figure 3.40a). Increase in the total cytochrome P450 level was accompanied by a 3-fold increase (from 0.08 ± 0.02 to 0.25 ± 0.03 O.D. units) in *CYP*1A1 content (Figure 3.40b).

3.4.2.2 Androst-4-ene-3,17-dione metabolism

The effect of β-NF treatment on cytochrome P450 activity was further assessed using the hepatic metabolism of androstenedione. This assay detects three cytochrome P450 isoenzymes: cytochromes P450 2A1 (7α-hydroxylase),2C11 (16α-hydroxylase), 3A1/3A2 (6β-hydroxylase).

β-NF-treatment caused ≈1.5-fold increase in 7α-hydroxylase activity ($P < 0.01$; Figure 3.41). In contrast, there was a significant($P < 0.001$) decrease in 16α-hydroxylase (CYP2C11) activity and no change in 6β-hydroxylase activity. Furthermore, β-NF caused significant increases in 17α(β)-hydroxysteroid dehydrogenase and 5α-reductase activities; these are however not cytochrome P450 isoenzymes.

Results obtained are consistent with reported effects of β-NF on hepatic levels of cytochrome P450; pretreatment with β-NF induces cytochromes P450 1A1 and 2A1 (Lau & Strobel, 1982; Murray & Reidy, 1990). However, increases in the levels of cytochromes P450 were modest compared to dramatic increases reported in literature and the decrease in cytochrome P450 3A1/3A2 activity was not expected.

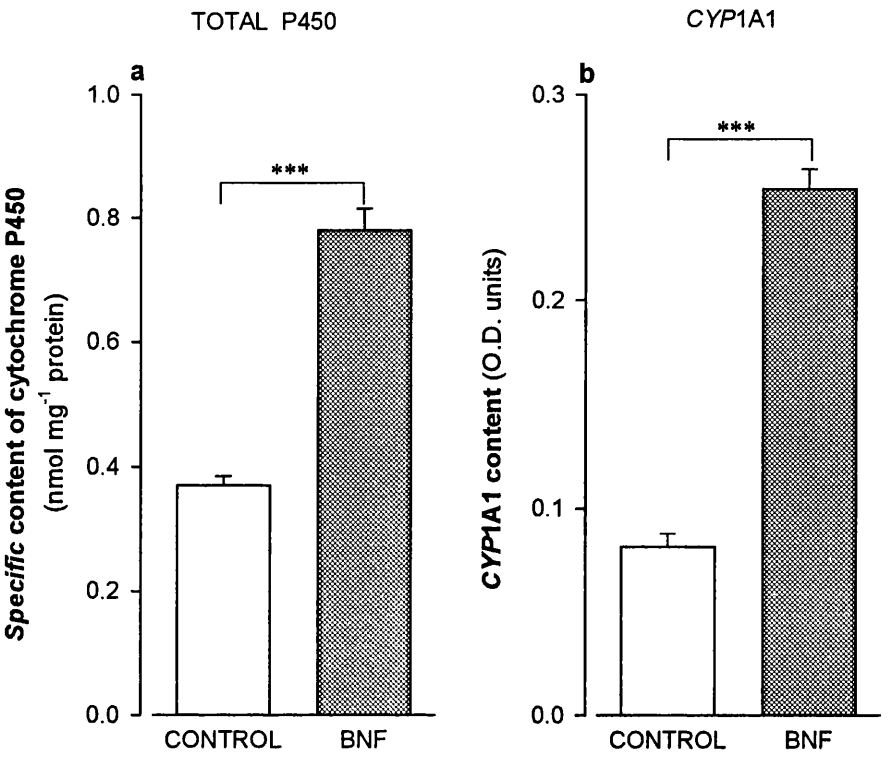


Figure 3.40 Effect of pretreatment of adult male rats with β-naphthoflavone on the induction of hepatic cytochrome P450 and *CYP*1A1. Groups of male rats were treated with either vehicle (corn oil) or β-NF (80 mg kg^{-1}) daily for 3 days prior to isolation of hepatocytes. Total cytochrome P450 and CYP1A1 were then assayed in microsomes obtained from the hepatocytes as described in *Materials and Methods*. Five replicate determinations were made for each microsomal sample. Values represent means ± s.d. (n=15). ***$P < 0.001$; Student's *t* test.

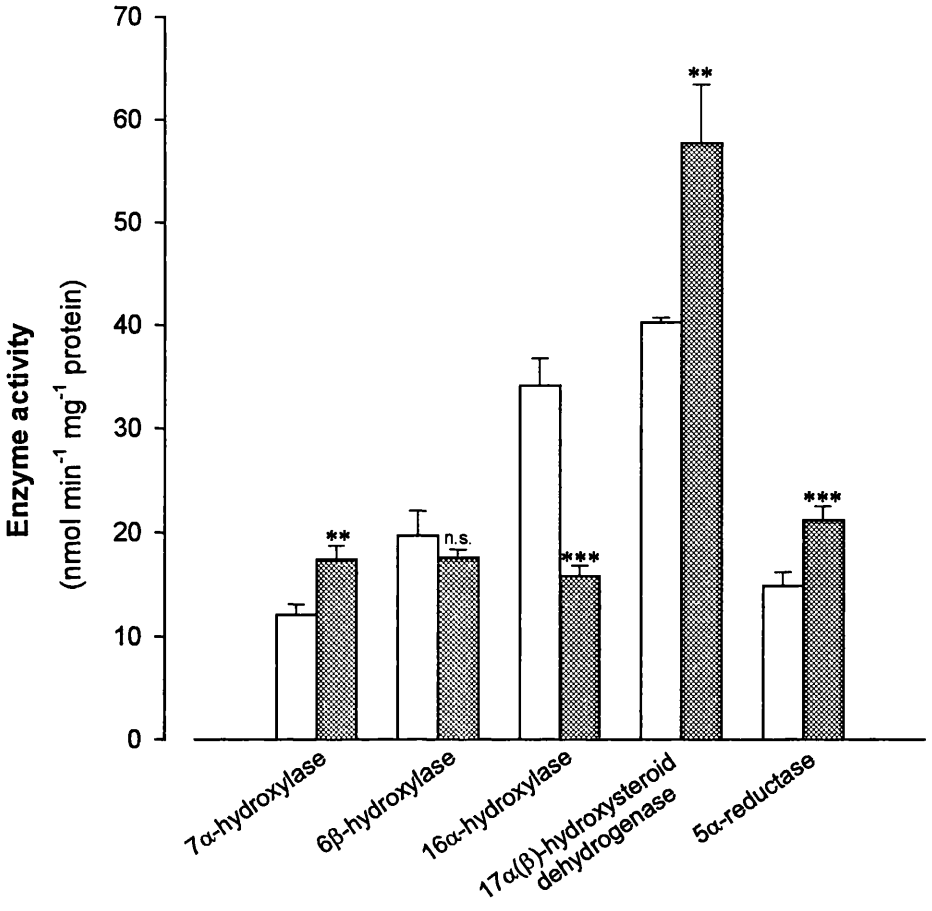


Figure 3.41 Effect of pretreatment of adult male rats with β-naphthoflavone on hepatic metabolism of androst-4-ene-3,17-dione. Groups of male rats were treated with either vehicle (corn oil) or β-NF (80 mg kg^{-1}) daily for 3 days prior to isolation of hepatocytes. Microsomes from each group were incubated with androstenedione and enzyme activities assayed as described in *Materials and Methods*. Five replicate assays were carried out for each microsomal sample. Values represent means ± s.d. (n=15). $^{**}P < 0.01$; $^{***}P < 0.001$ and $^{n.s.}P > 0.05$ (not significant).; Student's *t* test.

3.4.2.3 α- and β-adrenergic responses

Figure 3.42 shows the effect of α-naphthoflavone on phenylephrine- and isoprenaline-induced activation of glycogen phosphorylase *a* in hepatocytes isolated from normal male rats and rats treated with β-naphthoflavone (80 mg kg^{-1}, i.p., daily for 3 days). Animals in the control group were treated with the vehicle (corn oil) as an i.p. injection by the same schedule.

β-NF-pretreatment of the rats prior to isolation of the hepatocytes, led to a significant decrease in the potency (EC_{50}) of phenylephrine by 2.2-fold ($P < 0.001$) in comparison to responses in control rats (see Table 3.16). In addition, the maximal phenylephrine response was much lower ($P < 0.001$) in hepatocytes from the β-NF-treated group. The EC_{50} and E_{max} of isoprenaline, in contrast, were not affected by β-NF pretreatment. Thus isoprenaline was relatively more potent in the treatment group due the rightward shift in the concentration-response curve to phenylephrine (with corresponding decrease in E_{max}) in hepatocytes from this group.

We did not anticipate phenylephrine to be less effective in hepatocytes from the treated group since these experiments were done on the hypothesis that cytochrome P450-induction will potentiate the actions of phenylephrine. Moreover, α-NF, known to inhibit cytochromes P450 inducible by β-NF (Murray & Reidy, 1990), did not reverse the effects of β-NF-treatment (see below).

Consistent with our previous studies showing that cytochrome P450 inhibitors blocked α-responses (see section 3.4.1), the presence of α-NF (100 μM) in the culture medium caused a rightward shift in the concentration-response curve of phenylephrine in hepatocytes from control and treated rats (Figure 3.42**a** and **c**). Furthermore, the maximal phenylephrine response was depressed in hepatocytes from both groups. However, phenylephrine responses

were more susceptible to inhibition by α-NF in treated cells than in control cells—a 15-fold shift and a 60% decrease in maximal response in treated cells *vs.* 11-fold shift and a 40% decrease in control cells (see Table 3.16).

The concentration-response curve of isoprenaline was significantly shifted to the right by α-NF in hepatocytes from control rats accompanied by an increase in the efficacy (E_{max}) of isoprenaline (Figure 3.42**b** and Table 3.16). In contrast, α-NF did not displace the concentration-response curve to isoprenaline in the treated hepatocytes, however, it depressed the maximal isoprenaline response (Figure 3.42**d**). Differences therefore, exist between the actions of α-NF on the β-adrenergic-induced responses in the two groups.

In these experiments, α-naphthoflavone was added to the hepatocyte suspensions at 1000-fold the final concentration in 20 μl acetone 5 min before the effects of the agonists were measured. Final incubation volume was 2 ml. Addition of vehicle only (20 μl acetone) to the culture did not affect the basal activity significantly—25.2 ± 0.1 and 27.2 ± 1.2 nmol min^{-1} mg^{-1} protein in the absence and presence of 20 μl acetone.

Calcium ionophore, A23187

Recent evidence suggests that cytochrome P450 inhibitors suc as SKF-525A and imidazole antimycotics may inhibit agonist-stimulated Ca^{2+} entry in rat thymocytes (Alvarez *et al.*, 1991) and in neutrophils (Montero *et al.*, 1992). We therefore assessed the ability of α-NF to block the effect of A23187-induced increases in glycogen phosphorylase a activity in isolated rat hepatocytes. The results are shown in Figure 3.43.

α-NF (100 mM) caused a dramatic shift of the concentration-response curve of A23187 to the right—EC_{50} was 6.51 ± 0.13 and 4.76 ± 0.41 respectively in the absence and presence of α-NF.

