STUDY OF THE ROLE OF CYCLIC NUCLEOTIDES IN VASCULAR CELL PROLIFERATION

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by

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SUMMARY

1. Two cyclic nucleotide phosphodiesterase (PDE) activities were identified in pig aortic endothelial cell (PAEC) homogenates , a cyclic GMP- stimulated PDE (Type II) which hydrolyses both cyclic AMP and cyclic GMP and a cyclic AMP- specific PDE (Type IV). The role of these PDE isozymes present in PAEC was evaluated by examining the effects of selective PDE inhibitors on cyclic AMP and cyclic GMP content.

2. Inhibitors of the calcium/ calmodulin- dependent PDE (Type I) and of the cyclic GMP- inhibited PDE (Type III), M & B 22948 and SK & F 94120, respectively, only weakly inhibited the two PDE isozymes. In contrast, dipyridamole and trequinsin, two non-selective PDE inhibitors, potently inhibited both isozymes, whereas rolipram, selectively inhibited the cyclic AMP- specific PDE.

3. Incubation of intact cells with the non- selective PDE inhibitors, dipyridamole (25μM) and trequinsin (25μM), induced large increases in intracellular cyclic GMP content, which were completely blocked by the addition of haemoglobin (10μM). The selective cyclic AMP PDE inhibitor, rolipram (25μM), was without effect on the cyclic GMP content.

4. Dipyridamole (25μM) enhanced the increase in cyclic GMP content induced by the nitrovasodilator and stimulant of soluble guanylate cyclase, sodium nitroprusside (1μM).

5. Atriopeptin II (0.1-100nM), which activates particulate guanylate cyclase, increased the cyclic GMP content in a concentration- dependent manner. Dipyridamole (25μM) and trequinsin (25μM), but not rolipram (25μM), enhanced the increase

in cyclic GMP content induced by atriopeptin II (10nM).

6. The non- selective PDE inhibitor, dipyridamole increased cyclic AMP content at 100μM but not at 25μM. The selective cyclic AMP PDE inhibitor, rolipram (25μM) induced, a large increase in cyclic AMP content.

7. Dipyridamole (25μM and 100μM) enhanced the increase in cyclic AMP content stimulated by the β- adrenoceptor agonist, isoprenaline (25μM), or the activator of adenylate cyclase, forskolin (10μM). Furthermore, rolipram (25μM) enhanced the increase in cyclic AMP content induced by forskolin (30μM).

8. These results suggest that the cyclic GMP- stimulated PDE present in PAEC regulates the cyclic GMP content and the cyclic AMP PDE regulates the cyclic AMP content. Whether or not the cyclic GMP- stimulated PDE also contributes to the regulation of the cyclic AMP content could not be determined.

9. The effects of stimulation of protein kinase C and of cyclic nucleotides on proliferation of PAEC in culture was investigated.

10. Phorbol 12- myristate 13- acetate (PMA. 0.1nM-1μM), which activates protein kinase C, inhibited proliferation in a concentration- dependent manner. No early stimulation of proliferation was seen with PMA (0.3μM). The inactive phorbol ester, 4α- phorbol- 12,13- didecanoate (0.3μM), lacked the ability of PMA to inhibit proliferation of PAEC.

11. Staurosporine (10nM and 100nM) inhibited serum- induced proliferation of PAEC but had no effect on the antiproliferative effects of PMA (0.3μM).

12. PMA- induced inhibition of proliferation appeared not to be

due to stimulated production of destructive oxygen- derived free radicals since it was unaffected by the radical scavangers, superoxide dismutase (30 units/ ml) and catalase (30 units/ ml), vitamin E (30μM), or butylated hydroxytoluene (30μM). The anti-proliferative actions of paraquat (10μM), an agent which generates free radicals intracellularly, was, in contrast, inhibited by vitamin E (30μM) or butylated hydroxytoluene (30μM) but not by the extracellular radical scavangers, superoxide dismutase (30 units/ ml) and catalase (30 units/ ml).

13. Neither dibutyryl cyclic AMP (30μM), nor 8 bromo cyclic GMP (30μM) had any effect on the ability of PMA (0.3μM) to inhibit proliferation of PAEC.

14. Dibutyryl cyclic AMP (30μM) inhibited proliferation, but 8 bromo cyclic GMP (30μM) had no effect. Four other stimuli known to increase PAEC cyclic GMP content by stimulating particulate or soluble guanylate cyclase, atriopeptin II (10nM), bradykinin (0.1μM), sodium nitroprusside (1μM) and glyceryl trinitrate (1μM), were also without effect on proliferation.

15. Two agents known to inhibit soluble guanylate cyclase and lower intracellular cyclic GMP content, haemoglobin (10μM) and methylene blue (10μM), each inhibited proliferation of PAEC.

16. The inhibitory effect of haemoglobin (10μM) was mediated by inhibition of soluble guanylate cyclase since it was reversed by agents known to increase cyclic GMP content i.e. atriopeptin II (10nM), 8 bromo cyclic GMP (30μM) and sodium nitroprusside (1μM). The inhibitory effect of methylene blue (10μM) was not reversed by these agents.

17. L- NMMA (300μM), which blocks the synthesis of nitric oxide by inhibiting nitric oxide synthase, inhibited proliferation of PAEC. This appeared to be a non- specific effect, however, since the inactive D- enantiomer, D- NMMA (300μM) also inhibited proliferation.

18. The non- selective PDE inhibitor, dipyridamole (25μM), inhibited proliferation of PAEC. The antiproliferative effect of dipyridamole was not blocked by the addition of haemoglobin (10μM) and therefore probably resulted from elevation of cyclic AMP and not cyclic GMP content.

19. These results suggest that protein kinase C, cyclic AMP and cyclic GMP have powerful effects on the proliferation of PAEC.

20. The role of protein kinase C and cyclic nucleotides in controlling the proliferation of rat aortic smooth muscle cells (rat ASMC) in culture was investigated.

21. Serum (2-20%) stimulated proliferation of rat ASMC in a concentration- dependent manner.

22. PMA (0.3μM), which activates protein kinase C, had no effect on the proliferation of rat ASMC grown in a range of serum concentrations (4-20%).

23. Phenylephrine (0.1μM-1mM), α_1- adrenoceptor agonist, stimulated proliferation in a concentration- dependent manner, whereas the selective β_1 - and β_2- adrenoceptor agonist, dobutamine (10μM) and salbutamol (10μM), respectively, inhibited proliferation.

24.Dibutyryl cyclic AMP (30μM-1mM) inhibited proliferation in a

concentration- dependent manner. Furthermore, forskolin (1μM-100μM), which activates adenylate cyclase, inhibited proliferation in a concentration- dependent manner. The inactive isomer, dideoxy forskolin (1μM-30μM), lacked the ability of forskolin to inhibit proliferation of rat ASMC.

25. Forskolin- induced inhibiton of proliferation appeared to be mediated via an increase in cyclic AMP content since it was potentiated by the selective cyclic AMP PDE inhibitor, rolipram (30μM).

26. Histamine (1μM-1mM) inhibited proliferation in a concentration- dependent manner. The histamine (10μM)- induced inhibition of proliferation appeared to be mediated via H_2- receptors since the H_2- antagonist, cimetidine (10μM), blocked its antiproliferative effects.

27. 8 bromo cyclic GMP (1mM), glyceryl trinitrate (1mM) and sodium nitroprussside (0.1mM-1mM) inhibited proliferation of rat ASMC in a concentration- dependent manner. In contrast, atriopeptin II (0.1μM) had no effect on proliferation.

28. The sodium nitroprusside- induced inhibiton of proliferation appeared to be mediated by a mechanism independent of the activation of soluble guanylate cyclase, since haemoglobin (20μM) was without effect. Furthermore, M & B 22948 (30μM) failed to potentiate the antiproliferative effect of sodium nitroprusside (0.1mM-1mM). It is likely that the inhibition of proliferation resulted from a cytotoxic actions since sodium nitroprusside (0.1mM-1mM) stimulated uptake of trypan blue.

29. The inhibitory effect of sodium nitroprusside (0.1mM-1mM) was not mediated through the production of cyanide since

methaemoglobin (5μM), which binds cyanide avidly, failed to reverse the antiproliferative effect.

30. These results suggest that cyclic AMP powerfully inhibits the proliferation of rat ASMC. This inhibition of proliferation induced by nitrovasodilators appears to reflect a cytotoxic action which occurs independently of guanylate cyclase stimulation.

PUBLICATIONS

Several aspects of the work presented in this thesis have been published.