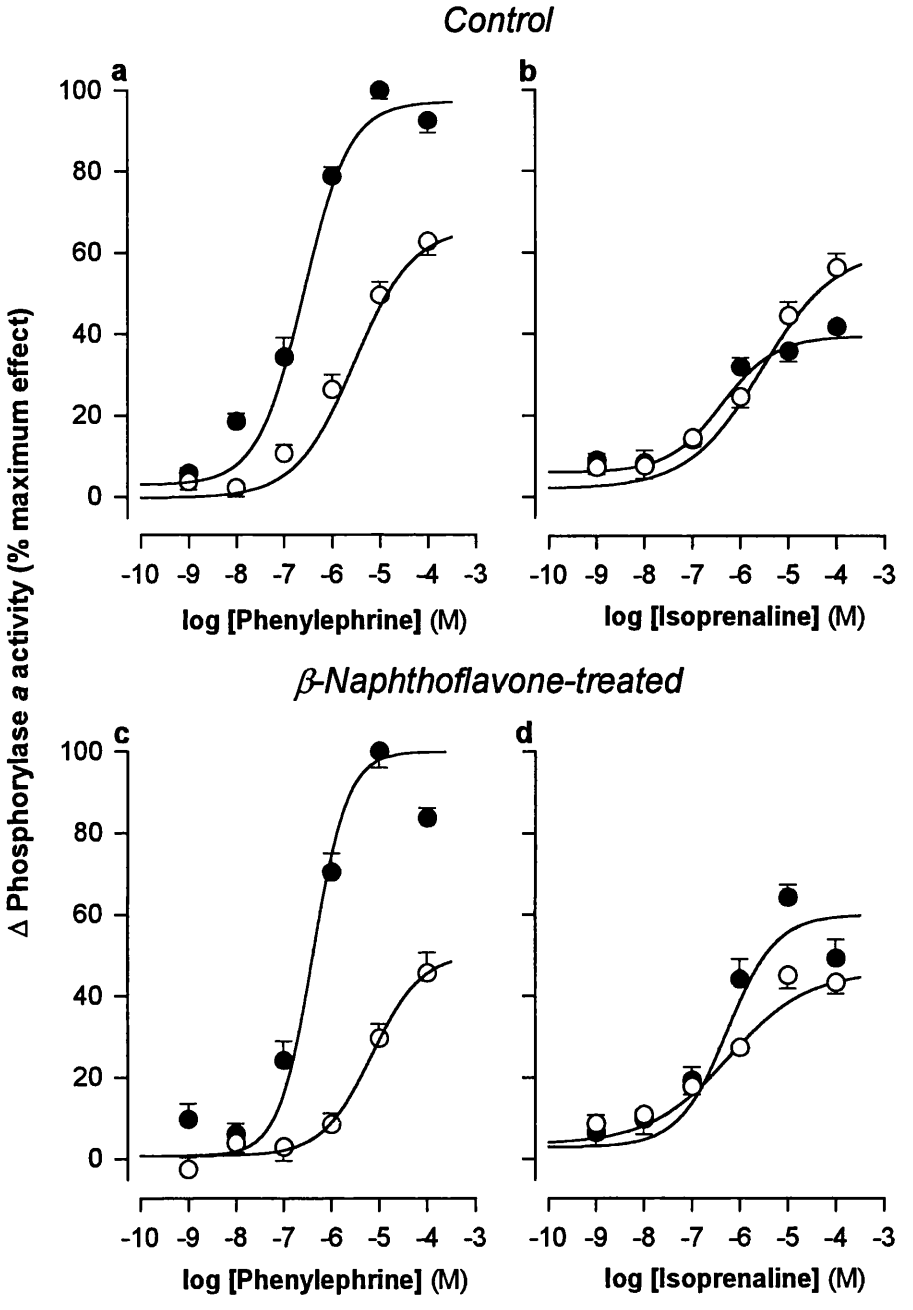


Figure 3.42 Effects of α-naphthoflavone on adrenergic-induced activation of glycogen phosphorylase *a* in isolated hepatocytes from control and β-NF-treated rats. Hepatocyte suspensions were preincubated in Williams' E medium for 30 min and then treated for 4 min with increasing concentrations of phenylephrine and isoprenaline in the absence (●) and presence of α-NF (100 μM, ○). α-NF was added to the 2 ml hepatocytes suspensions (see section 2.3 'Culture of Hepatocytes') in a total volume of 20 μl 5 min before the effects of the agonists were measured. Samples were then processed for phosphorylase *a* assay as described in *Materials and Methods.* Assays were done in duplicate and each data point represents the mean ± s.e. obtained from 2 rats (n=12). The curves are least square fits of the data to the logistic function expressed in Eqn 2.1. For comparative purposes, isoprenaline responses have been expressed as % of respective maximal phenylephrine responses. Basal activities and individual EC_{50} and E_{max} values are presented in Table 3.16.

Table 3.16 Effects of β-naphthoflavone-treatment on adrenergic responses in isolated rat hepatocytes: Basal glycogen phosphorylase *a* activities, EC_{50} and E_{max} values.

Treatment	Basal GPase *a* activity[a]	EC_{50} x 10^7 *(M)*		E_{max}	
		Phenylephrine	*Isoprenaline*	*Phenylephrine*	*Isoprenaline*
Untreated					
Control	25.24 ± 0.12	2.51 ± 0.42	4.07 ± 1.44‡	26.04 ± 2.12	11.24 ± 0.48†††
α-NF (100 μM)	26.15 ± 1.63$^{n.s.}$	28.84 ± 11.46***	27.54 ± 11.95*‡	16.64 ± 1.73**	14.83 ± 1.42$^{n.s.}$‡
β-NF-treated					
Control	17.28 ± 1.24	5.49 ± 0.92	4.17 ± 1.08‡	19.42 ± 2.14	12.64 ± 1.73†
α-NF (100 μM)	25.64 ± 1.12***	69.10 ± 29.37***	5.75 ± 2.73$^{n.s.}$††	8.92 ± 0.84***	8.22 ± 1.44 $^{n.s.}$‡

[a]Values are expressed as nmol min^{-1} mg^{-1} protein are means ± s.e. (n = 6). The values were obtained from the experiments shown in Figure 3.42.

EC_{50}s ± s.e. were obtained by least-square nonlinear regression as described in *Materials and Methods.*

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; $^{n.s.}P \geq 0.05$ represent significant differences from respective controls (Student's t test with Bonferroni's correction) and †$P < 0.05$; ††$P < 0.01$; †††$P < 0.001$; ‡$P \geq 0.05$ represent significant differences from corresponding values for phenylephrine.

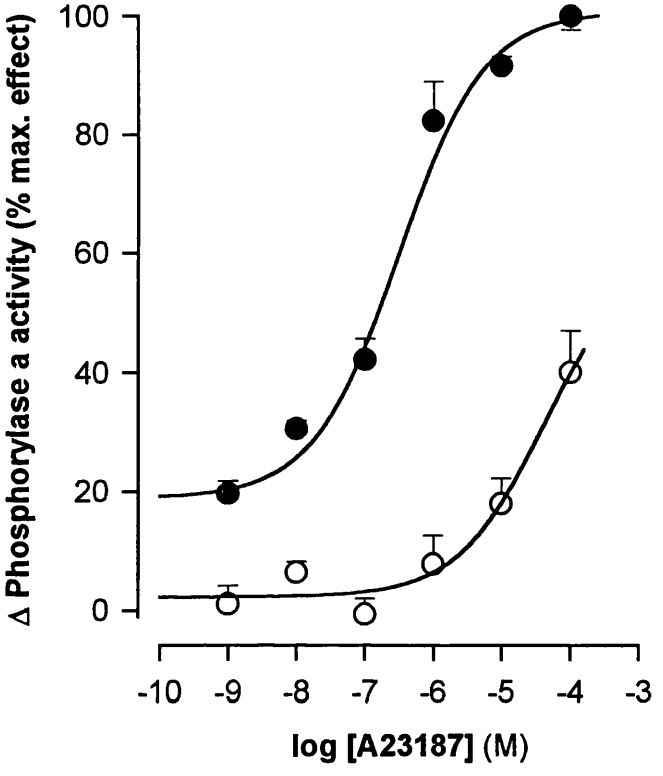


Figure 3.43 Inhibition of A23187-induced activation of glycogen phosphorylase induced by α-naphthoflavone in isolated hepatocytes. Hepatocyte suspensions were preincubated in Williams' E medium for 30 min and then treated for 4 min with increasing concentrations of A23187 in the absence (●) and presence of α-NF (100 μM, ○). α-NF was added to the 2 ml hepatocytes suspensions (see section 2.3 'Culture of Hepatocytes') in a total volume of 20 μl 5 min before the effects of the agonists were measured. Samples were then processed for phosphorylase *a* assay as described in *Materials and Methods.* Assays were done in duplicate and each data point represents the mean ± s.e. (n=6). The curves are least square fits of the data to the logistic function expressed in Eqn 2.1. Basal glycogen phosphorylase *a* activity was 23.6 ± 2.2 and E_{max} was 20.6 ± 1.1 nmol min^{-1} mg^{-1} protein.

3.5 Effect Of Sodium Nitroprusside (SNP) And Glyceryl Trinitrate (GTN) On Adrenergic Responses In Hepatocytes

Chronic endotoxemia induced by lipopolysaccharide leads to a shift from α- to β-adrenergic response in rats (Pittner & Spitzer, 1993) and it is well established that nitric oxide is one of the main mediators of the actions of endotoxin (Habrecht *et al.*, 1994; Milbourne & Bygrave, 1995). A series of experiments were therefore set up to explore the involvement of nitric oxide in the adrenergic response in isolated rat hepatocytes. Two nitric oxide donors, sodium nitroprusside (SNP) and glyceryl trinitrate (GTN) were used in the experiment.

SNP and GTN activated glycogen phosphorylase *a* in a dose-dependent manner in freshly isolated rat hepatocytes (see Figure 3.44). Basal phosphorylase a activity were increased 2-fold by 50 μM of SNP and GTN. Furthermore, SNP (Figure 3.45**a** & **b**) and GTN (Figure 3.45**c** & **d**) inhibited dose-dependently, the activation of glycogen phosphorylase induced by phenylephrine and isoprenaline. Thus the addition of nitric oxide donors did not potentiate the effects of isoprenaline as observed in chronically endotoxemic rats.

A striking feature of the effects of NO-donors in these experiments is the complete 'reversal' of the actions of isoprenaline—isoprenaline becomes inhibitory in the presence of the donors (see Figure 3.45**b** & **d**).

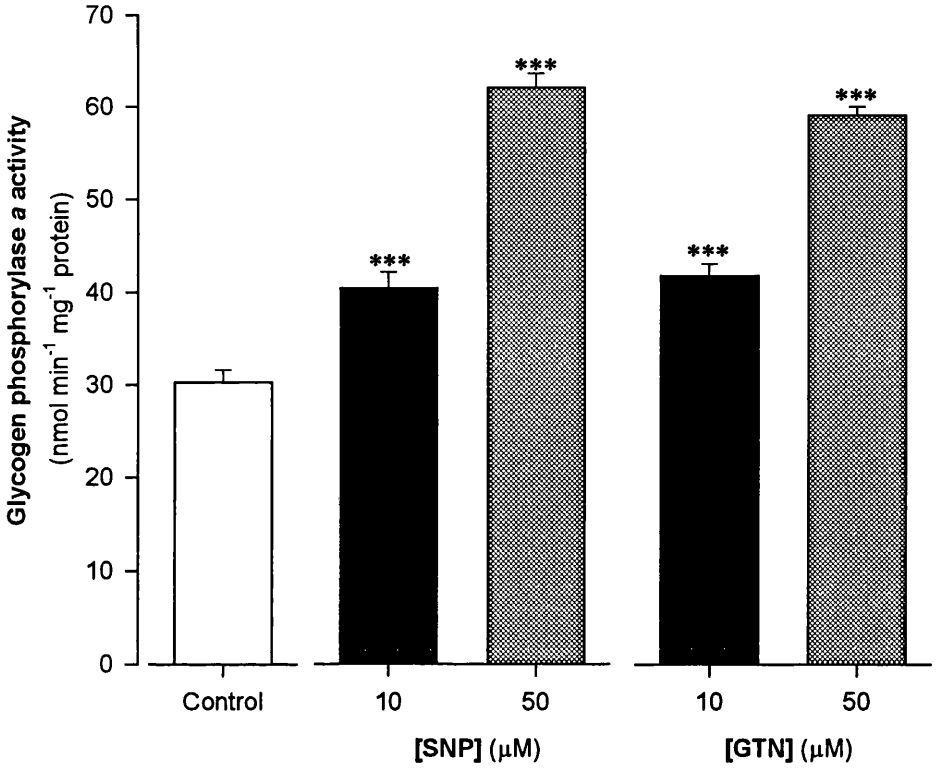


Figure 3.44 Activation of glycogen phosphorylase *a* activity by the nitric oxide donors, sodium nitroprusside (SNP) and glyceryl trinitrate (GTN). Hepatocyte suspensions were preincubated in Williams' E medium for 30 min and then treated for 4 min with 10 μM (solid columns) and 50 μM (cross-hatched columns) SNP or GTN. Samples were then processed for phosphorylase *a* assay as described in *Materials and Methods.* Assays were done in duplicate and each data point represents the mean ± s.e. (n=6). ***$P < 0.001$ compared to control (open column) - Student's *t* test with Bonferroni's correction.

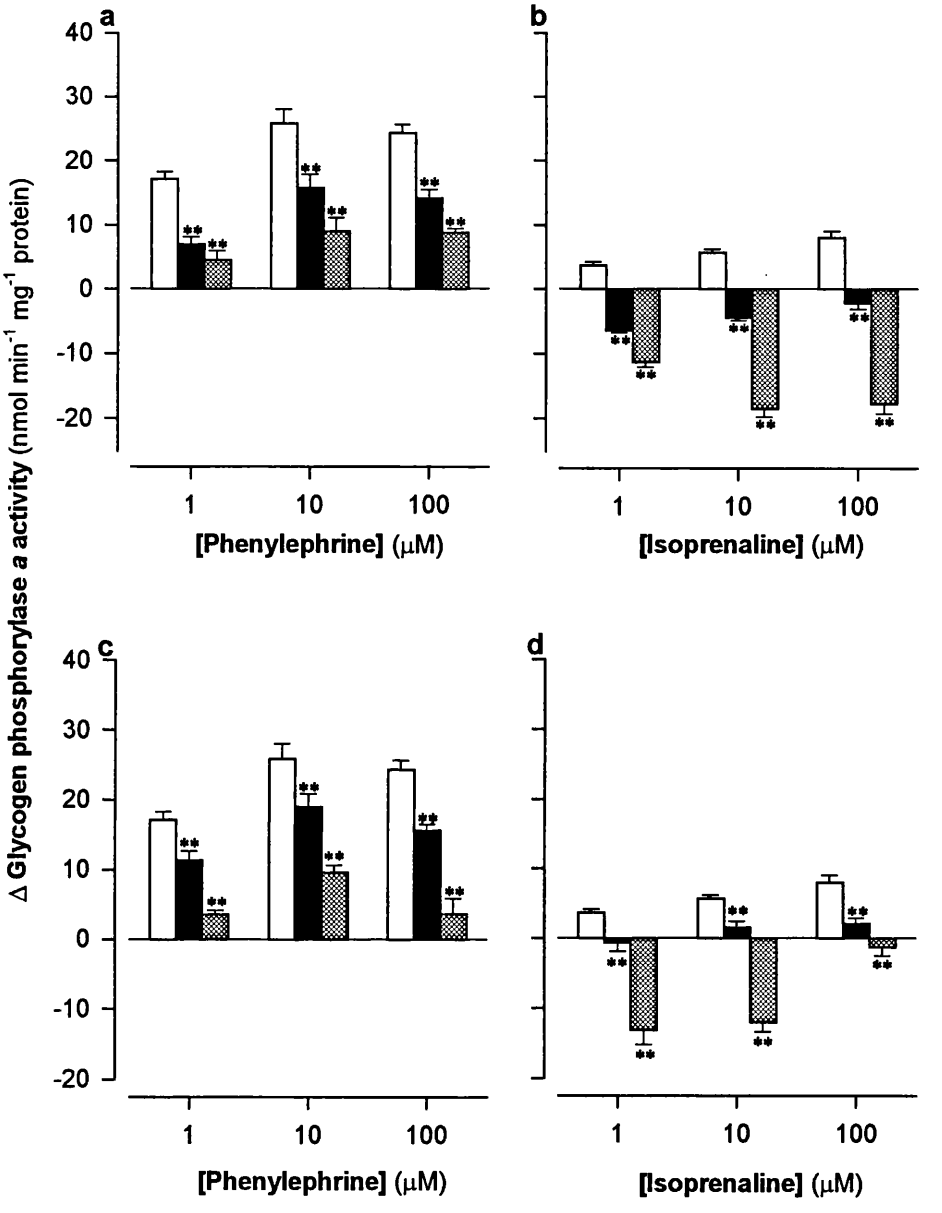


Figure 3.45 Inhibition of adrenergic responses by sodium nitroprusside and glyceryl trinitrate. Hepatocyte suspensions were preincubated in Williams' E medium for 30 min and then treated for 4 min with increasing concentrations of phenylephrine or isoprenaline in the absence (open columns) and presence of 50 μM SNP (solid columns) or 50 μM GTN (cross-hatched columns). SNP and GTN were added 5 min before the effects of the adrenergic agonists were measured. Samples were then processed for phosphorylase *a* assay as described in *Materials and Methods.* Assays were done in duplicate and each data point represents the mean ± s.e. (n=6). $^{**}P < 0.01$ compared to control (open column) - Student's *t* test with Bonferroni's correction.