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CONTENTS

INTRODUCTION

MATERIALS AND METHODS

DISCUSSION

LIST OF FIGURES

content of PAEC in the presence of haemoglobin.

17 Concentration- effect relationship showing the ability of atriopeptin II to elevate the cyclic GMP content of PAEC in the presence of haemoglobin.

18 Effect of diyridamole, rolipram and trequinsin on the atriopeptin II- induced increase in cyclic GMP content in PAEC.

19 Effects of dipyridamole and isoprenaline on the cyclic AMP content of PAEC.

20 Effects of dipyridamole and forskolin on the cyclic AMP content of PAEC.

21 Effects of rolipram and forskolin on the cyclic AMP content of PAEC.

22 Effects of the active phorbol ester, phorbol 12- myristate 13- acetate (PMA) and the inactive phorbol ester, 4α- phorbol didecanoate (4α-PDD), on the proliferation of PAEC.

23 Concentration- effect curve showing the ability of PMA to inhibit proliferation of PAEC.

24 Effects of PMA and paraquat on proliferation, attachment and on trypan blue uptake by PAEC.

25 Effects of staurosporine on the antiproliferative action of PMA on PAEC.

26 Effects of PMA and staurosporine on [^{3}H]-thymidine incorporation by PAEC grown in a range of serum concentrations.

27 Effects of PMA on [^{3}H]- thymidine incorporation by PAEC.

28 Effects of PMA and staurosporine on [^{3}H]-thymidine incorporation by PAEC.

29 Effects of superoxide dismutase on the antiproliferative action of PMA on PAEC.

30 Concentration- effect curve showing the ability of paraquat to inhibit proliferation of PAEC.

31 Effects of combined treatment with superoxide dismutase and catalase on the antiproliferative actions of PMA and paraquat on PAEC.

32 Effects of vitamin E and butylated hydroxytol-

LIST OF TABLES

ABBREVIATIONS

AP II	Atriopeptin II
BHT	Butylated hydroxytoluene
BK	Bradykinin
8BrcGMP	8 bromo guanosine 3':5'- cyclic monophosphate
C	Control
$CaCl_2$	Calcium chloride
CAT	Catalase
CIM	Cimetidine
CO_2	Carbon dioxide
c.p.m.	Counts per minute
CS	Calf serum
Cyclic AMP	Adenosine 3':5'- cyclic monophosphate
Cyclic GMP	Guanosine 3':5'- cyclic monophosphate
DABA	3,5, diaminobenzoic acid
DBcAMP	Dibutyryl adensoine 3':5'- cyclic monophosphate
DiP	Dipyridamole
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DOB	Dobutamine
d.p.m.	Distintegrations per minute
EDRF	Endothelium- derived relaxing factor
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ET	Endothelin
FGF	Fibroblast growth factor
fmol	femtomoles
FOR	Forskolin

GTN	Glyceryl trinitrate
Hb	Haemoglobin
HBGF	Heparin- binding growth factor
HCl	Hydrochloric acid
HIS	Histamine
IBMX	3-isobutyl-1-methylxanthine
IL-1	Interleukin-1
IP_3	Inositol trisphosphate
IPR	Isoprenaline
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
K_M	The substrate concentration at which the reaction rate is half of its maximal
L- CAN	L- canavanine
LDL	Low density lipoproteins
MB	Methylene blue
M & B 22948	2-0-propoxyphenyl-8-azapurin-6-one
mg	milligram
µg	microgram
µl	microlitre
$MgSO_4$	Magnesium sulphate
ml	millilitre
mM	millimolar
n	number of observations
NaCl	Sodium chloride
$NaHCO_3$	Sodium hydrogen carbonate
NaOH	Sodium hydroxide
NH_4OH	Ammonium hydroxide

nm	nanometre
nM	nanomolar
NO	Nitric oxide
D- NMMA	N^G- monomethyl D- arginine
L- NMMA	N^G- monomethyl L- arginine
L- NOARG	N^G- nitro L- arginine
NO synthase	Nitric oxide synthase
PAEC	Pig aortic endothelial cells
PAR	1,1'- dimethyl-4,4'- bipyridinium dichloride (Paraquat)
4α- PDD	4α- phorbol 12,13- didecanoate
PDE	Phosphodiesterase
PD- ECGF	Platelet- derived endothelial cell growth factor
PDGF	Platelet- derived growth factor
PGD_2	Prostaglandin D_2
PGE_1	Prostaglandin E_1
PGI_2	Prostacyclin
PKC	Protein kinase C
PMA	Phorbol 12- myristate 13- acetate
pmol	picomole
Rat ASMC	Rat aortic smooth muscle cells
RIA	Radioimmunoassay
ROL	4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone (Rolipram)
r.p.m.	Revolutions per minute
SAL	Salbutamol
SD	Seeding density
SK & F 94120	5-(4-acetaminidophenyl)pyrazin-2(1H)-one

SMC	Smooth muscle cells
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
STAUR	Staurosporine
TCA	Trichloroacetic acid
TGFβ	Transforming growth factor β
TNFα	Tumour necrosis factor α
TPA	12- σ-tetradecanoylphorbol-13-acetate
TREQ	9,10-dimethoxy-2-mesitylimino-3-methyl-3,4,6,7,-tetrahydro-2H-pyrimido(6,1-a)isoquinolin-4-one (Trequinsin)
VEGF	Vascular endothelial cell growth factor
ViTE	DL-α- tocopherol acetate (Vitamin E)
V_{MAX}	maximum rate of reaction
VPF	Vascular permeability factor

INTRODUCTION

.1. VASCULAR CELL BIOLOGY

dentification of the hormonal factors and intracellular pathways hat control the migration and proliferation of vascular endothe-ial and smooth muscle cells is important since dysfunction of oth cell types is observed in several cardiovascular diseases Hansson & Bonjers, 1986; Nilsson, 1986; Ross, 1986b; Schwartz et l., 1986). A example of such a disease is atherosclerosis.

.2. ATHEROSCLEROSIS

he term atherosclerosis was originally devised to describe a egenerative process involving the progressive hardening of the lood vessel wall. Today's description of this disease is more omplex: a fatty streak is observed to be present in the early evelopment of atherosclerosis. Furthermore, these lesions are onsidered to be the precursor for the more advanced state of his disease. The fatty streak is characterized by the presence f smooth muscle cells, T- lymphocytes and macrophages. These ells are located at a subendothelial level upon an extracellular atrix composed of lipids, collagen, elastin and proteoglycans. he predominant cell type found here is the macrophage which eadily accumulates lipids and takes on the appearance of foam ells.

he fibrous plaque represents the more advanced state of the isease. These plaques are found to have a cellular core ontaining smooth muscle cells, macrophages and T- lymphocytes, hich is capped by a layer of smooth muscle cells, macrophages nd T- lymphocytes in a dense matrix of connective tissue. urther degeneration of the plaque can occur as the disease rogresses and this includes calcification and ulceration (Bocun

et al., 1986; Small, 1986; Munro et al., 1987; Munro & Cotran, 1988).

1.2.1. Pathogenesis of atherosclerosis

In 1976, Ross & Glomset proposed the 'response-to-injury' model which stated that injury (mechanical, LDL, homocysteine, immunological) to and subsequent denudation of the endothelium was the initiating event for the disease. Injury to the endothelium may involve the loss of either individual cells or in extreme damage, loss of large areas of the endothelium. The endothelial cells respond by either spreading, or by the proliferation and migration of neighbouring endothelial cells to re- endothelialize the denuded area.

Normally the endothelium is an intact monolayer which is nonthrombogenic and secretes various antithrombotic substances, for example, endothelium- derived relaxing factor and prostacyclin, which prevent the adhesion of platelets to the arterial wall. Damage to the endothelial cell monolayer may promote the adhesion and aggregation of platelets to the arterial wall thereby allowing the platelets to release the contents of their α- granules. This includes several factors which are chemotactic and mitogenic for the underlying smooth muscle cells.

Modification of this earlier 'response-to-injury' model is necessary since several studies have indicated that endothelial denudation or platelet adhesion is neither necessary nor sufficient to explain atherosclerosis (Schwartz & Reidy, 1987; Munro & Cotran, 1988; Hansson & Bondjers, 1986). Recently, the involve-

ment of other cells of the immune system, in particular monocytes/ macrophages has been proposed (reviewed in Hansson et al., 1989).

The revised hypothesis is that formation of the fatty streak and the more advanced fibrous plaque can occur via two pathways: the first highlighted in models of experimentally induced hypercholesterolemia. In animals which have been fed a high fat diet it was demonstrated that alterations in the viscosity of the plasma membrane occurs. This increases the attachment of monocytes to the endothelium (Alderson et al., 1986). Furthermore, macrophages enhance this process of adhesion of monocytes to the arterial wall by the localised release of interleukin-1 (Bevilacqua et al., 1985). After attachment, these cells move between the endothelial cells and become localized in the subendothelium. This is observed to be the earliest event in the formation of a fatty streak. The monocytes become activated and secrete numerous chemoattractants and mitogens, such as transforming growth factor β, tumour necrosis factor α and interleukin-1, which maybe sufficient for stimulation of smooth muscle accumulation in the arterial intima (Libby et al., 1988; Raines et al., 1989; Majack et al., 1990). The opportunity for platelets to attach to the endothelium increases thereby allowing the localised release of the contents of the α- granules. Furthermore, it has been shown that macrophages can damage neighbouring endothelial cells by the formation of toxic free radicals. Thus, endothelial injury could result from any or all of three sources, namely, the endothelial cells themselves, macrophages or platelets.

The second revised hypothesis for development of atherosclerosis involves stimulation of the endothelium to release growth factors. This too is an example of a non- denuding injury. The release of these factors induces smooth muscle migration and proliferation, and further growth factor release by the stimulated smooth muscle cells themselves. Figure 1 describes the development of an advanced intimal lesion of atherosclerosis by the different pathways outlined above.

1.2.2. Production of growth factors by cells implicated in atherosclerosis

There is a multidude of different growth promoting and growth inhibitory factors secreted by the various cell types present in the atherosclerotic lesion (endothelial cells, smooth muscle cells, monocytes-macrophages and platelets) (Bowen-Pope et al., 1985; Ross, 1986a; Klagburn & Edelman, 1989). The effects of these growth factors upon the cells located within the arterial wall is important in the development of atherosclerosis. Table 1 summarizes only four of these growth factors secreted by the different cell types: platelet- derived growth factor, fibroblast growth factor, transforming growth factor β and epidermal growth factor. All of these growth factors are present in serum. Fibroblast growth factor is the primary mitogen responsible for the migration and proliferation of endothelial cells whereas all four of the growth factors have been observed to be mitogenic for smooth muscle cells in culture.

The following introductory chapters will discuss the effect of these growth factors upon the migration and proliferation of

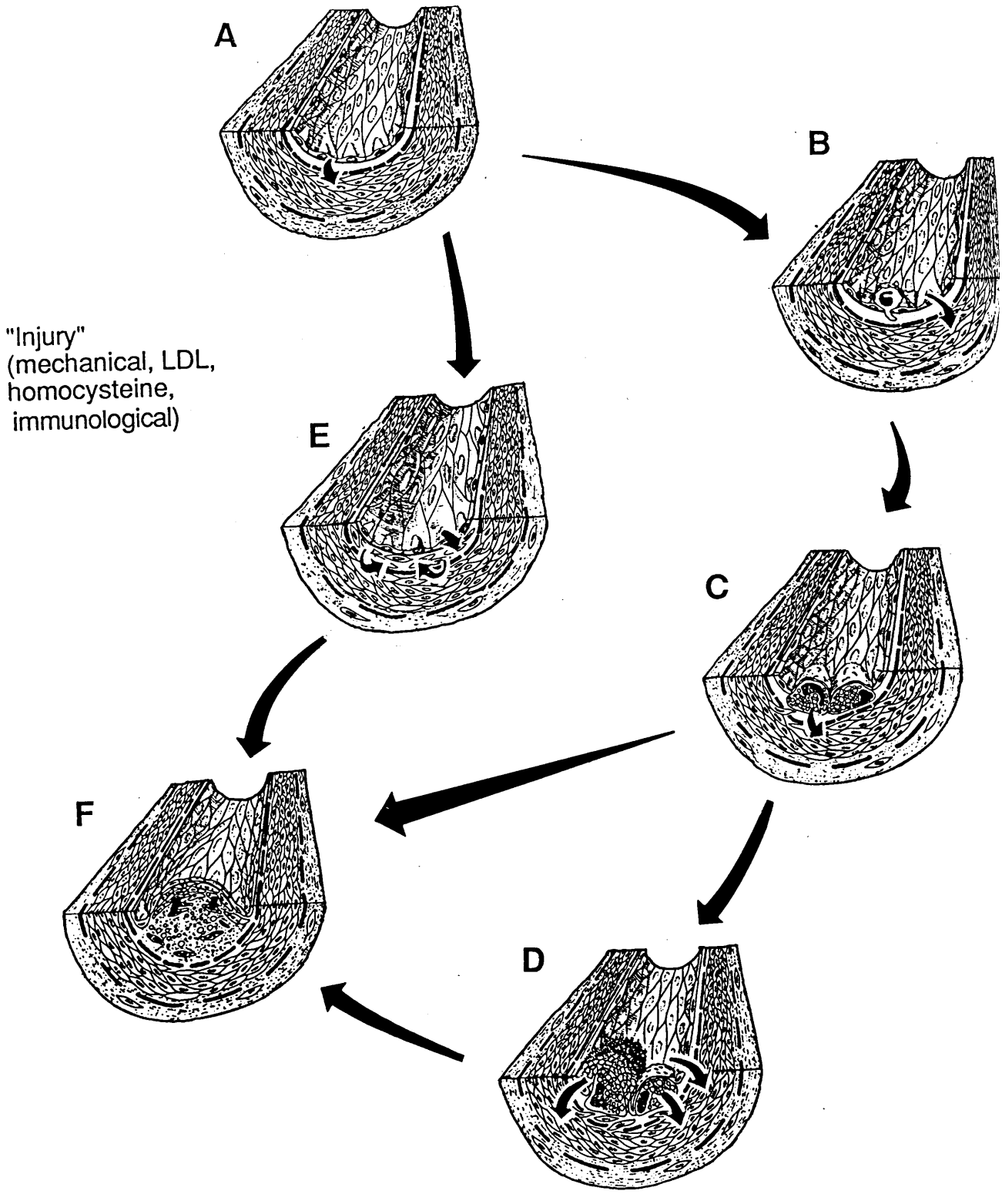

A
B
"Injury"
(mechanical, LDL,
homocysteine,
immunological)
E
C
F
D

Figure 1: Schematic diagram of the Responses to Injury Hypothesis. Advanced intimal lesions of atherosclerosis may occur by at least two pathways. The pathway demonstrated by the clockwise arrows to the right has been observed in experimentally induced hypercholesterolemia. Injury to the endothelium (A) may induce growth factor release (short arrow). Monocytes attach to endothelium (B) and release growth factors. Subendothelial cell migration of monocytes (C) occurs and this leads to the formation of the fatty streak and release of growth factors such as PDGF (short arrow). The appearance of fibrous plaque occurs through the release of growth factors from macrophages or endothelial cells or both (long arrow from C to F). Macrophages may also stimulate or injure surrounding endothelial cells. The loss of endothelial cells allows the attachment of platelets to the arterial wall (D). This allows the localised release of growth factors from platelets. Some of the smooth muscle cells in the lesion (F) may release growth factors such as PDGF (short arrows). An alternative pathway for the development of advanced lesions of atherosclerosis is shown by the arrows from A to E to F. In this case, the endothelium may be injured but remains intact. Increased proliferation of endothelial cells may result in increased growth factor release by the endothelial cells themselves (A). This may stimulate the migration and proliferation of the underlying smooth muscle cells from the media into the intimal. This is accompanied by the endogenous production of PDGF by the smooth muscle cells (E). These interactions could then lead to the formation of the fibrous plaque and further lesion progression (F). This diagram is reproduced from New Eng. J. Med., 314, 488-500, 1986.

vascular cells. They will also highlight the intracellular messengers and pathways that are utilised to commit these cells to intiate DNA synthesis.

Table 1: Production of growth factors by cells associated with atherosclerosis.

CELL	PDGF	FGF	TGFβ	EGF
Platelets	+	-	+	+
Monocytes-Macrophages	+	+	+	+
Endothelial cells	+	+	+	+
Smooth muscle cells	+	+	+	-

1.3. PLATELET- DERIVED GROWTH FACTOR (PDGF)

PDGF is a dimeric polypeptide with a relative molecular mass of 32000 daltons and was initially purified from platelets (Antoniades, 1981). The predominant form of PDGF released by α- granules in platelets is a heterodimer consisting of an A chain and a B chain, but A-A and B-B homodimers are also produced by other cell types (Antoniades, 1981). There is about a 60% sequence homology between A and B chains (Heldin & Westermark, 1987). PDGF is the main mitogenic component found in serum and is known to be a potent mitogen for a diverse array of cells including cells of mesenchymal origin (Kohler & Lipton, 1974; Ross et al., 1974; Seppä et al., 1982; Diliberto et al., 1990).