3.5.1 Effect of protein kinase G inhibitor, KT5822 on the actions of sodium nitroprusside and glyceryl trinitrate

Since most of the actions of NO are mediated *via* the activation of guanylate cyclase , we examined the interaction between NO-donors and KT5822 in freshly isolated and 6-hour old hepatocyte suspensions. KT5822 is a highly specific inhibitor of protein G (K*i* = 234 nM) (Kase *et al.*, 1997; Grider, 1993).

In contrast to earlier observation (see Figure 3.44), the addition of GTN (50 mM) to the medium did not activate glycogen phosphorylase *a* activity in the cell cultures at 0 h (Table 3.17). Also, KT5822 (50 nM) did not have any significant ($P > 0.05$) effect on the basal activity of phosphorylase *a*. However, a combination of both agents caused a significant ($P < 0.01$) increase in the glycogen phosphorylase a activity. After 6 hours in culture, basal activity was increase by all 3 treatments i.e. GTN, KT5822, and GTN + KT5822 to the same extent. Thus the effect of GTN was not inhibitable by KT5822

At 0 h, 50 nM KT5822 significantly ($P < 0.01$) displaced the concentration-response curve of phenylephrine to the left (decreased EC_{50}) with a significant ($P < 0.05$) decrease in the maximal response to phenylephrine (Figure 3.45**a** and Table 3.17). GTN or GTN + KT5822 had no effect on the potency or efficacy. In comparison, the potency of isoprenaline was not affected by KT5822 but was significantly decreased by GTN and increased by GTN + KT5822 (Figure 3.45**b** and Table 3.17). Furthermore, GTN decreased the E_{max} of isoprenaline by ≈2-fold while GTN + KT5822 increased significantly ($P < 0.001$) increased the E_{max}. KT5822 had no effect on the maximum response to isoprenaline.

At 6 h, KT5822 again displaced the concentration-response of phenylephrine to the left, though to a lesser extent (Figure 3.45**c**). However, KT5822 significantly increased (≈2-fold) the maximal response to

phenylephrine compared to the decrease at 0 h. Furthermore, KT5822 completely blocked the stimulatory response to isoprenaline. GTN, either alone or in combination with KT5822, had no effect on the potency of phenylephrine or isoprenaline and halved the maximal response to isoprenaline. When added in the absence of KT5822, GTN increased the efficacy of isoprenaline at 6 h.

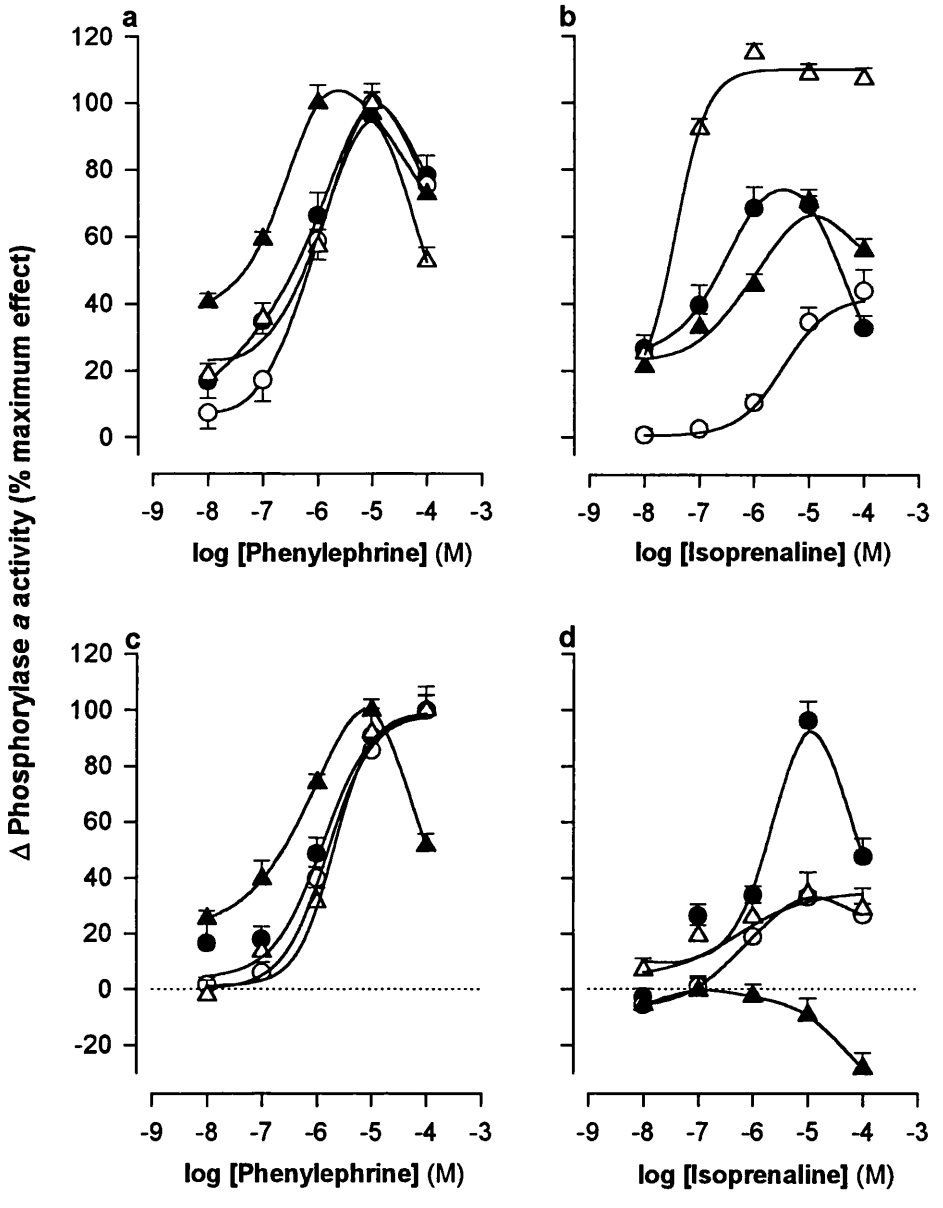


Figure 3.46 Effect of glyceryl trinitrate (GTN) and protein kinase G inhibitor , KT5822 on adrenergic induced activation of glycogen phosphorylase *a* in isolated hepatocytes. Hepatocyte suspensions were preincubated in Williams' E medium for 30 min and then treated for 4 min with increasing concentrations of phenylephrine or isoprenaline in the absence (●) and presence of 50 μM GTN (○), 50 nM KT5822 (▲) or 50 mM GTN + 50 nM KT5822. (Δ). See text for order and times of drug addition. Samples were then processed for phosphorylase *a* assay as described in *Materials and Methods.* Assays were done in duplicate and each data point represents the mean ± s.e. (n=6). The curves are least square fits of the data to the logistic function expressed in Eqn 2.1. For comparative purposes, isoprenaline responses have been expressed as % of respective maximal phenylephrine responses. Basal activities, EC_{50} and E_{max} values are presented in Table 3.17.

Table 3.17 Effect of glyceryl trinitrate (GTN) and KT5822 on the activation of glycogen phosphorylase *a* induced by adrenergic agonists in isolated rat hepatocytes.

Addition	Basal GPase *a* activity[a]	EC_{50} x 10^7 *(M)*		E_{max}[a]	
		Phenylephrine	*Isoprenaline*	*Phenylephrine*	*Isoprenaline*
0 h					
None	32.01 ± 1.34	5.13 ± 1.18	2.69 ± 0.86‡	26.78 ± 0.70	18.30 ± 1.35†††
GTN (50 μM)	34.71 ± 1.22$^{n.s.}$	8.12 ± 0.18$^{n.s.}$	34.67 ± 12.28**††	23.30 ± 1.13*	10.46 ± 0.86***†††
KT5822 (50 nM)	34.1 ± 0.80$^{n.s.}$	2.04 ± 0.41**	7.94 ± 2.93$^{n.s.}$††	27.98± 0.54$^{n.s.}$	20.32 ± 0.37$^{n.s.}$†††
GTN + KT5822	38.47 ± 1.25**	10.23 ± 2.64$^{n.s.}$	0.37 ± 0.06*†††	27.15 ± 0.67$^{n.s.}$	29.52 ± 0.63***†
6 h					
None	23.37 ± 0.53	12.88 ± 3.71	18.20 ± 9.34‡	10.87 ± 0.67	10.46 ± 0.55‡
GTN (50 μM)	31.18 ± 0.77***	15.49 ± 3.46$^{n.s.}$	6.45 ± 2.25$^{n.s.}$‡	17.84 ± 0.76***	5.91 ± 0.27***†††
KT5822 (50 nM)	35.49 ± 1.07***	4.68 ± 1.08**	n.d.	24.62 ± 0.78***	-6.95 ± 1.06***†††
GTN + KT5822	33.89 ± 0.91***	10.71 ± 5.52$^{n.s.}$	5.37 ± 2.76$^{n.s.}$‡	12.69 ± 0.85$^{n.s.}$	4.35 ± 0.80***†††

[a]Values are expressed as nmol min^{-1} mg^{-1} protein are means ± s.e. (n = 6). The values were obtained from the experiments shown in Figure 3.46..

EC_{50}s ± s.e. were obtained by least-square nonlinear regression as described in *Materials and Methods.*

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; $^{n.s.}P \geq 0.05$ represent significant differences from respective controls (Student's t test with Bonferroni's correction) and †$P < 0.05$; ††$P < 0.01$; †††$P < 0.001$; ‡$P \geq 0.05$ represent significant differences from corresponding values for phenylephrine.

3.5.2 Effect of Protein Kinase Inhibitors on Adrenergic Responses in Isolated Hepatocytes

Results presented in this section are from experiments to study further the effects of the protein kinase G inhibitor, KT5822. The effects were compared to those of K-252a (Figure 3.47) and K-525b (Figure 3.48). K-252a is a general protein kinase inhibitor while K-252b is a protein kinase C inhibitor [K_i = 20 nM] (Kase *et al.*, 1987).

Effects of KT5822 and K-252-a

Figure 3.47 shows the effects of the protein kinase inhibitors on adrenergic-induced activation of glycogen phosphorylase *a* in isolated hepatocyte suspensions at 0 and 6 h. In this experiment, responses to phenylephrine were antagonized by KT5822 at 0 h (Figure 3.47**a**). In contrast, the same responses were potentiated by KT5822 in 6-hour old cultures (Figure 3.47**c**). Thus inhibition of protein kinase G (PKG) had opposite effects on phenylephrine responses in 0 h and 6 h cultures. The general protein kinase inhibitor, K-252a antagonized the responses to isoprenaline in both 0 h and 6 h hepatocyte suspensions.

At 0 h, KT5822 inhibited responses to isoprenaline while K-252a caused a reversal of the responses to isoprenaline (from stimulatory to inhibitory) (Figure 3.47**b**). At 6 h, the kinase inhibitors appear to exchange roles, with KT5822 causing the reversal of isoprenaline actions (Figure 3.47**d**).

Effects of KT5822 and K-252b

The effects of KT5822 on responses to phenylephrine in this experiment were not consistent with results presented above. In this experiment, KT5822 potentiated responses to phenylephrine at 0 h (Figure 3.48**a**) and antagonized the responses at 6 h (Figure 3.48**c**). Also, KT5822 potentiated the responses to isoprenaline at 0 h. These results are exactly opposite to the effects observed in

the previous experiment. The protein kinase C inhibitor, K-252b had effects similar to KT5822 on the responses to the agonists at both times .

The reason for this varied effects of KT5822 are not apparent. However, the two cell populations differ in the magnitude of the β-adrenergic response at 0 h. In Figure 3.47, the β-response is 40% of the α-response compared to >80% in Figure 3.48.