PDGF has been implicated in the abnormal proliferation of vascular cells that occurs in the development of atherosclerosis (Ross, 1986b). The effect of PDGF is discussed below.

1.3.1. Effect of PDGF on endothelial cell proliferation

With the exception of cells from the microvasculature (Bar et al., 1989), endothelial cells exhibit a growth independence from PDGF by being able to grow equally well in serum- or plasma-supplemented medium (Kazlauskas & DiCorleto, 1985). Even though vascular endothelial cells do not respond mitogenically to PDGF, they synthesize and secrete PDGF or a PDGF- like material (DiCorleto & Bowen-Pope, 1983; Vlodavsky et al., 1987). It is possible, therefore, that the endothelial cell layer is involved in the modulation of events (migration and proliferation) via a paracrine action on adjacent smooth muscle cells in the vascular wall.

1.3.2. Effect of PDGF on smooth muscle cell proliferation

Unlike endothelial cells which exhibit a growth- independence from PDGF in culture, smooth muscle cell growth depends upon the presence of platelet factors as observed by Fager et al. (1988). In this study, human aortic smooth muscle cells were observed not to grow in the absence of PDGF or when an antiserum to PDGF was added to the culture medium. PDGF is a potent mitogen for the vascular smooth muscle cells derived from aortas of many other species including bovine (Banskota et al., 1989), rat (Tomita et al., 1987; Takagi et al., 1988; Abell et al., 1989; Kihara et al., 1989; Majack et al., 1990), and rabbit (Kariya et al., 1987a).

PDGF is produced and secreted not only by platelets, activated monocytes and endothelial cells, but by the smooth muscle cells themselves (Sjölund et al., 1988). The production and secretion

of a PDGF- like material was observed in cultured aortic smooth muscle cells derived from rat pups but not from 3 month old animals (Seifert et al., 1984). Even though these latter cells did not produce or secrete PDGF, they were found to possess cell surface receptors for it thus making them responsive to the mitogen. Furthermore, Walker et al. (1986) reported that smooth muscle cells from the arterial intima produced higher levels of PDGF in culture when compared to medial smooth muscle cells cultured from control uninjured arteries.

1.3.3. Mechanism of PDGF- induced signal transduction

The mechanism of PDGF induced signal transduction has been investigated in several cell types. After binding, PDGF is rapidly internalized and degraded (Bowen-Pope & Ross, 1982). This triggers several biochemical changes that lead individually or collectively to various changes in cellular physiology and to commit the cell to the initiation of DNA synthesis.

These responses include receptor- mediated tyrosine kinase phosphorylation of cellular and nuclear proteins (Cooper et al., 1982; Fields et al., 1990), increases in intracellular pH by activation of Na^+/H^+ exchange and increase in cytosolic Ca^{2+} (Ives & Daniel, 1987; Tucker et al., 1989; Diliberto et al., 1990). On this last point, in rat aortic smooth muscle cells it was found that calcium antagonists and calmodulin inhibitors blocked the PDGF- induced mitogenic response (Tomita et al., 1987). Furthermore, activation by PDGF induces cytoskeletal changes (Bockus & Stiles, 1984) and hydrolysis of phosphatidyinositol thus generating the second messengers

diacyglycerol and inositol trisphosphate (Tsuda et al., 1986). Addition of phorbol esters or a PKC inhibitor, H7, to rabbit and rat aortic smooth muscle cells reportedly blocks the PDGF-induced mitogenic response (Kariya et al., 1987a; Takagi et al., 1988), whereas another investigation found that inhibition of PKC failed to suppress the DNA synthesis induced by PDGF in rat aorta smooth muscle cells (Kihara et al., 1989).

Increased intracellular cyclic AMP concentrations and expression of the proto- oncogenes, c-fos and c-myc (Kelly et al., 1983; Rozengurt et al., 1983a; Armelin et al., 1984; Kruijer et al., 1984; Coughlin et al., 1985; Banskota et al., 1989; Williams, 1989; Diliberto et al., 1990) have also been observed in response to PDGF.

1.4. HEPARIN- BINDING GROWTH FACTOR (HBGF)

The main member of this family is the fibroblast growth factors (FGF). Acidic and basic FGF share a strong sequence homology (55%) and have similiar molecular mass (18000 daltons) but differ on isoelectric points and chromosome position (Gospodarowicz, 1989).

1.4.1. Effect of HBGF on endothelial cells

HBGF are the best characterized and most potent of the endothelial cell mitogens and depending on type, a difference in potency can be observed: acidic FGF is approximately 30- to 100- fold less potent than that of basic FGF (Gospadorowicz, 1989). Both factors induce endothelial cell proliferation, migration and increased production of the enzyme, protease, in culture and are

found to be angiogenic in vivo.

1.4.2. Effect of HBGF-I on endothelial cell proliferation

HBGF-I is a family of acidic polypeptides that stimulate the proliferation of endothelial cells in vitro (Schreiber et al., 1985; Gospodarowicz et al., 1986; Herbert et al., 1988; Hoshi et al., 1988a; 1988b). The main member of this family is acidic FGF but others include endothelial cell growth factor, brain- derived growth factor I and eye- derived growth factor II. Vascular endothelial cells have been shown not to express or produce active HBGF-I (Schweigerer et al., 1987b; Winkles et al., 1987; Bikfalvi et al., 1990).

All these growth factors share similiar chemical and physical characteristics: molecular mass, amino acid composition, isoelectric point (pI 5-6) and possess the ability to strongly bind to heparin.

1.4.3. Effect of HBGF-I on smooth muscle cell proliferation

Unlike vascular endothelial cells which were observed not to actively produce or secrete HBGF-I, human aortic and umbilical vein smooth muscle cells were seen to express the HBGF-I mRNA transcipt (Winkles et al., 1987). HBGF-I has been found to be mitogenic for smooth muscle cells (Mioh & Chen, 1987; Winkles et al., 1987; Hoshi et al., 1988a).

1.4.4. Mechanism of HBGF-I- induced signal transduction

It has been shown that in capillary endothelial cells the binding of HBGF-I to its receptor is followed by rapid internalization

(Friesel & Macaig, 1988). Upon internalization, HBGF-I induces several cellular responses.

In murine 3T3 fibroblasts, the response includes tyrosine phosphorylation of cellular proteins (Huang & Huang, 1986). Addition of purified HBGF-I to quiescent Chinese hamster lung fibroblasts leads to the reinitiation of DNA synthesis without activation of phosphatidylinositol hydrolysis or increased cytosolic calcium concentrations (Magnaldo et al., 1986). This report is in agreement with Tucker et al (1989) who showed that an increase in cytosolic calcium was not necessary for FGF-induced mitogenesis in Balb/ c3T3 cells.

In human aortic endothelial and smooth muscle cells, HBGF-I was found to stimulate the activity of adenylate cyclase, and a concurrent rise in cyclic AMP content was observed (Mioh & Chen, 1987; 1989). Recent reports indicate that HBGF-I increases the cellular levels of several proto- oncogenes: c-fos, c-jun amd c-myc in human umbilical vein endothelial cells (Gay & Winkles, 1990; Lampugnoni et al., 1990). These cellular oncogenes are known to encode for DNA binding proteins which function as transcriptional activators and therefore maybe involved in the mitogenic response to HBGF-I.

1.4.5. HBGF-II

The main growth factor in this group is basic FGF. As discussed earlier, basic FGF possesses 55% sequence homology with acidic FGF but differs on isoelectric point and position on chromosomes (Gospodarowicz, 1989). Basic FGF was initially isolated and

purified from several distinct tissues, including cultured pituitary follicular cells, brain, and placenta (Ferrara et al., 1987; Moscatelli et al., 1986).

1.4.6. Effect of HBGF-II on endothelial cell proliferation

Basic FGF is a potent mitogen for vascular endothelial cells. This mitogenic activity is not species dependent nor is it dependent upon vascular origin of endothelial cells unlike PDGF which is mitogenic only for microvascular endothelial cells (Bar et al., 1989). Bovine aortic and capillary endothelial cells (Gospodarowizc et al.,1978; 1986; Schweigerer et al., 1987a; 1987b; Herbert et al., 1988; Sato & Rifkin, 1988; Presta et al., 1989a, 1989b; De Cristan et al., 1990), human umbilical vein endothelial cells (Gospodarowizc et al., 1978; Hasegawa et al., 1988; Herbert et al., 1988) and rat carotid artery endothelial cells (Lindner et al., 1990) are known to respond mitogenically to basic FGF.

In contrast to HBGF-I, it is known that vascular endothelial cells express, synthesize and secrete their own basic FGF (Schweigerer et al., 1987b; Vlodavsky et al., 1987; Winkles et al., 1987; Sato & Rifkin, 1988; Presta et al., 1989b; Bikfalvi et al., 1990) and are capable of responding to basic FGF through the interaction of the mitogen with high affinity cell surface receptors (Neufield & Gospodarowicz, 1988; Bikfalvi et al., 1989).

1.4.7. Effect of HBGF-II on smooth muscle cell proliferation

Basic FGF is a mitogen for vascular smooth muscle cells and this

mitogenic activity has been observed in bovine aortic (Gospodarowicz et al., 1981) and rat aortic smooth muscle cells (Kihara et al., 1989; Majack et al., 1990).

1.4.8. Mechanism of secretion of HBGF-I and basic FGF from vascular cells

It is proposed that HBGF-I and basic FGF act in an autocrine manner to control smooth muscle and endothelial cell growth, respectively, despite both HBGF-I and basic FGF lacking the classical signal sequences required for secretion (Abraham et al., 1986a; 1986b). This is consistent with the finding that cultured endothelial cells that synthesize basic FGF appear to release little or none into the surrounding bathing medium (Vlodavsky et al., 1987).

Whether or not basic FGF was secreted remained unknown until recently when it was shown that it binds to the endothelial cell-derived glycosaminoglycan, heparan sulphate, a major structural component of the underlying basement membrane, and is secreted as a complex. These complexes of basic FGF and heparan sulphates then become integrated into the extracellular matrix (Saksela & Rifkin, 1990; Baird & Ling, 1987). Furthermore, at areas of regrowth after endothelial cell injury, secretion of the proteolytic enzymes, protease and collagenase, degrades the basement membrane thereby releasing the complexed basic FGF (Saksela & Rifkin, 1990). The released basic FGF induces growth and migration of surrounding endothelial cells thereby promoting the restoration of the endothelial cell layer.

This scheme may indicate that basic FGF has a potential role as a growth regulator of endothelial cells in the arterial wall (reviewed by Klagsbrun & Edelman, 1989). It is possible, therefore, that the release of HBGF-I from smooth muscle cells occurs through a similiar mechanism by complexing with heparan-related glycosaminoglycans.

1.4.9. Mechanism of HBGF-II- induced signal transduction

Basic FGF has been shown to interact with specific cell- surface receptors in human capillary and umbilical vein endothelial cells and this is followed by rapid internalization (Neufeld & Gospodarowicz, 1988; Bikfalvi et al., 1989). This triggers several biochemical changes leading individually or collectively to commit the cell to initiation of DNA synthesis.

The signal transduction pathway utilised by basic FGF has been investigated in a number of cell systems. There is, however, no clear understanding of the primary transducing mechanism involved. Unlike with PDGF, increases in cytosolic calcium are not necessary for mitogenesis in Balb/ c3T3 fibroblasts stimulated with basic FGF (Tucker et al., 1989). Activation of the calcium, phospholipid dependent kinase, protein kinase C (PKC) and increases in cyclic AMP are not consistently observed. In Swiss 3T3 fibroblasts, basic FGF at mitogenic concentrations activates protein kinase C (Blackshear et al., 1985; Moscatelli et al., 1986), whereas as in hamster fibroblasts the basic FGF-receptor signalling pathway was found to be independent of phospholipase C- activated phosphatidylinositol hydrolysis or protein kinase C activation (Magnaldo et al., 1986).

In rat aortic smooth muscle cells, bovine cerebral cortex capillary endothelial cells, normal and transformed foetal bovine aortic endothelial cells, activation of protein kinase C is likely to be responsible for the chemotactic and mitogenic activity of basic FGF. This is suggested since pretreatment of the cells with H7 or staurosporine (protein kinase C inhibitors) or prolonged pretreatment with phorbol esters, abolishes the chemotactic and mitogenic activity of basic FGF in these cells (Kihara et al., 1989; Presta et al., 1989a; 1989b; Daviet et al., 1990). Increased expression of the proto-oncogenes, c-fos and c-myc, have been observed in response to basic FGF (Lampugnani et al., 1990).

1.4.10. Additional members of FGF family

Additional members of the FGF family have recently been identified: int-2, hst/k53 and FGF-5 (Yoshida et al., 1987; Zhan et al., 1988). All three proteins share considerable sequence homology (between 35 and 55%) with acidic and basic FGF. One major structural difference is the presence of a hydrophobic leader sequence that facilitates secretion (Delli- Bovi et al., 1988) and therefore permits access of to the plasma membrane receptors thus closing the autocrine loop. The targets of these growth factors are not yet fully characterized.

1.5. EPIDERMAL GROWTH FACTOR (EGF)

EGF is a polypeptide with a relative molecular mass of 6000 daltons and was first discovered because of its presence in high concentrations within the submaxilary glands of mice (Savage & Cohen, 1972). It is now known that platelets, monocytes-

macrophages and endothelial cells produce and release EGF (Bowen-Pope et al., 1985; Assoian et al., 1984).

1.5.1. Effect of EGF on endothelial cell proliferation

EGF has been shown to have differing effects upon the proliferation of endothelial cells in culture: stimulation of proliferation was observed in human aortic and umbilical vein endothelial cells (Gospodarowicz et al., 1978; Hoshi et al., 1988a) and in a transformed foetal bovine aortic endothelial cell line (Presta et al., 1989a). In contrast, no effect was observed in bovine aortic, bovine umbilical vein or foetal bovine aortic endothelial cells. These cells were shown to lack the receptors for EGF (Gospodarowicz et al., 1978).

1.5.2. Effect of EGF on smooth muscle cell proliferation

EGF is a mitogen for vascular smooth muscle cells including those from bovine aorta, human aorta and umbilical vein, and rat aorta (Gospodarowicz et al., 1981; Owen, 1985; Tomita et al., 1987; Hoshi et al., 1988a; Takagi et al., 1988).

1.5.3. Mechanism of EGF- induced signal transduction

The signal transduction pathway utilised by EGF has been investigated in a number of cell types. There is, however, no clear understanding of the primary transducing mechanisms involved.

After binding EGF triggers receptor- mediated tyrosine kinase phosphorylation of cellular substrates including its own receptor (Cooper et al., 1982; Margolis et al., 1989). Pretreatment of NIH 3T3 cells with a protein tyrosine kinase inhibitor abolished the

mitogenic activity of EGF (Margolis et al., 1989). Stimulation of Na^+/ H^+ antiport activity, of amino acid and glucose transport and of increased c-myc expression have also been observed in response to EGF (Tsuda et al., 1986). Phosphatidylinositol hydrolysis is not consistently observed upon stimulation by EGF. In human foreskin fibroblasts and A431 human epidermoid cells, a phosphatidylinositol hydrolysis was observed (Sawyer & Cohen, 1981; Meisenhelder et al., 1989; Thompson et al., 1989). In contrast in Swiss 3T3 cells there was no hydrolysis of phosphatidylinositol in response to EGF (Tsuda et al., 1986). Furthermore, addition of calcium antagonists, calmodulin inhibitors, phorbol esters, or a PKC inhibitor blocked the EGF- induced stimulation of DNA sythesis in rat and bovine aortic smooth muscle cells (Owen, 1985; Tomita et al., 1987; Takagi et al., 1988).

1.6. INTERACTIONS OF GROWTH FACTORS AND VASCULAR CELL PROLIFERATION

It is apparent that the control of vascular cell proliferation requires complex interactions between growth factors. Control may be mainly under the influence of a specific growth factor whose actions is then modified by others. Several substances have been identified that are known to modulate proliferation of endothelial cells in response to FGF, or of smooth muscle cells in response to serum. These include heparin, transforming growth factor β, tumour necrosis factor and interleukin-1. Several of these factors are released by the numerous cell types present within the atherosclerotic lesions (Table 2), and are discussed below.

Table 2: Production of heparin, transforming growth factor β, tumour necrosis factor α and interleukin- 1 by cells associated with atherosclerosis.