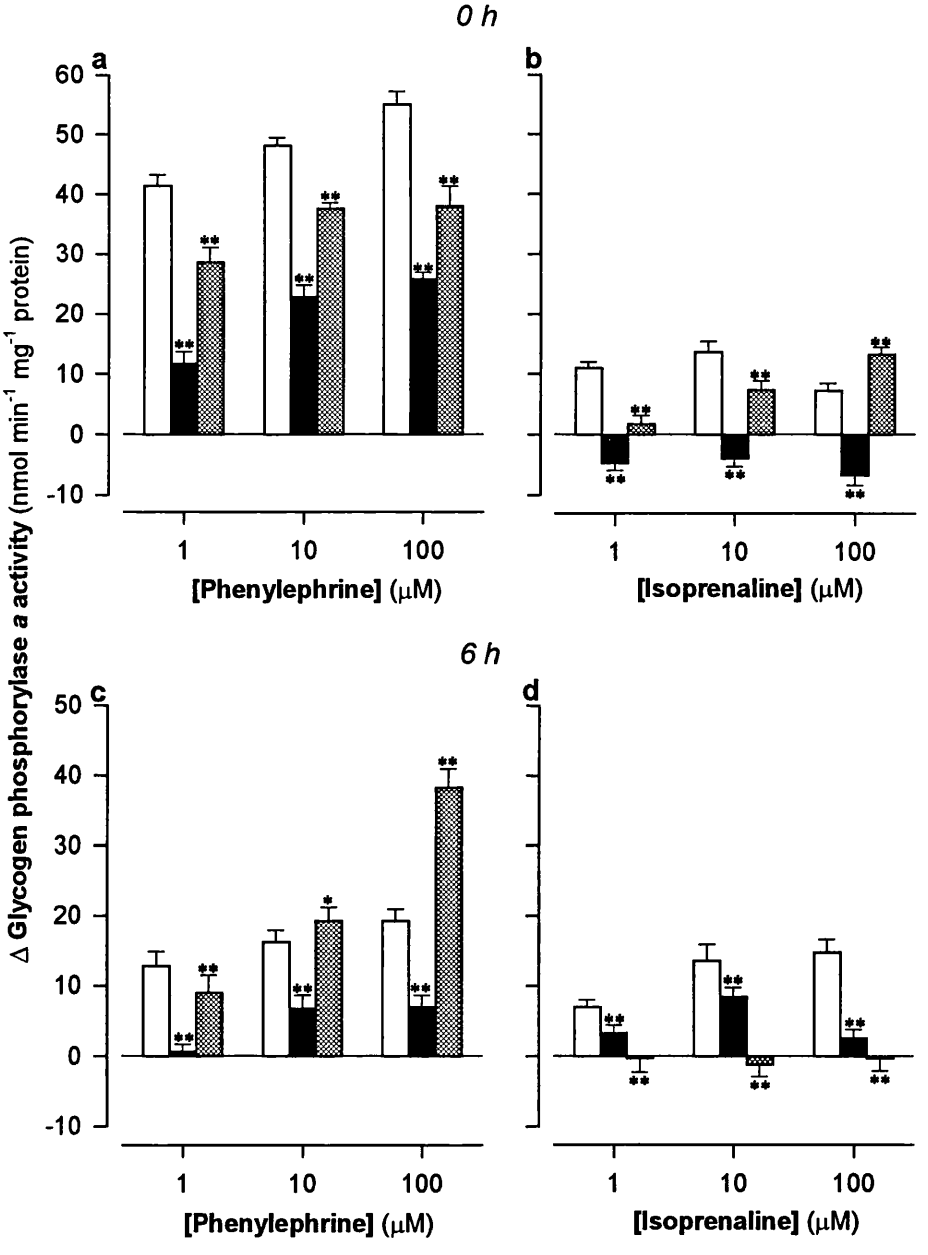


Figure 3.47 Antagonism of phenylephrine- and isoprenaline-induced activation of glycogen phosphorylase *a* by the protein kinase inhibitors, K252a and KT5822. Hepatocyte suspensions were preincubated in Williams' E medium for 30 min and then treated for 4 min with increasing concentrations of phenylephrine (panel **a** and **d**) or isoprenaline (panels **b** and **d**) in the absence (open columns) and presence of 16 nM K252a (solid columns) or 0.2 μM KT5822 (cross-hatched columns). K252a and KT5822 were added 5 min before the effects of the adrenergic agonists were measured. Samples were then processed for phosphorylase *a* assay as described in *Materials and Methods.* Assays were done in duplicate and each data point represents the mean ± s.e. (n=6). **P*<0.5 ,***P*<0.01 compared to control -(open columns) -Student's *t* test with Bonferroni's correction..

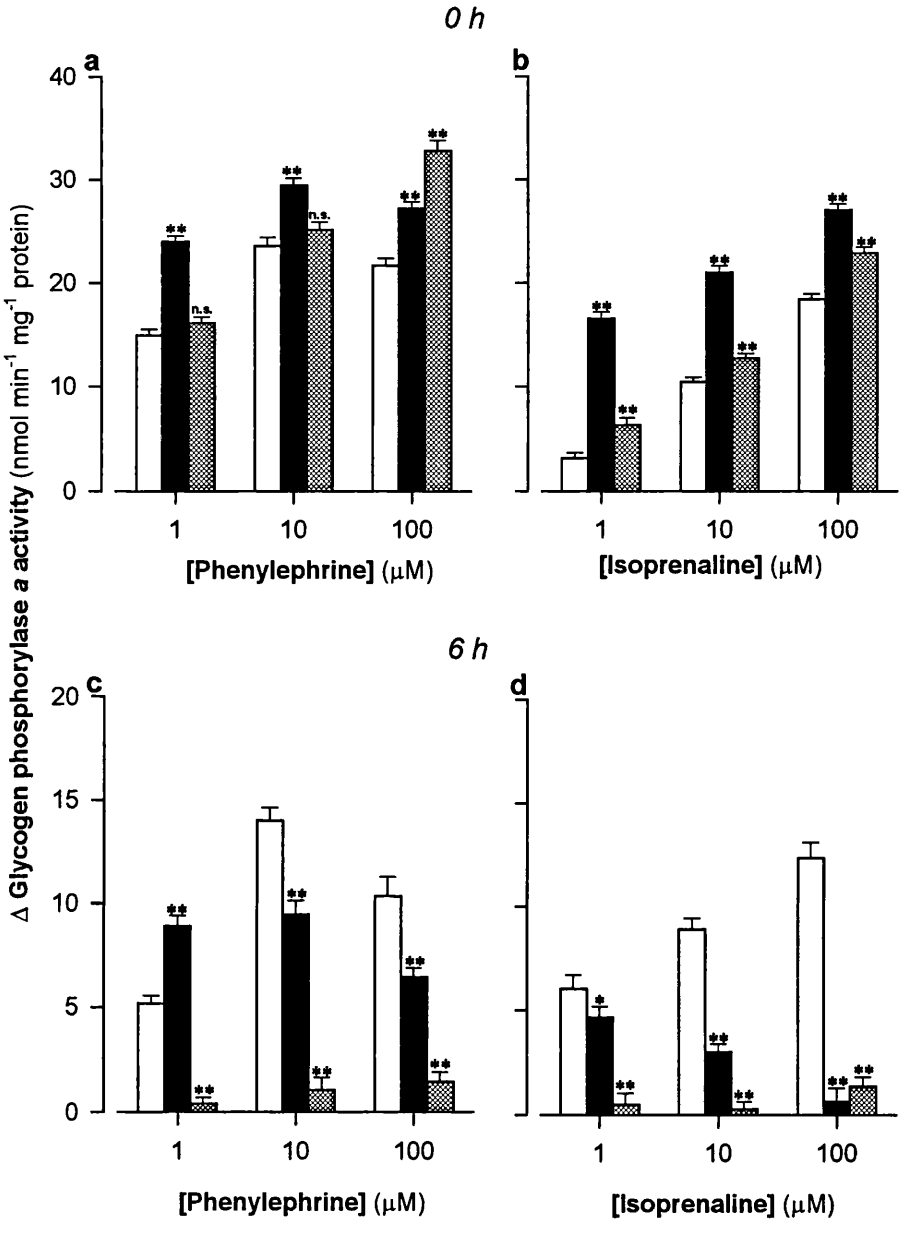


Figure 3.48 Antagonism of phenylephrine- and isoprenaline-induced activation of glycogen phosphorylase *a* by the protein kinase inhibitors, K252b (solid columns) and KT5822 (cross-hatched columns). Hepatocyte suspensions were preincubated in Williams' E medium for 30 min and then treated for 4 min with increasing concentrations of phenylephrine (panel **a** and **d**) or isoprenaline (panels **b** and **d**) in the absence (open columns) and presence of 0.5 nM K252b (solid columns) or 50 nM KT5822 (cross-hatched columns). K252b and KT5822 were added 5 min before the effects of the adrenergic agonists were measured. Samples were then processed for phosphorylase *a* assay as described in *Materials and Methods.* Assays were done in duplicate and each data point represents the mean ± s.e. (n=6). $^{*}P < 0.5$, $^{**}P < 0.01$, $^{n.s.}P > 0.5$ compared to control values (open columns) -Student's *t* test with Bonferroni's correction.

DISCUSSION

4.1 VALIDATION EXPERIMENTS

A major problem associated with the use of crude collagenase in isolation procedures is the damage caused by the presence of other proteolytic enzymes that may damage the surface properties of the cells (Marteau *et al.*, 1988; Meredith, 1988). To ascertain the integrity of the cell membrane, especially regarding receptors, we carried out preliminary experiments to verify the usefulness of the isolation procedure adopted. Hormones, neurotransmitters and growth factors activate glycogen phosphorylase in the liver *via* two main receptor-coupled pathways—a Ca^{2+}/PtdInsP_3-dependent pathway (e.g. adrenaline, arginine-vasopressin, angiotensin II, histamine, ATP, steroids and epidermal growth factor) and a cAMP-dependent pathway (e.g. glucagon, isoprenaline and prostaglandins) (Gomez-Muñoz *et al.*, 1989, Kanemaki *et al.*, 1993, Bocckino & Blackmore, 1993). Glucagon is also reported to act by increasing $[Ca^{2+}]_i$ through a second type of glucagon receptors (Wakelam *et al.*, 1986; Mallat *et al.*, 1987; Kristensen & Gammeltoft, 1987). The results from this investigation clearly indicate cells used in these studies were viable: the cells could respond to the activation of glycogen phosphorylase on stimulation by the hormones used in the study. The hepatocytes also responded to stimulation by dibutyryl cAMP (Bt_2cAMP), a cell-permeant analogue of cAMP (activates PKA), and the calcium ionophore A23187. Ionophore A23187 acts by transporting Ca^{2+} across cell membranes thus increase $[Ca^{2+}]_i$ (Pfeiffer *et al.*, 1974; Pressman, 1976). However, the cells were not particularly sensitive to insulin. There are conflicting reports on insulin's effect on carbohydrate metabolism in hepatocytes. While some report observable responses, other workers have reported the effect of insulin only in the presence of steroids or dependent on the physiological state (fed or fasted) of the donor animal (Agius *et al.*, 1990). A recent report has shown that stimulation of DNA synthesis in

response to insulin in hepatocytes is dependent on the culture medium (Hasegawa *et al.*, 1994). This observation could possibly account for the different effects of insulin on carbohydrate metabolism.

4.2 Activation of Glycogen Phosphorylase By Adrenergic Agonist in Rat Hepatocytes

It is well established that both α- and β-adrenergic agonists induce activation of glycogen phosphorylase *a* in the liver (Blackmore *et al.*, 1978; Exton, 1985; Ishac *et al.*, 1992; Bocckino & Blackmore, 1993).

These results are consistent with that obtained by Tsujimoto *et al.* (1989) who observed that methoxamine selectively stimulated the α_1-adrenergic receptor subtype, α_{1A}. A different α_1-adrenergic subtype, α_{1B}, activates glycogen phosphorylase in rat hepatocytes (Ishac *et al.*, 1992). The low efficacy of methoxamine in activating glycogen phosphorylase in hepatocytes has also been reported by other workers (Aggerbeck *et al.*, 1980). Also García-Sáinz *et al.* (1985) showed that though methoxamine could interact with α_1-adrenoceptors in the liver, it was not able to activate the receptors.

4.3 Effect of Culture Medium on Adrenergic Responses

Activation of both α- and β-adrenoceptors in the liver leads to the same response. The adrenergic response in the adult male rat is mediated by an α_{1B}-adrenergic pathway the β-effect is undetectable or absent (García-Sáinz *et al.*, 1992; Ishac & Kunos, 1987; Studer & Ganas, 1988; Woode *et al.*, 1995). Catecholamines increase glycogenolysis and in turn glucose output in the liver via two main pathways:- a cAMP-dependent β_2-adrenoceptor and a Ca2+/PtdIns-dependent α_1-adrenoceptor pathways (Exton, 1982). The extent to which each pathway is involved depends on age, sex and pathophysiological factors (Ishac *et al.*, 1992). The β-response is almost absent in the liver and freshly isolated hepatocytes of adult male rats. This study shows that the size of

the β-response may depend on the medium used to suspend the cells. The β-response in Williams' E medium was always greater than the response in Krebs-Henseleit buffer.