CELL	HEPARIN	TGFβ	TNFα	IL-1
Platelets	-	+	-	-
Monocytes- Macrophages	-	+	+	+
Endothelial cells	+	+	-	+
Smooth Muscle cells	+	+	+	-

1.6.1. Effect of heparin on endothelial cell prolferation

Heparin and its related glycosaminoglycan, heparan sulphate, have been shown to have differing effects on endothelial cell growth: stimulation of growth was observed in human umbilical vein endothelial cells, inhibition of growth was observed in bovine capillary and human omental microvascular endothelial cells, and no effect was observed in human umbilical vein endothelial cells (Gospodarowicz et al., 1986; Gospodarowicz & Cheng, 1986; Bikfalvi et al., 1988; Herbert et al., 1988). In bovine aortic, human capillary and umbilical vein endothelial cells, HBGF-I was seen to become more potent in the presence of heparin, thus making it as potent as basic FGF. It was suggested that this reflected the ability of heparin to stabilize the tertiary structure of acidic FGF (Schreiber et al., 1985; Gospodarowicz et al., 1986; Herbert et al., 1988). In contrast, in the presence of heparin, basic FGF had an either an antiproliferative effect or no effect on human umbilical vein endothelial cell growth (Herbert et al., 1988; Hasegawa et al., 1988).

1.6.2. Effect of heparin on smooth muscle cell proliferation

Heparin and its related glycosaminoglycan, heparan sulphate, have been shown to have an inhibitory effect on the proliferation of vascular smooth muscle cells. This inhibition is not species dependent nor is it dependent upon vascular origin of the smooth muscle cells: heparin inhibited the serum-, PDGF-, HBGF- and phorbol ester- induced growth of bovine and human aortic (Clowes & Karnowsky, 1977; Reilly et al., 1988; Hoshi et al., 1988a; Castellot et al., 1989), rat aortic (Hoover et al., 1980; Guyton et al., 1980; Castellot et al., 1989), rabbit aortic (Herbert & Maffrand, 1989) and human saphenous vein smooth muscle cells (Castellot et al., 1989).

Heparin was found to inhibit the mitogenic response to growth factors which utilise the activation of protein kinase C as a mechanism of signal transduction. The stimulation of proliferation by EGF is independent of activation of protein kinase C and is therefore insensitive to heparin (Castellot et al., 1989). A possible mechanism by which heparin inhibits growth of smooth muscle cells is by its ability to selectively inhibit the expression of the proto- oncocgenes, c- fos and c- myc, induced by a protein kinase C dependent pathway (Castellot et al., 1989).

Furthermore, production of an antiproliferative heparan sulphate by bovine aortic smooth muscle cells has been demonstrated (Fritze et al., 1985). This suggests that smooth muscle cells themselves can control their own growth.

1.6.3. Effect of transforming growth factor β (TGFβ) on endothelial cell proliferation

TGFβ is a polypeptide with a relative molecular mass of 25000 daltons which is secreted by platelets, monocytes- macrophages, endothelial cells and medial smooth muscle cells (Assoian et al., 1984; Sarzani et al., 1989; Antonelli- Orlidge et al., 1989; Assoian & Sporn, 1986).

TGFβ has been shown to act as a bifunctional regulator of endothelial cell growth. TGFβ inhibits the growth of bovine aortic and adrenal cortex capillary and human arterial endothelial cells induced by serum and basic or acidic FGF (Fràter-Schröder et al., 1986; Baird & Durkin, 1986; Hoshi et al., 1988a; Bell & Madri, 1989; Mioh & Chen, 1989). In contrast to bovine aortic and adrenal cortex capillary endothelial cells, TGFβ is mitogenic for bovine corneal endothelial cells and has a synergistic effect on the mitogenic action of basic FGF (Plouët & Gospodarowizc, 1989).

1.6.4. Effect of TGFβ on smooth muscle cell proliferation

TGFβ has been shown to act as a bifunctional regulator of smooth muscle cell growth. TGFβ potentiated the mitogenicity of serum, PDGF-BB and basic FGF in cultures of quiescent, confluent smooth muscle cells, whereas in sparse, proliferating smooth muscle cells, it inhibited growth induced by these stimuli (Hoshi et al., 1988a; Majack et al., 1990).

1.6.5. Mechanism of TGFβ- induced signal transduction

TGFβ has been shown to display a variety of activities: decreased

synthesis of the proteases, collagenase and plasminogen activator, elevated synthesis of inhibitors of both collagenase and plasminogen activator, and the induction of matrix macromolecule synthesis (Saksela & Rifkin, 1990). These actions would tend to decrease the amount of complexed basic FGF released from the subendothelium extracellular matrix, but in bovine corneal endothelial cells TGFβ exerted its mitogenic effect via the increased intracellular content of basic FGF (Plouët & Gospodarowicz, 1989).

TGFβ has been observed to decrease the migration of bovine aortic endothelial cells, whereas it stimulates the migration of bovine aortic smooth muscle cells (Bell & Madri, 1989).

Addition of TGFβ to HBGF-I stimulated human adult endothelial cells induced a reduction in the intracellular cyclic AMP content by decreasing adenylate cyclase activity (Mioh & Chen, 1989). It is likely that therefore that TGFβ exerts its effects on proliferation at least partially through modulation of cyclic AMP content.

1.6.6. Tumour necrosis factor α (TNFα)

TNFα is released from monocytes- macrophages and smooth muscle cells (Warner & Libby, 1989) and has been reported to have several actions on endothelial cells: increased neutrophil adhesion, induction of pro-coaggulant activity, cytoskeletal rearrangement, and stimulated production of PDGF, prostacyclin, interleukin-1 and granulocyte- monocyte- colony stimulating factor (GM-CSF) (Bevilacqua et al., 1986; Nawroth et al., 1986; Hajjar et al.,

1987; Endo et al., 1988; Cotran & Pober, 1989). Furthermore, TNFα induces the expression of interleukin-1 by smooth muscle cells (Warner & Libby, 1989). TNFα is known to inhibit the proliferation of human umbilical vein endothelial cells grown in serum and of capillary endothelial cells stimulated to grow with basic FGF (Schweigerer et al., 1987a; Shimada et al., 1990).

1.6.7. Effect of Interleukin-1 (IL-1) on endothelial cell proliferation

IL-1 is a cytokine which is involved in enabling endothelial cells to participate actively in immune and inflammatory responses (Cotran & Pober, 1989; Martin et al., 1988a).

IL-1 was found to inhibit thymidine incorporation into human umbilical vein endothelial cells (Cozzolino et al., 1990). IL-1 is secreted not only by activated macrophages, but by endothelial cells themselves. Endothelial cells respond following the binding of IL-1 to cell- surface receptors, thus indicating that IL-1 may have an autocrine role in controlling endothelial cell growth (Cozzolino et al., 1990). IL-1 inhibits endothelial growth in response to basic FGF (Cozzolino et al., 1990).

1.6.8. Effect of IL-1 on smooth muscle cell proliferation

IL-1 has been shown to act as a growth factor for vascular smooth muscle cells (Libby et al., 1988; Raines et al., 1989). The mitogenic activity of IL-1 was found to be associated with increased expression and secretion of PDGF from the smooth muscle cells themselves. This was demonstrated by the observation that addition of antibodies against PDGF completely blocked the mito-

genic response to IL-1 (Raines et al., 1989).

1.7.1. INTERACTIONS BETWEEN ENDOTHELIAL CELLS AND SMOOTH MUSCLE CELLS

As introduced earlier, smooth muscle cell migration and proliferation plays an important role in the formation of an atherosclerotic lesion. Upon re- endothelialization of the endothelial cell monolayer there is some indication that regression of the intimal thickening occurs (Ross, 1986b). This would suggest that vascular endothelial cells produce and secrete growth inhibitors for the underlying smooth muscle cells. Several studies have indicated that endothelial cells not only produce growth inhibitory substances, but under certain conditions also growth stimulatory substances, and chemotactic factors for smooth muscle cells.

Endothelial cells are known to produce and secrete a PDGF or a PDGF- like substance which is a potent mitogen for smooth muscle cells (DiCorleto & Bowen- Pope, 1983; Vlodavsky et al., 1987; Staiano- Coico et al., 1988). Other growth promoting substances have been detected in the conditioned medium of bovine aortic endothelial cells (Wang et al., 1981).

On the other hand, the conditioned medium from confluent monolayers of either bovine aortic or human umblilical vein endothelial cells have been found to contain a growth inhibitory substance for actively dividing smooth muscle cells (Castellot et al., 1981; 1982; Willems et al., 1982; Herbert & Maffrand, 1989). It is possible that endothelial cells secrete factors which are not only mitogenic but are chemotactic for the underlying smooth

muscle cells. A chemotactic factor has been detected in the conditioned medium from confluent monolayers of bovine aortic endothelial cells (Autio et al., 1989).