To our knowledge, the present study is the first instance that the effect of adrenergic agonists on glycogen phosphorylase activity has been investigated in a medium other than simple balanced salt solutions. Previous studies on glycogen phosphorylase activation and/or glucose release induced by adrenergic agonists have used balanced salt solutions such as Hanks' (Nakamura *et al.*, 1983), Earle's salts (Itoh *et al.*, 1984), Krebs-Henseleit (Preiksaitis *et al.*, 1982; Ishac & Kunos, 1987; Hermsdorf & Dettmer, 1992; Ishac *et al.*, 1992). In all these solutions, isoprenaline failed to activate glycogen phosphorylase or cause the release of glucose in isolated hepatocytes. In contrast, other workers (Aggerbeck *et al.*, 1980; Tsujimoto *et al.*, 1986; Schwartz *et al.*, 1985) detected significant responses to isoprenaline even in these solutions. In the paper by Schwartz *et al.* (1985), the maximal response to isoprenaline in Krebs-Henseleit was about 60% of the response to phenylephrine and the potencies of the two agonists were similar. Similarly, in Krebs-Ringer (Aggerbeck *et al.*, 1980) the maximal isoprenaline response was 80% of the maximal response to phenylephrine though isoprenaline was about 24-fold less potent. The presence of the β-response to varying extent in Krebs-Henseleit buffer was also observed in our work. However, in the same pool of cells, the β-response in cells kept in Williams' E was always greater than cells in Krebs'. Reasons for the presence of β-responses are not clear but it is not inconceivable to speculate that this could be due to subtle differences in isolation procedures probably caused by different batches of collagenase.

The difference may be attributable to the presence of amino acids are but the actual physiological significance of this in relation to the results here is not clear. Amino acids however generally stimulate glycogen synthesis (Carabaza *et*

al., 1992; Flückigler-Isler & Walter, 1993) and are known to inhibit hepatic glycogenolysis by causing cell swelling (Häussinger & Lang, 1991, 1992; Baquet *et al.*, 1990). We examined the effects of eight amino acids because of the limited time available and the sheer numbers of amino acids present in Williams' E. Amino acids used in the study were chosen at random starting with those reported to modify some pharmacological or biochemical actions in hepatocytes.

Proline and glutamic acid have been shown to stimulated EGF-induced synthesis in isolated hepatocytes (Nakamura *et al.*, 1984a; Hasegawa *et al.*, 1982, 1994; Houck and Michalopoulos, 1985). Also proline stimulates glycogen synthesis from fasted rats (Baquet *et al.*, 1990). Interestingly, a recent study by Hasegawa *et al.* (1994) shows that the effects of glutamic acid on the stimulation of DNA synthesis depended on the culture media used. Their results showed that when hepatocytes were cultured in Leibovitz L-15 medium was low compared to responses in Williams' medium E or Koga's medium L. Furthermore, they showed that, though EGF was without effect in female hepatocytes cultured in L-15 medium, the responses were increased by more than 10-fold on addition of glutamic acid to the medium. Glutamic acid also increased the EGF response in male rats. Proline also has similar effects on the responses to EGF but was less potent than glutamic acid. Also, the study showed that hepatocytes did not respond well to insulin when cultured in L-15 and, in contrast, Koga's medium L and Williams' medium E permitted the hepatocytes to respond to insulin resulting in stimulation of DNA synthesis. In the present study, the addition of proline to Krebs-Henseleit medium enhanced the responses to isoprenaline: EC_{50} and E_{max} of isoprenaline were both increased. Glutamic also increased the affinity of isoprenaline but not the efficacy. Furthermore, the addition of glutamic acid to the medium caused a decrease in the affinity of phenylephrine. These results together with those

obtained by Hasegawa and co-workers (1994) therefore suggest that proline and glutamic acid may contribute to unmasking the response of the hepatocytes to β-adrenergic stimulation. There is also a possibility that components other than proline and glutamic acid or other mechanisms are involved since increased β-adrenergic responsiveness was also observed in Dulbecco's modified Eagle's medium (DMEM) in this study. Although, DMEM and Williams' E both contain amino acids, the compositions of amino acids are markedly different (see Appendix B). Notably, the quantities of amino acids are several-fold higher in DMEM than in Williams' E and, moreover, DMEM does not contain any proline or glutamic acid.

A recent study has shown that glycine improves hepatocyte viability by inhibiting Ca^{2+}-dependent degradative, nonlysosomal proteases (Nichols *et al.*, 1994). In the present study, the addition of glycine to Krebs-Henseleit buffer had no effect on both α- and β-responses; therefore this beneficial effect of glycine could not be assessed. Moreover, the work by Nichols and co-workers (1994) found glycine to be particularly useful during anoxia.

The ability of valine and lysine to inhibit arginase activity (Boucher *et al.*, 1994) could possibly underlie the results obtained with these amino acids. β-Adrenergic response was barely detectable in the presence of lysine and was about a thousand-fold less than responses to α-adrenergic stimulation. The possible involvement of arginase in the expression of adrenergic responses is discussed in section 4.8. Interestingly arginine, the substrate for arginase, markedly increased the EC_{50} of isoprenaline.

Recent evidence has shown cell-volume changes as potent modulators of cellular function (Häussinger & Lang, 1991, 1992; Häussinger *et al.*, 1994). Hypo-osmotic liver-cell swelling inhibits glycogenolysis, glycolysis and proteolysis but stimulates glycogen and protein synthesis, flux through the pentose phosphate pathway, amino acid uptake and utilization. Therefore

hepatocyte swelling shifts metabolism to an anabolic, proliferative pattern. This observation, together with the conclusion that the isolation procedures 'primed' the normally quiescent hepatocytes to enter the cycle and dedifferentiate (Padgham *et al.*, 1993), could offer a possible explanation for the increased β-adrenergic responsiveness in the presence of amino acids. There is a possibility that a combination of both factors results in an addition or potentiation of the individual effects. That cells isolated by disruption of the liver with collagenase or other procedures are stimulated to proliferate, is supported by the induction of mRNA encoding the transcription factor c-*jun* (Padgham *et al.*, 1993) and c-*myc* and c-*fos* transcripts (Etienne *et al.*, 1988) in such cells. The immediate early genes, c-*myc* and c-*fos* are also expressed in liver cells with high proliferative activities such as after partial hepatectomy or after the administration of carbon tetrachloride (Goyette *et al.*, 1983; Makino *et al.*, 1984 Thompson *et al.*, 1986; Kruijer *et al.*, 1986). These conditions are also associated with increased β-adrenergic responsiveness (Huerta-Bahena *et al.*, 1983; Sandnes *et al.*, 1986; Hatta & Oshika, 1990; Kost *et al.*, 1992).

4.4 Time-Dependent Changes in α- and β-Adrenergic Responses

Results in the present study show, in agreement with previous workers (Nakamura *et al.*, 1984, Tsujimoto *et al.*, 1986; Sandnes *et al.*, 1986 Ishac *et al.*, 1992; Kajiyama & Ui, 1994), a time-dependent increase in β-adrenergic responsiveness. However, while some earlier reports (Okajima & Ui, 1982; Kunos & Ishac, 1985; Schwartz *et al.*, 1985; Ishac *et al.*, 1992; Kajiyama *et al.*, 1994) observed a complete switch from an α_1- to β_2-adrenergic response, our results show that the α-response does not completely disappear with time after the 6 h incubation period. Our findings are consistent with the results of Nakamura *et al.* (1984) and Hermsdorf & Dettmer (1992). In the report by Nakamura *et al.* (1984), the ability of phenylephrine to stimulate glycogenolysis

(measured as glucose release) increased from 0.56 μmol min^{-1} mg^{-1} protein in freshly isolated hepatocytes to 0.79 μmol min^{-1} mg^{-1} protein in 7-h cultured hepatocytes. Similarly, the results of Scheglmann & Dettmer (1992) show little change in responses to phenylephrine in 3-h old culture and a slight increase in the responses in 24-h old cultures. These effects of phenylephrine cannot be attributed to its β-adrenergic actions that have been reported in some tissues (Hoffmann & Lefkowitz, 1990), since the effects of phenylephrine on glycogenolysis in the liver have been shown not to involve cAMP (Assimacopoulous-Jeannet *et al.*, 1977; Exton, 1981).

The apparent differences in the results regarding the α-adrenergic responses may have arisen from the approach each group of workers took in assessing the phenomenon. Most workers have monitored the changes mainly by either changes in the number and affinity of receptors or measurement of the second messengers ($InsP_3$ and cAMP) generated. This approach may give a wrong picture of the situation since there is no general agreement about which step(s) of the signal transduction pathway is involved in the change in adrenergic responses. Also, the α-adrenergic response may be transduced *via* the PKC pathway or through the generation of Ins(1,4,5)P_3 - two distinct pathways (García-Sáinz, 1993). Moreover, recent work supports the existence of at least two α_1-adrenoceptor signalling pathways in the rat liver that can operate independently- one of them is PKC sensitive and Ca^{2+} independent; and the other one is Ca^{2+}-sensitive and PKC independent (Butta *et al.*, 1993; Urcelay *et al.*, 1993, 1994; Ciprés *et al.*, 1995). Thus, a more appropriate way of assessing the change is probably by measuring the physiological response.

Considering the results obtained, there are two 'factors' or 'components' to the reciprocal changes observed- *i)* the potency (EC_{50}) and *ii)* the efficacy (E_{max}). A change in the potency is not always accompanied by a corresponding

change in the efficacy. The 'classical' inversion (complete switch from a- to b-adrenergic response) was observed in the Krebs-Henseleit buffer i.e. after six hours in culture both the E_{max} and EC_{50} of isoprenaline were greater than for phenylephrine. Changes in Williams' E were less dramatic in comparison to Krebs-Henseleit. Also, there was a dramatic decrease in the potency of phenylephrine without any significant changes in the maximal responses to both phenylephrine and isoprenaline. The difference in pattern of changes may have nothing to do with the type of culture medium used since the various patterns were observed throughout this project: Williams' E was the main medium used. A striking observation was that both the α- and β-responses were always present.

4.5 Accumulation of cAMP and Glycogen Phosphorylase Activation in Response to Isoprenaline

Results from the cyclic AMP assay were quite unusual because there was no correlation between the effect of isoprenaline on cAMP accumulation and glycogen phosphorylase *a* activity. The results, therefore appear to suggest that either there is some 'cross-talking' involved in the isoprenaline signal or isoprenaline may be acting by more than one signal pathway. This hypothesis is also supported by results obtained in section 3.5.2, the assay of cyclic AMP levels and by the effects of the nitric oxide donors, SNP and GTN, and protein kinase inhibitors (see sections 4.10). In the cAMP assay, isoprenaline had no effect on basal cAMP levels but stimulated glycogen phosphorylase *a* activity. Furthermore, the effects of isoprenaline were inhibited by SNP and GTN suggesting that guanylate cyclase/cGMP may contribute significantly to the actions of isoprenaline in freshly isolated hepatocytes. 'Cross-talk' between second messenger systems have been observed for the actions of glucagon in hepatocytes (Wakelam *et al.*, 1986; Whipps *et al.*, 1987; Bygrave & Benedetti, 1993) and for isoprenaline in a transfected cell line (Horn *et al.*, 1991) and in

turkey erythrocyte membranes (Rooney *et al.*, 1991). In these cells, glucagon and isoprenaline not only stimulate adenylate cyclase but also cause a rapid rise in InsP_3 with a consequent increase in Ca^{2+} mobilization.

4.6 Effect of Dimethyl Sulfoxide and Sodium Butyrate on Adrenergic Responses

The results show that the use of DMSO or sodium butyrate is not beneficial when used over a short period as with hepatocyte suspensions and that these effects require a much longer period of cultivation. The mechanisms by which DMSO and sodium butyrate promote or maintain differentiation in hepatocytes are not clear. DMSO is thought to act by activating protein kinase C (Yamamoto, 1989; Morley & Whitfield, 1993; Arterburn *et al.*, 1995). Both agents have been used to preserve the high-affinity EGF receptor which is otherwise downregulated during culture (Gladhaug *et al.*, 1988, 1989; 1992). Also, sodium butyrate has been shown to increase the number of β-adrenoceptors in HeLa cells (Tallman *et al.*, 1977, 1978; Lin *et al.*, 1979; Kassis, 1985), in fetal hepatocytes (Lin *et al*, 1979), and to increase the level of thyroid hormone nuclear receptor in primary cultures of adult rat hepatocytes (Mitsuhasi *et al.*, 1987). HeLa cells, like adult male rat hepatocytes, have a substantial number of β-receptors and adenylate cyclase that is responsive to nonhormonal effectors such as guanine nucleotides, forskolin, and cholera toxin but is poorly stimulated by catecholamines (Lin *et al.*, 1979; Kassis *et al*, 1984, Kassis, 1985). When the cells are cultured in 0.6-5 nM sodium butyrate, the number of β-adrenoceptors increases and qualitative changes in G_s occurred which improved their ability to interact with the receptors. The changes occur rapidly reaching a peak after 2-4 hours. These actions of butyrate appear to be distinct from those associated with cell proliferation and differentiation i.e. hyperacetylation of histones and are greatly reduced by inhibitors of protein synthesis (Mitsuhasi *et al.*, 1987). Several possible mechanisms for these actions

have been proposed including: a) an alteration of the nucleotide regulatory component either directly by modification of the protein or indirectly by changes in the lipid environment of the adenylate cyclase system and b) the formation of a new component that serves as a coupling factor between newly formed hormone receptor and the existing nucleotide regulatory component.

DMSO failed to prevent the time-dependent increase of the β-adrenergic response in hepatocytes suspension probably due to the absence of cell-cell contact that is present in monolayer cultures. A recent observation has shown that DMSO induces the gap junctional protein connexin32 and thus causes the reappearance of extensive gap junctional intercellular communications in adult rat hepatocyte cultures (Kojima *et al.*, 1995). Gap junctional intercellular communication is thought to play a crucial role in cell differentiation and growth control in multicelluar organisms (Lang *et al.*, 1991, Mesnil & Yamasaki, 1993).

Butyrate has been shown to cause dramatic accumulation of pyrophosphates which can trap mitochondrial Ca^{2+} especially after addition of calcium mobilizing hormones (Davidson & Halestrap, 1988).