Such interactions between endothelial cells and smooth muscle cells might contribute to the proliferation and migration of smooth muscle cells into the intima during the development of atherosclerosis.

1.7.2. Endothelin

Recently it has been demonstrated that various endothelial cells possess the ability to synthesize and release a vasoconstrictor peptide now recognised as endothelin (ET). These include pig aortic (Yanagisawa et al., 1988) and bovine adrenal cortex capillary and aortic (Hexum et al., 1990; Vigne et al., 1990) endothelial cells. It has been shown, however, that not all endothelial cells possess the ability to produce ET. One such cell type is bovine brain capillary endothelial cells (Vigne et al., 1990).

The release of ET by endothelial cells in culture occurs spontaneously, but can also be stimulated by a variety of substances including IL-1 (Yoshizumi et al., 1990), TGFβ, PDGF and EGF (Resink et al., 1990a), thrombin and the phorbol ester, TPA (Emori et al., 1989). The primary sources of IL-1 and thrombin in the arterial wall are monocytes and platelets, respectively. It has been demonstrated that an important complication in atherosclerosis is the increased vasoconstricted state of affected arteries. A recent study indicated that the ET- induced vasoconstrictor response was potentiated in atherosclerotic arteries

(Lopez et al., 1990). Furthermore, a recent report has demonstrated that cyclic GMP- elevating agents such as atrial natriuretic peptide and the nitrovasodilator, sodium nitroprusside, reduce basal and stimulated ET production from human umbilical vein endothelial cells (Saijunmaa et al., 1990).

Several studies have suggested a possible role for ET in vascular endothelial cell proliferation. ET was observed to stimulate DNA synthesis in bovine brain capillary endothelial cells with greater potency than basic FGF. These cells were found to possess a large number of high affinity cell surface receptors for ET (Vigne et al., 1990). A second study confirmed the growth promoting action of ET (Takagi et al., 1990). Here it was reported that serum- induced proliferation of human umbilcal vein endothelial cells was inhibited by anti- ET antibodies. Furthermore, ET has been shown to stimulate proliferation of rat aortic smooth muscle cells in culture (Komuro et al., 1988; Bobik et al., 1990).

1.7.3. Mechanism of ET- induced signal transduction

The mechanism by which ET induces vasoconstriction and DNA synthesis has been investigated in vascular cells. ET has been shown to interact with high affinity specific cell- surface receptors on endothelial (Vigne et al., 1990) and smooth muscle cells (Hirata et al., 1988; Resink et al., 1990c). The signalling pathways involved include intracellular alkalinization, activation of S6- kinase, activation of phopsholipase C, which stimulates the formation of the second messengers, inositol trisphosphate and diacyglycerol, and mobilization of intracellular calcium (Komuro et al., 1988; Resink et al., 1988; 1990b; Griendling

et al., 1989; Vigne et al., 1990). Increased expression of the proto-oncogenes, c-fos and c-myc, have been observed in response to ET (Komuro et al., 1988; Bobik et al., 1990).

1.8. NEW ANGIOGENIC FACTORS

Acidic and basic FGF are known to be angiogenic in vivo, that is they stimulate the migration and proliferation of endothelial cells, but evidence suggests that other factors may have a role in triggering angiogenesis. Firstly, FGF lacks the hydrophobic signal sequence that governs secretion, but if a factor is to be angiogenic, it must be able to diffuse freely. Secondly, basic FGF is synthesized by the endothelial cells themselves, so if basic FGF is present in and around the endothelial cells at all times but in spite of this the cells are quiescent, then the possibility exists that other factors may initiate angiogenesis. Recent work has identified and characterized several new angiogenic factors. These include:-

1.8.1. Platelet-derived endothelial cell growth factor (PD-ECGF)

This is a platelet- derived endothelial cell mitogen, distinct from PDGF that has recently been identified and characterized (Miyazano et al., 1987; Miyazano & Heldin, 1989). PD-ECGF is a single polypeptide with a relative molecular mass of 45000 daltons.

It has been shown to stimulate migration and proliferation of porcine aortic endothelial cells in culture and angiogenesis in vivo (Ishikawa et al., 1989). The mitogenic activity of PD-ECGF has been examined on a diverse array of cell types and was found

to be mitogenic only for vascular endothelial cells. This mitogenic activity was not species dependent nor dependent upon the vascular origin of the cells (Ishikawa et al., 1989).

1.8.2. Vascular endothelial cell growth factor(VEGF)

This is a family of mitogens recently characterized from the conditioned culture media of either rat glioma cells (Conn et al., 1990a), or of bovine folliculo stellate cells (Ferrara & Henzel, 1989; Gospodarowicz et al., 1989; Plouët et al., 1989). These mitogens appear to be members of a distinct family of growth factors which have been characterized as dimeric polypeptides with a relative molecular mass of 45-46000 daltons and exhibit sequence homology to the human PDGF A and B chains (Tischer et al., 1989; Conn et al., 1990b).

VEGF possesses the ability to bind to heparin and like basic FGF, is complexed with heparan sulphates in the basement membrane. The mitogenic activity of VEGF has been examined on a diverse array of cell types and was found to be mitogenic only for vascular endothelial cells (Ploüet et al., 1989; Gospodarowicz et al., 1989; Ferrara & Henzel, 1989). This mitogenic activity is not dependent on species nor is it dependent upon the vascular origin of the endothelial cells. This is in contrast to PDGF which is mitogenic only for endothelial cells derived from the microvasculature (Bar et al., 1989).

Although PD-ECGF and VEGF have the same apparent unique cell specificity and similiar molecular mass, the two mitogens differ by a 20- fold difference in potency (VEGF is the more potent

mitogen) and by their secondary structure (PD-ECGF is a single polypeptide whereas VEGF is a dimer).

1.8.3. Vascular permeability factor(VPF)

VPF was isolated and purified from the conditioned medium of a guinea pig line 10 tumour cells (Senger et al., 1990). VPF has been characterized as a dimeric polypeptide with a relative molecular mass of 40000 daltons and was originally described as a permeability factor since it promoted the leakage of plasma fluids and proteins across the endothelial cell layer (Senger et al., 1990).

Recently VPF was found to be angiogenic when injected intradermally into guinea pigs and to be mitogenic for human umbilical vein endothelial cells and bovine aortic and adrenal capillary endothelial cells in culture (Connolly et al., 1989; Ferrara & Henzel, 1989).

Recent work has indicated that VPF possesses identical NH_2-terminal sequences to VEGF. Therefore, VPF might be considered to be an additional member of the mitogenic family of VEGF (Connolly et al., 1989; Ferrara & Henzel, 1989).

1.9. PROTEIN KINASE C (PKC) AND CELLULAR PROLIFERATION

PKC was first reported in 1977 as a proteolytically- activated protein kinase located in many tissues with no obvious role in signal transduction (Inoue et al., 1977). Later it was shown that it was a calcium- activated, phospholipid- dependent enzyme which was firmly linked to signal transduction (Nishizuka, 1984). PKC

is widely distributed in many tissues and organs of mammals and is involved in mediating an intracellular signal that triggers various cellular responses including those elicited by the growth factors PDGF and basic FGF.

PKC is transiently activated by diacylglycerol, which is one of two second messengers produced by the degradation of phosphatidylinositol bisphosphate in response to stimulation of phospholipase C (Bell, 1986). The other messenger, inositol trisphosphate, activates the release of calcium stored in the endoplasmic reticulum (Putney, 1987; Rana & Hokin, 1990). More recent work has discovered an alternative source of diacylglycerol. This occurs via the degradation of phosphatidylcholine by phospholipase C or D to yield phosphatidic acid which is further degraded to diacyglycerol by phosphatidic acid phosphohydrolase (Pelech & Vance, 1989).

1.9.1. Molecular Heterogenity and Structure

PKC is a single polypeptide chain with an approximate molecular mass of 77000 daltons and is composed of two functionally different domains: a hydrophobic domain that binds to the plasma membrane and hydrophilic domain that contains the catalytically active centre.