4.7 Effect of Dexamethasone on Adrenergic Responses

One of the several physiological/pathological factors *in vivo* which lead to a switch from α- to β-adrenergic response is glucocorticoid insufficiency (Brønstad and Christofferssen, 1980; Goodhardt *et al.*, 1982; Borle & Studer, 1990). Goodhardt *et al.*, (1982) achieved some success in preventing the changes that occur in the liver by replacement therapy. Glucocorticoids in combination with insulin have also been used, *in vivo*, to counteract the elevated noradrenaline-sensitive adenylate cyclase activity that develops after adrenalectomy (Bitensky *et al.*, 1970; Leray *et al.*, 1973; Hutson *et al.*, 1976). However, their results showed that dexamethasone, in combination with insulin, partially prevented the emergence of the β-response. However, arresting the

switch from α- to β-adrenergic response in primary cultures has met with little success when the culture medium is supplemented with dexamethasone (Christofferssen *et al.*, 1984; Goodhardt *et al.*, 1984; Nakamura *et al.*, 1984).

In the present studies we have reexamined the effects of dexamethasone on α- and β-adrenergic responses in hepatocyte suspensions and in short-term primary cultures. Unlike previous workers, our studies were carried out in serum-free culture medium as serum contains several soluble factors which may modulate or modify the actions of dexamethasone (Barnes and Sato, 1980; Michalopoulos *et al.*, 1982;). However, the concentrations of dexamethasone used were within the range reported in the literature.

As found by previous workers, dexamethasone failed to suppress the emergence of the β-response as measured by the activation of glycogen phosphorylase *a* in response to stimulation by isoprenaline. In fact, the presence of dexamethasone in the culture medium appeared to accelerate the emergence of the β-response. These effects of dexamethasone, though surprising compared to reported effects *in vivo*, are not unexpected. Dexamethasone is well known to cause upregulation of β-receptors in several other tissues and cells (Collins *et al.*, 1991) as well in hepatocytes (Goodhardt *et al.*, 1984; Nakamura *et al.*, 1984).

The results also indicated that the addition of dexamethasone to freshly isolated hepatocyte cultures increased the potency of phenylephrine while response to isoprenaline. This initial beneficial effect of dexamethasone was however lost when the suspensions were kept in culture for 6 hours. The initial effects may be due to inhibition by dexamethasone of inflammatory reactions, probably resulting from enzymatic (e.g., proteases present in the collagenase preparation) destruction of membrane structure during the isolation of the cells (Wang *et al.*, 1985; Meredith, 1988; Liu *et al;* 1994). Also, it been shown that acute phase proteins synthesized *in vivo* during stress periods, are constitutively

produced by hepatocytes in suspension or monolayer cultures (Wolffe & Tata, 1994; Koj *et al.*, 1984) These results suggest that cultured hepatocytes obtained from normal animals are similar to hepatocytes in the intact liver of a stressed animal.

Although dexamethasone has been used successfully to maintain the viability and some liver-specific function of hepatocytes (Laishes & Williams, 1976), the exact mechanisms of action are not clear. However, our results suggest that the synthesis of new protein(s) is involved in the increase in β-responsiveness, since the addition of cycloheximide to the culture inhibited the increased expression of the β-adrenergic response. The synthesis may involve synthesis of β-adrenergic proteins. There are conflicting reports on changes in receptor numbers as a cause for the increased β-responsiveness and, as we have not carried out any test to determine the type of protein(s) involved we cannot confirm or conflict any of the observation made by other workers. However, dexamethasone is known to modulate the proliferation and differentiation of various tissues and cell types (De Juan *et al.*, 1992). The effects of dexamethasone in the liver/hepatocytes appear to depend on, among other factors, the age of the rat and the presence of several soluble factors in the culture medium. In the liver, dexamethasone has been shown to suppress α-fetoprotein and DNA synthesis and together with cAMP inhibits the entrance of cultured adult rat hepatocytes into the S-phase (Vintermyr *et al.*, 1989). Together with EGF, dexamethasone inhibits the initiation of DNA synthesis in cultures of suckling rat hepatocytes (Baribault & Marceau, 1986). In contrast, dexamethasone stimulates DNA synthesis in proliferating hepatocytes (Richman *et al.*, 1976; Brønstad & Christoffersen, 1980; McGowan, 1988).

4.8 Effects of Putrescine and (+)-S-2-Amino-5-Iodo-acetamidopentanoic Acid (AIAP) on Adrenergic Responses

The switch from the α_1- to β-subtypes in adrenergic response in the male rat liver and isolated hepatocytes is associated with conditions associated with cell proliferation (Kunos & Ishac, 1987; Kajiyama & Ui, 1994). Maintenance of hepatocytes at the growth-arrested quiescent stage appears to provide conditions favourable for α_1-receptor-mediated functions. Induction of polyamine biosynthesis is one of the first events in cell proliferation (Morgan, 1994; Hayashi and Murakami, 1995), and the inhibition of polyamine synthesis has been used successfully to arrest cells at the resting (G_0) state (Pegg, 1988; McCann & Pegg, 1992).

The inhibitors of polyamine synthesis used in this investigation were (+)-S-2-amino-5-iodoacetamidopentanoic acid (AIAP) and putrescine. AIAP, an analogue of arginine, is a site-directed inhibitor of arginase (Mendez *et al.*, 1986; Trujillo *et al.*, 1991) and putrescine, a polyamine, is reported to inhibit polyamine biosynthesis *via* a negative feedback mechanism (Fong *et al.*, 1976; Marton & Morris, 1987).

The results suggest that synthesis of polyamines may play a role in the increased β-adrenergic responsiveness since the presence of the inhibitors completely inhibited the increase in the β-response. α-Adrenergic responses were maintained over the incubation period. The mechanisms by which polyamines interfere with adrenergic responses are not clear. However, polyamines have been shown to interact with large anionic molecules such as DNA, RNA, and phospholipids (reviewed in McCann *et al.*, 1987 and Schuber; 1989). Of the effects listed in the literature above, the following may play a role in the diminished α-adrenergic response in hepatocyte cultures and proliferating cells: a) impairment of the activities of PLC and PLA_2, b) stimulation of Ca^{2+} uptake by mitochondria, c) an InsP_3-independent inhibition of Ca^{2+}-efflux from

the endoplasmic reticulum induced by Ca^{2+}-mobilizing agonists. Also, polyamines have been shown to have differential effects on several kinases involved in the biosynthesis of PtdInsP_2, InsP_3 and other polyphosphoinositides (Lundberg *et al.*, 1987; Singh *et al.*, 1995) and may act to modulate the α-adrenergic signal. Interestingly, in the presence of the inhibitors, isoprenaline (β-agonist) inhibited the activity of glycogen phosphorylase *a*. i.e. a reversal of its effects. The liver is one of few organs in which α- and β-adrenergic stimulation leads to the same response (Ruffulo *et al.*, 1991). The results therefore suggest that the inhibitors may cause change(s) in the hepatocytes that enable isoprenaline to play its 'classical' role i.e. oppose the actions of α-adrenergic stimulation. Although not previously investigated or reported in the literature, there is a likelihood that very low concentrations (>10 nM) of isoprenaline may inhibit the glycogen phosphorylase *a* activity.

In contrast to the actions above, polyamines have been shown to play a 'second messenger' role. Isoprenaline has been shown to induce Ca^{2+} fluxes across the plasma membranes of isolated rat ventricular myocytes (Koenig *et al.*, 1983, 1988). These actions were seemingly mediated by a rapid (< 2 min) and sustained increase in cellular polyamines and were blocked by α-difluoromethylornithine, an inhibitor of ODC. This observation implies a receptor-mediated activation of a preexisting, latent form of ODC, presumably associated with the membrane. Similar results were obtained by other group of workers (Mustelin *et al.*, 1986, 1987) working on the mode of action of mitogens in human T lymphocytes. The exact mechanism of ODC activation was obscure but it has been suggested that it could involve G proteins (Mustelin *et al.*, 1987). If such mechanisms exist for the actions of isoprenaline in the liver, then it could possibly explain the little or absence of the β-response in the liver in the quiescent state.

The studies also looked at the effect of AIAP on arginase activity in the hepatocytes. In contrast to inhibition of arginase observed in the uterus and (Mendez *et al.*, 1986), AIAP stimulated arginase in the hepatocyte cultures. Recent reports have shown that AIAP and closely related compound, (+)-S-2-amino-6-iodoacetamidohexanoic acid (AIHA), to be inhibitors of extrahepatic arginase (Trujillo *et al.*, 1992; Chamorro *et al.*, 1996). Although the highest levels of mammalian arginase is found in the liver, the enzyme activity is also widely distributed in extrahepatic tissues including the red blood cells, lactating mammary gland, kidney, lymphocytes and macrophages (Reckowski & Ash, 1994; Wang *et al.*, 1995). The extrahepatic tissues contain an arginase isoform that is distinct from hepatic arginase, though they share certain physico-chemical properties (Herzfeld & Raper, 1976). The two isoforms are termed arginase I and arginase II for the hepatic and extrahepatic forms respectively (Herzfeld and Raper, 1976; Grody *et al.*, 1985). The function of arginase in extrahepatic tissues (these lack the complete urea cycle) is not clear but is thought to be the production of L-ornithine, which serves as a biosynthetic precursor of polyamines (Tabor & Tabor, 19894). Since AIAP was synthesized as an active-site-directed inhibitor (Trujillo et al., 1991), our results suggest differences in the configuration of the active site of hepatic and extrahepatic isoforms.

The actual role of arginase regarding adrenergic responses is not clear. However, if NO plays a significant role in the adrenergic signal then this area is worth pursuing for the following reasons:-

1. arginase and nitric oxide synthase share the same substrate (Robertson *et al.*, 1993; Daghigh *et al.*, 1994)
2. the arginine/NOS and arginine/arginase pathways are mutually antagonistic (Robertson *et al.*, 1993; Boucher *et al.*, 1994; Daghigh *et al.*, 1994)

3. hepatocytes are depleted of arginine during isolation and culture (Paine, 1990).
4. in the intact liver NO plays a very important role in glycogenolysis (Moy *et al.*, 1991).
5. levels of cytochrome P450, capable of catalyzing the formation of NO from NOHA and other substrates (Andronik-Lion *et al.*, 1992; Boucher *et al.*, 1992; Renaud *et al.*, 1993), are altered during cell culture (Bequé *et al.*, 1984; Padgham *et al.*, 1993).

The effect of AIAP on arginase was not consistent with work reported by (Mendez *et al.*, 1986; Trujillo *et al.*, 1991). Recent reports have however shown that AIAP is an arginase II inhibitor and our study appears to be first reported work of AIAP on arginase I in the liver. Thus, the effect of AIAP is not clear. nitric oxide synthase inhibition and more work will have to be done in this respect.

4.9 Cytochrome P450 and Adrenergic Responses

The actions of phenylephrine on glycogen phosphorylase *a* in isolated hepatocytes may involve cytochrome P450 as these actions were inhibited by both metyrapone and SKF525A. These results are consistent with the reported effects of cytochrome P450 on Ca^{2+} fluxes in several cell types. However, the inhibition of responses to isoprenaline by metyrapone was not as expected since it is well established that isoprenaline acts via the adenylate cyclase/cAMP signal pathway (Exton, 19985). This observation further supports the hypothesis that there might be some cross-talking with other pathways or that there is another pathway for the β-adrenergic signal (see section 4.3).

To further examine the involvement of cytochrome P450 in the responses to adrenergic agonists, the effect of β-naphthoflavone-inducible cytochromes P450 were investigated. As expected, treatment of the rats with β-naphthoflavone resulted in increased levels of hepatic CYP1A1 and CYP2A1,

(Lau & Strobel, 1982; Murray & Reidy, 1990). In contrast, βNF treatment decreased the microsomal levels of *CYP*2C11. This is consistent with the observation that several inducers of liver P450 enzymes suppress CYP2C11 (Waxman & Chang, 1995). Inducers of hepatic known to suppress CYP2C11 include ethanol (Badger *et al.*, 1993), 3-MC (Yeowell *et al.*, 1987; Shimada *et al.*, 1989), TCDD (Gustafsson & Ingelman-Sundberg, 1979), phenobarbitone (Waxman, 1984, Shimada *et al.*, 1989), and dexamethasone (Levin *et al.*, 1987). The mechanism(s) by which the drugs and xenobiotics are not clear, however, 3-MC, TCDD, and dexamethasone are each known to decrease serum testosterone levels (Waxman & Chang, 1995). Testosterone regulates the expression of CYP2C11. Surprisingly, these changes in the inventory of cytochrome P450 resulted in a decreased potency of phenylephrine. An increase in potency was expected as responses to phenylephrine were inhibitable by α-naphthoflavone and SKF525A which are known to inhibit CYP1A1 (Murray & Reidy, 1990). This observation may be because βNF induces not only CYP1A1 but also CYP1A2 (not determined by our assays) and CYP2A1. CYP2A1 is present in the neonate male and female rats and female rats but the concentration declines in the male rat after sexual development (Waxman *et al.*, 1985, Imaoka *et al.*, 1991)- microsomal level of CYP2A1 in adult female rat is fourfold that of the adult male rat.

Thus, our initial hypothesis that CYP1A1 was involved in the adrenergic response was not supported by this observation. Nevertheless, a review of the literature together with our result indicates that CYP2C11 could be a likely candidate for changes in the relative expression of α- and β-adrenergic responses in rat hepatocytes. Levels of CYP2C11 are decreased or suppressed during primary culture (Padgham *et al.*, 1993), in chemical induced hepatocarcinogenesis or partial hepatectomy (Padgham *et al.*, 1993; Habib *et al.*, 1994), cholestasis (Chen *et al.*, 1995), and in hyperplasia (Chen *et al.*,

1993). All the conditions listed above also lead to increased β-adrenergic responsiveness with parallel decreases in α-adrenergic responsiveness. Incidentally, CYP2C11 is a male-specific cytochrome P450 (Ryan *et al.*, 1984; Waxman, 1984; Morgan *et al.*, 1985) — α-adrenergic response predominates in the male rat liver. Nonetheless, the involvement of other isoforms, particularly the CYP2Bs, should not be discounted since the levels of these are also decreased under these conditions. Interestingly, the levels of CYP2B and *CYP*1A seem to follow a reciprocal pattern similar to the expression of α- and β-adrenergic responses. CYP2B levels are higher in the liver of male rat and low in neonates increasing with age until adulthood; while CYP1A levels are higher in the female, predominate in the neonate and decrease with age (Ioannides & Parke, 1990). A postulate, drawn from study and results from elsewhere, is illustrated schematically in Figure 4.1.

Interestingly, induction of hepatic cytochrome P450 by β-naphthoflavone alter the composition of eicosanoids generated by hepatic microsomes. Treatment with β-NF increases the production of HETEs (19- and 20-HETEs) by hepatic microsomes, while reducing the formation of EETs (Falck *et al.*, 1990). The major route of cytochrome P450-mediated metabolism of arachidonic acid is epoxidation (Capdevila *et al.*, 1995) and therefore any factor affecting the levels of CYP2C11, the main isoenzyme involved, is likely to adversely affect the production of EETs.

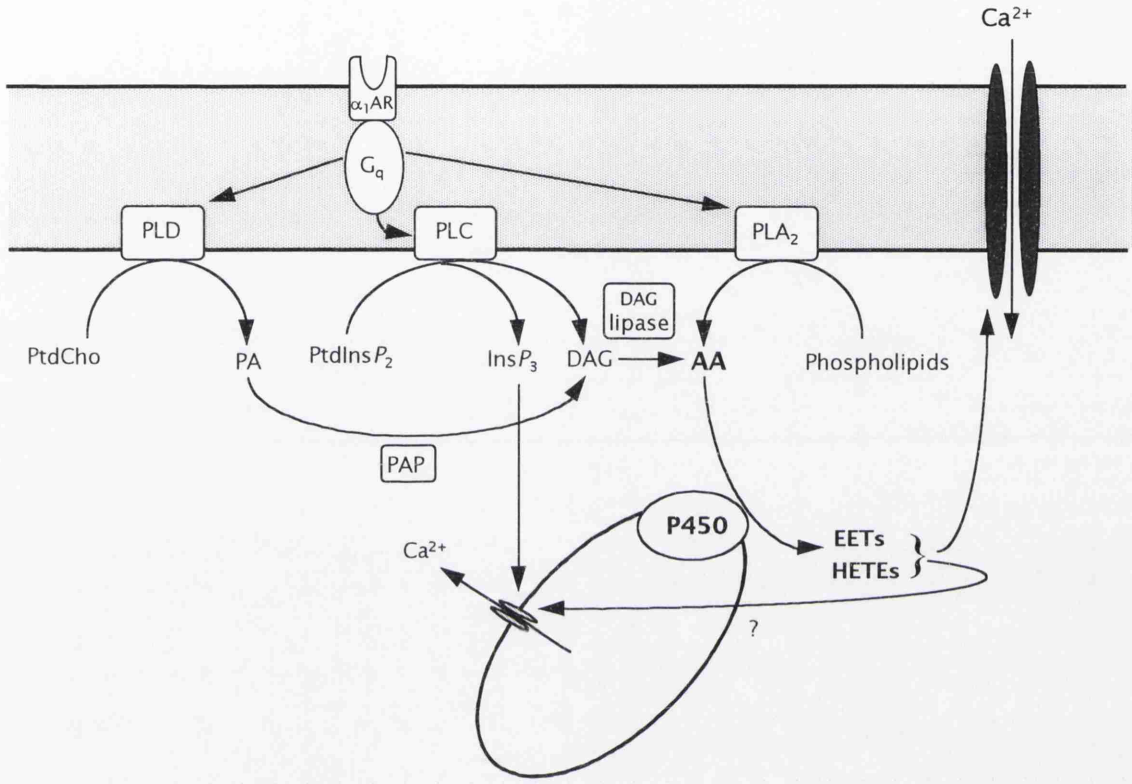


Figure 4.1 Possible mechanisms by which cytochrome P450 may modify α-adrenergic responses.

4.10 Effect of Sodium Nitroprusside, Glyceryl Trinitrate, and Protein Kinase Inhibitors on Adrenergic Responses

Acute and chronic endotoxemia induces several, profound alterations of liver metabolism. In the context of this study, a recent study has shown that chronic endotoxemia -by continuous infusion of *E. coli* endotoxin increased the β-adrenergic responsiveness accompanied by a decrease in the α-adrenergic responsiveness in hepatocytes isolated from such rats (Pittner & Spitzer, 1993a). The same group of workers had earlier shown that chronic endotoxemia caused significant impairments in several aspects of intracellular Ca^{2+} homeostasis including lowered ability to mobilize intracellular Ca^{2+} and concomitantly to activate glycogen phosphorylase in response to Ca^{2+}-mobilizing agonists (Deacuic & Spitzer, 1986, 1989). Recent studies into the mechanisms of hepatocellular dysfunction during sepsis and endotoxemia suggest longer-term effects involve the release of cytokines from Kupffer cells which interact with hepatocytes to induce nitric oxide synthase (NOS) with concomitant nitric oxide production (Billiar *et al.*, 1989, 1990; Curran *et al.*, 1990). Nitric oxide is also released in regenerating liver (Hortelano *et al.*, 1995), one of the several conditions in which there is increase β-adrenoceptor response. The aim of this study was therefore to investigate the effect of nitric oxide on adrenergic responses by using the nitric oxide donors, GTN and SNP.

In the present study, both NO donors, GTN and SNP, activated glycogen phosphorylase activity presumably by activating guanylate cyclase. It is generally accepted that glyceryl trinitrate and sodium nitroprusside act *via* biotransformation to NO which then activates guanylate cyclase with concomitant formation of cyclic GMP. Enzymes suggested for bioactivation of GTN in vascular tissues include glutathione S-transferase and cytochrome P450 (Yeates *et al.*, 1989; Schröeder, 1992, McDonald & Bennet, 1993; Bennet *et al.*, 1994). However, evidence from several studies suggests that the bioactivation of

GTN may involve enzymes that differ between tissues and species (Li & Rand, 1996). The bioactivation of SNP differs from that of GTN (Bates *et al.*, 1991; Kowaluk *et al.*, 1992). Bioactivation of GTN has been shown to involve cytochromes 2C11 and 3A1 (McDonald *et al.*, 1994).

Most of the reported work on the effects of nitric oxide on hepatic glucose metabolism has been carried out using perfused livers with conflicting results. While some workers report no effect on basal glucose output (Moy *et al.*, 1991), others report of inhibition of glucose output resulting probably from inhibition of gluconeogenesis (Brass *et al.*, 1993; Horton et al., 1994, Stadler *et al.*, 1995; Titheradge *et al.*, 1995) and, by contrast, NO has recently been reported to increase hepatic glucose output involving the covalent activation of glycogen phosphorylase (Borgs *et al.*, 1996). The NO-induced increase in hepatic glycogenolysis was blocked by co-administration of cyclooxygenase inhibitors, suggesting a mediatory role of prostanoids from nonparenchymal cells (Borgs *et al.*, 1996). Also, Borgs *et al.* (1996) observed that NO paradoxically inhibited glycogenolysis when the liver was perfused with Ca^{2+}-free medium. In contrast to our results, most of these workers (except Brass *et al.*, 1993 and Stadler *et al.*, 1995) did not observe any effect of NO on glucose metabolism in isolated hepatocytes. Lack of NO effect in isolated hepatocytes but in perfused livers has been explained by the observation that the control of hepatic glycogenolysis involves an intricate paracrine communication between nonparenchymal cells and hepatocytes (Altin & Bygrave, 1988; Kuiper *et al.*, 1988, García-Sáinz, 1989).

Most of the actions of NO have been attributed to its ability to activate soluble guanylate cyclase resulting in the formation of cyclic GMP (Moncada, 1993, Murad *et al.*, 1993). NO donors have been shown to elicit accumulation of cyclic GMP in liver or hepatocytes (Arnold *et al.*, 1987; Brass *et al.*, 1993; Wettstein *et al.*, 1994). A recent work by Rooney *et al.* (1996) has shown that

the cAMP analogues, 8-bromo-cGMP and dibutyryl cGMP as well as the NO-donors, SNP and SIN-1, produced oscillatory $[Ca^{2+}]_i$ increases in rat hepatocytes. Furthermore, they showed that cGMP phosphorylated the InsP_3 receptor in hepatocyte and thereby increased the sensitivity to InsP_3 for $[Ca^{2+}]_i$ release and the subsequent generation of $[Ca^{2+}]_i$ oscillations. Also, by increasing the levels of cGMP, NO oxide could act by increasing the levels of cAMP through the cGMP-mediated inhibition of cyclic nucleotide phosphodiesterases (Manganiello *et al.*, 1992, Sonnenburg & Beavo, 1994). Cyclic-GMP-inhibited cAMP phosphodiesterases (cGI PDEs, Type III cAMP PDEs) have been purified from rat liver (Pyne *et al.*, 1987; Boyes & Loten, 1988). Moreover, the activity of cGI PDEs is rapidly increased in during the incubation of hepatocytes with agents or hormones that increase cAMP (Manganiello *et al.*, 1992).

Contrary to our working hypothesis, the NO donors inhibited the activation of glycogen phosphorylase induced by the β-adrenergic agonist isoprenaline. We had expected NO to enhance the β-adrenergic responses at the expense of α-adrenergic responses as observed in endotoxemia or treatment of hepatocytes with endotoxin (Pittner & Spitzer, 1993). This observation probably suggests that isoprenaline may be acting *via* a cAMP-independent signal pathway (see section 4.5). In the presence of the KT5822, a cGMP-dependent protein kinase inhibitor (Kase *et al.*, 1987; Grider, 1993), NO did not inhibit responses to isoprenaline (the responses were enhanced) suggesting the involvement of cyclic GMP. NO has also been reported to inhibit glucagon-stimulated glycogenolysis in perfused liver and isolated hepatocytes (Brass *et al.*, 1993). Glucagon, like isoprenaline, stimulates glycogenolysis *via* an adenylate cyclase/cAMP-dependent pathway. Although not proven, it has been proposed that NO inhibited glucagon by stimulating the hydrolysis of cAMP by cGMP-mediated stimulation of cAMP phosphodiesterase (Brass *et al.*, 1993; Borgs *et al.*, 1993).

A striking feature of the effect of NO on β-responses is the reversal of the effects of isoprenaline. 'Reversal' of the isoprenaline effect was also observed on addition of putrescine and AIAP to the culture medium.

The effect of NO on phenylephrine-stimulated glycogenolysis was, however, not consistent - NO did not have any effect on phenylephrine-induced glycogenolysis; confirming results obtained in perfused livers by Moy *et al.*, 1991. The inhibition of phenylephrine effects could be explained considering results obtained by Spitzer and coworkers (Spitzer & Deacuic,1986, 1987; Deacuic & Spitzer, 1989), *i.e.* assuming NO is the mediator in chronic and acute endotoxemia. They showed that endotoxemia caused impairments in several aspects of Ca^{2+} homeostasis including lowered hepatocyte ability to mobilize intracellular Ca^{2+} and concomitantly, to activate glycogen phosphorylase in response to Ca^{2+}-mobilizing agonists (Deacuic & Spitzer, 1986). They further showed that the changes in Ca^{2+} homeostasis could be due to changes at the level of the plasma membranes (Roth & Spitzer, 1987) or by the subcellular structures to handle calcium e.g. decreased binding of InsP_3 to the endoplasmic reticulum (Spitzer & Deacuic, 1986, 1987; Deacuic & Spitzer, 1989).

Inhibition of phenylephrine response could also be due to the other actions associated with NO such as:

1. inhibition of hemoproteins involved with cell-signalling events including cytochromes P450 and lipoxygenases (Kanner *et al.*, 1992; Khatsenko *et al.*, 1993; Stadler et al., 1994; Gross & Wolin, 1995). In fact, the soluble form of guanylate cyclase contains a heme group; one of the most sensitive and important sites of action of NO (Gross & Wolin, 1995).
2. nitrosation of thiol-containing proteins such as receptors, and protein phosphatases (Gross & Wolin, 1995). It has also been suggested that NO could modulate protein function via ADP-ribosylation of some proteins. Interestingly, ADP-ribosylation of the inhibitory guanine nucleotide, G_i, has

been proposed as a mechanism for the inverse changes in α- and β-adrenergic responses (Itoh *et al.*, 1984; Ui *et al.*, 1985).

In the context of our studies, the inhibition of cytochromes P450 by NO is intriguing in view of the proposal made earlier (see section 4.9) that cytochrome P450 may be involved the α-adrenergic signal. Cytochromes P450 reported to be inhibited by NO in hepatocytes include CYP1A1 and CYP1A2 (Khatsenko *et al.*, 1993; Stadler et al., 1994)- two of the 'suspects' in our proposal. Though nitric oxide is reported to form spontaneously in primary cultures of hepatocytes (Pittner & Spitzer, 1993), the presence of constitutive nitric oxide synthase (cNOS) has not been immunologically detected in hepatocytes. It is tempting to suggest that cytochrome P450 may play a role in the 'unprimed' hepatocytes since it has been recently shown that cytochrome P450 could catalyze the formation NO from N^{ω}-hydroxy-L-arginine and other substrates (Boucher *et al.*, 1992; Andronik-Lion *et al.*, 1992).

Inhibition of protein kinases in the hepatocytes did not have consistent effect on phenylephrine- and isoprenaline-induced responses. For instance, in a set of experiments, the inhibition of protein kinase G by KT5822 led to and inhibition and potentiation of phenylephrine-induced responses respectively in freshly isolated cells and in 6-hour old cultures. On the contrary, the opposite effects (a potentiation then an inhibition) were observed with the same treatment in another set of experiments. Despite these inconsistencies, it is obvious that cGMP plays a role in the signal of both agonists and that this role may depend on the age of the culture. Also, it is inconceivable to suggest that increased levels of cGMP may partly contribute to the increased β-adrenergic responsiveness in the 6-hour culture; since these cells isoprenaline responses were particularly sensitive to the PKG inhibitor, KT5822.

4.11 General Discussion

It is well established that changes occur in the relative expression of α- and β-receptors in isolated hepatocyte suspensions and monolayer cultures (Schwartz et al., 1985; Sandnes *et al.*, 1986; Kunos & Ishac, 1987). Although several mechanisms have been proposed for these changes in the adrenergic responses, it is still not clear which of these are the major players. It is conceivable that, under a set of conditions, more than one of these mechanisms may act in concert to shift the predominantly α-response to a β-response. Besides showing conditions under which the shift in the changes may be arrested, this study also sheds more light on the possible causes of increased β-adrenergic responses in isolated hepatocyte cultures. The key points or findings in this study are summarized below:

1. besides the well-established regulation of adrenergic receptors and/or responses by hormones (Bohme *et al.*, 1986; Collins *et al.*, 1991), the adrenergic responses may be also modulated by constituents of culture medium e.g. amino acids in William E
2. other transduction pathway(s) may exist for the β-adrenoceptor signal apart from the adenylate cyclase/cAMP pathway
3. DMSO and sodium butyrate inhibited the shift from α to β-adrenoceptor response in monolayer cultures but not in hepatocyte suspensions
4. polyamines or polyamine biosynthesis plays a very important role in the shift in adrenergic responses. This important because a proliferative state is common denominator in all conditions associated with changes in the relative expression of α and β-adrenergic response.
5. cytochrome P450 may be involved in the α-adrenergic signal and that changes in the inventory of the cytochrome P450 isoenzymes during culture may play an important role in the reciprocal changes
6. dexamethasone appears to accelerate the changes in adrenergic responses

Interestingly, polyamines have been shown to markedly affect the metabolism of xenobiotics and oestrogens by microsomal mixed-function oxidases (Schuber, 1989). Also, some inducers of cytochrome P450 in the liver stimulate polyamine biosynthesis (Costa *et al.*, 1976). Studies have shown that the locus of polyamine action is cytochrome P450 itself and that the stimulation could result either from an increased stability of the oxyferrous intermediate of cytochrome P450 or from an acceleration of the second electron transfer from the NADP-cytochrome P450 reductase to cytochrome P450 (Andersson *et al.*, 1981; Dalet *et al.*, 1983). In this study, induction of cytochrome P450 with β-NF blunted the α-adrenergic response. If β-NF did stimulate polyamine biosynthesis, then this could possibly account for the decreased α-adrenergic response because inhibition (presumably) of polyamine biosynthesis by AIAP retained the α-adrenergic responses. However, further investigation is required to fully understand how polyamines and cytochrome P450 interact to modulate α-adrenergic response. It is also interesting to note that the aromatic hydrocarbon receptor, A_h, which regulate genes such as CYP1A1 [the gene responsible for the production of cytochrome P4501A1(CYP1A1)] also plays a key role in proliferation and differentiation of cells exposed to certain chemicals, and perhaps, to endogenous ligands (Okey *et al.*, 1994).

The recent observation that the α-adrenoceptor is also coupled to another nucleotide binding protein, G_h (Achyuthan & Greenberg, 1987, Nakaoka *et al.*, 1994, Huang *et al.*, 1995), distinct from the well-established G_q family (Taylor *et al.*, 1991, Sternweis *et al.*, 1992), may yet provide a clue to the diminished α-response under certain conditions. The α-subunit the G_h protein is unique in that it exhibits two distinct enzyme activities: a) as a guanosine triphosphatase with a signal transduction role and b) as a TGase II, involved with cell growth and activation of phospholipase A_2 (Piacentini *et al.*, 1991; Huang *et al.*, 1995).

Although the functional significance of α-adrenoceptor coupling to two distinct G proteins is yet to be determined, it is tempting to suggest that a signal triggers a switch from α_1-receptor coupling from α_q to α_h. Under this circumstance, the transglutaminase property of α_h may probably prevail over its signal transduction role. This may also explain the lack of glycogenolytic response to α_1-agonist at a time when responses associated with cell proliferation such as DNA syntheses are still present. Experiments designed to answer the questions below may probably solve this mystery.

1. to which G protein is the α_1-adrenoceptor preferentially coupled?
2. does the state of the cell (e.g., quiescent or proliferating) affect the type of coupling?
3. what conditions or factors determine the function (signalling or transglutamination) of $G\alpha_h$?

Also, recent work by Butta *et al.* (1996), has shown that cytoskeletal structures modulate the α-adrenergic responses in the liver; treatment of liver with colchicine blocked glycogenolysis and other α_1-mediated actions. An earlier work by Feng and Kraus-Friedmann (1993) showed that, InsP_3 receptors were associated with cytoskeletal element instead of the endoplasmic reticulum. These observations may be significant because morphologies of cytoskeletal structures are known to be greatly altered in isolated hepatocytes (Arterburn *et al.*, 1995). This could also explain to the beneficial effect of DMSO in this study; DMSO restores to some extent the original morphology in culture (Arterburn *et al.*, 1995).

APPENDICES

APPENDIX A

PERFUSION BUFFERS

Perfusion buffers were prepared from a fivefold stock solution containing the ingredients listed below in 500 ml of distilled water.

NaCl	17.10 g
$NaHCO_3$	5.25 g
Glucose	5.00 g
HEPES	6.50 g
KCl (10% w/v soln)	8.75 ml
$MgSO_4 \cdot 7H_2O$ (10% w/v soln)	7.25 ml
KH_2PO_4 (10% w/v soln)	4.00 ml

The stock solution was stored at 2-4 °C and was not used if more than a week old.

a) Ca^{2+}-Free Krebs-Henseleit solution (with 10 mM EGTA)

Ca^{2+}-free buffer was prepared by dissolving 1.9 g EGTA in 100 ml of the stock solution that has been diluted to 450 ml with distilled water. The pH was adjusted to 7.4 with 0.1 M NaOH and the volume made up to 500 ml with dH_2O.

b) Ca^{2+}-Free Krebs-Henseleit solution (without EGTA)

Twenty milliliter of the stock solution was diluted to ≈90 ml with distilled water. The pH was adjusted to 7.4 with 0.1 M NaOH and the solution made up to 100 ml with dH_2O.

c) Collagenase buffer

As above (a) but containing 4.2 mM $CaCl_2$ (0.42 ml of a 1M $CaCl_2$ stock solution/100 ml of buffer) and the pH adjusted to 7.6. Due to the high concentration of Ca^{2+}, precipitation of calcium salts may occur in the collagenase buffer. This is undesirable and to avoid precipitation, the $CaCl_2$ solution was added gradually to the solution while stirring.

d) Washing buffer

As in (a) but containing 1.5 mM $CaCl_2$.

APPENDIX B

COMPOSITIONS OF CULTURE MEDIA

The compositions of Dulbecco's modified Eagle's medium and Williams' medium E were taken from Catalogue for Cell Culture (1994), Life Technologies Ltd., Paisley, Scotland.

a) Krebs-Henseleit buffer

Component	*g/L*
Inorganic salts	
$CaCl_2 \cdot 2H_2O$	0.22
NaCl	6.84
KCl	0.35
$MgSO_4 \cdot 7H_20$	2.90
KH_2PO_4	1.6
$NaHCO_3$	2.10
Other components	
D-Glucose	2.00
HEPES	2.60

b) Dulbecco's Modified Eagle Medium

Component	*mg/L*
Inorganic salts	
$CaCl_2 \cdot 2H_2O$	264.00
$Fe(NO_3)_3 \cdot 9H_2O$	0.1000
KCl	400.00
$MgSO_4$ (anhyd.)	97.70
NaCl	3500.00
$NaHCO_3$	3700.00
$NaH_2PO_4 \cdot 2H_2O$	141.00
Vitamins	
D-Ca Pantothenate	4.00
Choline Chloride	4.00
Folic Acid	4.00
i-Inositol	7.20
Nicotinamide	4.00
Pyridoxal HCl	4.00
Riboflavin	0.40
Thiamine HCl	4.00
Other components	
D-Glucose	1000.00
HEPES	5958.00
Phenol Red	15.00
Sodium Pyruvate	110.00

Component	*mg/L*
Amino acids .	
L-Arginine • HCl	84.00
L-Cystine	48.00
L-Glutamine	584.00
Glycine	30.00
L-Histidine HCl • H_20	42.00
L-Isoleucine	105.00
L-Leucine	105.00
L-Lysine HCl	146.00
L-Methionine	30.00
L-Phenylalanine	66.00
L-Serine	42.00
L-Threonine	95.00
L-Tryptophan	16.00
L-Tyrosine	72.00
L-Valine	94.00

References: Dulbecco & Freeman, 1959; Smith *et al.*, 1960;

c) Williams' Medium E

Component	*mg/L*
Inorganic salts	
$CaCl_2 \cdot 2H_2O$	264.00
$CuSO_4 \cdot 5H_2O$	0.0001
$Fe(NO_3)_3 \cdot 9H_2O$	0.0001
KCl	400.00
$MgSO_4 \cdot 7H_20$	200.00
$MnCl_2 \cdot 4H_2O$	0.0001
NaCl	6800.00
$NaHCO_3$	2200.00
$NaH_2PO_4 \cdot 2H_2O$	158.00
$ZnSO_4 \cdot 7H_2O$	0.0002
Amino acids	
L-Alanine	90.00
L-Arginine	50.00
L-Asparagine $\cdot H_2O$	20.00
L-Aspartic Acid	30.00
L-Cysteine	40.00
L-Cystine	20.00
L-Glutamic Acid	50.00

Component	*mg/L*
Amino acids contd.	
L-Glutamine	
Glycine	50.00
L-Histidine	15.00
L-Isoleucine	50.00
L-Leucine	75.00
L-Lysine HCl	87.00
L-Methionine	15.00
L-Phenylalanine	25.00
L-Proline	30.00
L-Serine	10.00
L-Threonine	40.00
L-Tryptophan	10.00
L-Tyrosine	35.00
L-Valine	50.00
Vitamins	
Ascorbic Acid	2.00
Biotin	0.50
D-Ca Pantothenate	1.00

Component	*mg/L*
Vitamins contd.	
Choline Chloride	15.00
Ergocalciferol	0.10
Folic Acid	1.00
i-Inositol	2.00
Menadione Sodium	0.01
Nicotinamide	1.00
Pyridoxal HCl	1.00
Riboflavin	0.10
α-Tocopherol	0.01
Thiamine HCl	1.00
Vitamin A Acetate	0.10
Vitamin B_{12}	0.20
Other components	
D-Glucose	2000.00
Glutathione	0.05
Methyl Linoleate	0.03
Phenol Red	10.00
Sodium Pyruvate	25.00

Reference: Williams & Gunn, 1974.

APPENDIX C

BUFFER AND RADIOACTIVE MIXTURES FOR GLYCOGEN PHOSPHORYLASE ASSAY

a) Cell disruption (MOPS) buffer

	CONC (mM)
MOPS	100
NaF	200
NaEDTA	30
Dithiotreitol (DTT)	10

The pH was adjusted to 6.5 with 0.1M KOH. Buffer was stored at 2-4°C and discarded if more than a week old.

b) Radioactive assay mixture for glycogen phosphorylase *a* assay

	Conc.
Glycogen	2%
Caffeine	1 mM
α-D-Glucose 1-phosphate	100 mM
α-D-[U-^{14}C]Glucose 1-phosphate, potassium salt (sp. activity: ≈10.5 Gbq mmol^{-1})	50 nmol

Glycogen, caffeine, and unlabelled Glc1*P* were dissolved in diluted MOPS buffer (1:1) with GENTLE shaking. The pH of the solution was adjusted to 6.5 with 0.1 N HCl, the labeled Glc1*P* added and mixed thoroughly. One-milliliter aliquots were transferred to microcentrifuge tubes and stored at -20°C.

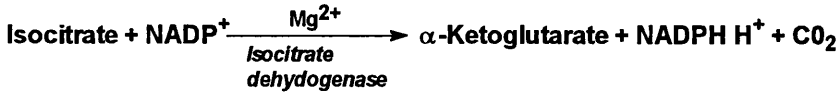
c) Radioactive mixture for the assay of total glycogen phosphorylase (*a* + *b*) activity

Caffeine was omitted from the formula above and replaced with 1 mM AMP and 0.5 M Na_2SO_4.

APPENDIX D

NADPH-GENERATING COFACTOR SOLUTION

The solution was prepared by the method of Gibson & Skett (1994) and involves the use of an enzymatic reaction as follows:



The solution was made by mixing the following components:

0.1 M Tris buffer	8.5 ml
0.15 M $MgCl_2$	1.0 ml
0.5 M Nicotinamide	1.0 ml
Trisodium isocitrate	40 mg
Isocitrate dehydrogenase	2 units
$NADP^+$	8 mg

Nicotinamide is included to prevent the destruction of pyridine nucleotide by tissue nucleosidases. The components are thoroughly mixed just before use, since the generated NADPH breaks down if left standing for more than a few minutes.

1 ml of the cofactor solution is used per assay.

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