Initially four species emerged from the screening of a variety of complementary DNA libraries: α, βI, βII and gamma. The enzymes with βI- and βII- sequences are derived from a single RNA transcript by alternative splicing, with βII being the most abundant. Each of the four PKC subspecies consists of a single

polypeptide with four conserved (C1- C4) and five variable (V1-V5) regions. The amino- terminal containing the C1 and C2 regions is the regulatory domain and interacts with calcium, phospholipid and either endogenous diacyglycerol or exogenous phorbol esters. The C1 region consists of a tandem repeat of cysteine- rich zinc-finger like sequences which seem to be essential for binding of phorbol esters implying its involvement in the membrane- PKC interaction (Ono et al., 1989b). C2 may be necessary for the calcium sensitivity of the enzyme (Ono et al., 1988; 1989a). The carboxyl- terminal containing the C3 and C4 regions show sequence homology with many other protein kinases (reviewed in Nishizuka, 1988). C3 contains the catalytic site of the enzyme. More recently at least three other subspecies have been identified: δ, ε and ζ. These subspecies have a common structure closely related to, but clearly distinct from the four subspecies described above. These additional members lack the C2 region of the regulatory domain and the requirement of calcium for enzymatic activity. It has been suggested that these subspecies of PKC may be responsible for the large diversity of responses observed upon activation of these enzymes (Coussens et al., 1986; Nishizuka, 1988; Farago & Nishizuka, 1990).

1.9.2. Phorbol esters

Phorbol esters are potent tumour promoters that were originally isolated from the oil of Euphorbiacea Croton tiglium and identified by Van Duuren & Orris (1965) and Hecker (1968). Phorbol esters are now known to activate PKC by interacting with the enzyme at the same site as diacyglycerol (Ashendel, 1985).

structural analysis indicates that the phorbol ester, phorbol 12-myristate 13-acetate, has a diacyglycerol- like structure which enables it to substitute for the endogenous activator at extremely low concentrations. There is an approximate correlation between the ability of the individual phorbol ester to promote tumours and to activate the enzyme (Castagna et al., 1982).

1.9.3. Function of PKC

Upon activation of PKC by either phorbol esters or by diacyglycerol, a rapid translocation of the enzyme occurs from the soluble to the particulate fraction without any change in PKC activity. This is associated with their role in cellular responses (Blackshear, 1988). Prolonged treatment with phorbol esters leads to a decrease or a down- regulation in PKC activity which is distinguishable from the rapid translocation of the enzyme.

Activation of PKC and subsequent phosphorylation of proteins (Takai et al., 1985; Kuroki & Chida, 1988) has been found to be involved in several cellular responses: modulation of ion conductance (Nishizuka, 1986); down- regulation of receptors (Hoshi et al., 1988b); stimulation of gene expression (Colotta et al., 1988; Murphy et al., 1988; Reuse et al., 1990); stimulation of protein synthesis (Brostrom et al., 1987); stimulated release of enzymes and growth factors (Nishizuka, 1986; Murphy et al., 1988) and interference in progression of the cell cycle (Fukumoto et al., 1988). These diverse functions may well involve activaion of the different PKC subspecies as described earlier (Coussens et al., 1986; Nishizuka, 1988; Farago & Nishizuka, 1990).

In addition to these stimulatory actions of PKC there is accumulating evidence to indicate that PKC provides an inhibitory control of cell signalling. It has been reported that PKC inhibits calcium mobilization by blocking the receptor- mediated hydrolysis of inositol phospholipid and hence the production of diacyglycerol and inositol trisphosphate (Nishizuka, 1988). This inhibitory role of PKC may be extended to include cellular proliferation.

1.9.4. Effect of phorbol esters on cellular proliferation

Phorbol esters, through activation of PKC have been shown to have a dualistic effect on DNA synthesis and proliferation in a variety of cell types examined. A stimulation of proliferation was observed in Swiss 3T3 cells (Collins & Rozengurt, 1982), lymphocytes (Kaibuchi et al., 1985), thyroid cells (Bachrach et al., 1985), primary adrenocortical cells (Menapace et al., 1987) and an interleukin-1 dependent T cell line (Goto et al., 1988).

In contrast, an inhibition of proliferation was observed in human lung carcinoma (Gescher & Reed, 1985), human melanoma (Huberman et al., 1979), human epithelial (Mckay et al., 1983) and human breast cancer cells (Osborne et al., 1981).

1.9.5. Effect of phorbol esters on endothelial cell proliferation

Activation of PKC by phorbol esters has been shown to have differing actions on the proliferation of endothelial cells from identical or from different sources in culture: inhibition of proliferation of human aortic endothelial cells (Hoshi et al., 1988b) and bovine adrenal capillary endothelial cells (Doctrow &

Folkman, 1987); stimulation of proliferation of bovine cerebral cortex capillary endothelial cells (Daviet et al., 1989; 1990), human umbilical vein endothelial cells (Dupuy et al., 1989), and of a transformed foetal bovine aortic endothelial cell line (Presta et al., 1989a) and had no effect on proliferation of foetal bovine aortic endothelial cells (Presta et al., 1989a), human omental microvascular endothelial cells (Dupuy et al., 1989), bovine aortic endothelial cells (Doctrow & Folkman, 1987) and bovine adrenal capillary endothelial cells (Morris et al., 1988).

In pig aortic endothelial cells, phorbol esters have been reported to produce an initial stimulation of proliferation followed within hours by an inhibition (Uratsuji & DiCorleto, 1988). In this latter study the presence of multiple subspecies of PKC was demonstrated in endothelial cells. It is possible that each of these subspecies of PKC has a different role in regulating the proliferation of endothelial cells.

1.9.6. Effect of phorbol esters on smooth muscle cell proliferation

Activation of PKC by phorbol esters has been shown to have differing actions on the proliferation of smooth muscle cells from identical or from different sources in culture. A stimulation of proliferation has been reported for smooth muscle cells obtained from bovine pulmonary artery and aorta (Dempsey et al., 1990; Doctrow & Folkman, 1987), rat aorta (Owen, 1985) and rabbit aorta (Kariya et al., 1987a).

In contrast, an inhibition of proliferation has been observed for smooth muscle cells obtained from rat aorta (Kihara et al., 1989) and in rabbit aorta (Kariya et al., 1987b; Fukumoto et al., 1988).

One study demonstrated the ability of PMA to stimulate or inhibit the proliferation of rabbit aortic smooth muscle cells when the cells were either grown in plasma- derived serum or whole blood serum, respectively (Kawahara et al., 1988). The dualistic action of PMA on rabbit aortic smooth muscle cells was suggested to reflect the ability of phorbol esters to either down- regulate growth factor receptors (Owen, 1985), or to reflect the activation of the numerous PKC isozymes present in smooth muscle cells (Kawahara et al., 1988).

1.9.7. Inhibitors of PKC

Much of our knowledge of the actions of PKC has been obtained by the use of activators of this enzyme such as phorbol esters. The role of PKC in cellular physiology has been further elucidated, however, by the recent development of potent inhibitors of PKC such as, staurosporine, a microbial alkaloid (Tamaoki et al., 1986).

Staurosporine blocks phorbol ester- and basic FGF- induced growth of bovine cerebral cortex capillary endothelial cells (Daviet et al, 1989; 1990) and serum- and phorbol ester- induced growth of rabbit and rat aortic smooth muscle cells (Matsumato & Sasaki, 1989; Takagi et al., 1988),and, phorbol ester- and PDGF-induced growth of NIH/3T3 fibroblasts (Fields et al., 1990).

Inhibitiors of protein kinase C are therefore powerful tools with which to investigate the role of this enzyme in cellular responses.

1.10. CYCLIC NUCLEOTIDES AND CELLULAR PROLIFERATION

Prior to 1968, there was little interest in the possibility that cyclic nucleotides were involved in cell proliferation. This lack of interest was altered following the observations of several groups (Bürk, 1968; Ryan & Heidrick, 1968; MacManus & Whitfield, 1969). These studies demonstrated that addition of either cyclic AMP itself or cyclic AMP phosphodiesterase inhibitors inhibited the growth of BHK 21/13 hamster cells, HeLa cells and rat thymic lymphocytes. This led to extensive research into the possibility that cyclic AMP, and subsequently cyclic GMP, had a role in cellular proliferation.

1.10.1. Role of cyclic AMP in cellular proliferation

Early observations indicated that cultured fibroblasts made quiescent by serum deprivation possessed a markedly elevated cyclic AMP content which fell upon release from quiescence (Anderson et al., 1973; Kram et al., 1973; Moens et al., 1975). In other extensive studies cyclic AMP analogues or agents that cause an elevation in intracellular cyclic AMP content were found to have either a growth inhibitory or stimulatory effect (reviewed in Pastan et al., 1975): stimulation of proliferation was observed in mammary epithelial cells (Yang et al., 1980), hepatocytes (McGowan et al., 1981) and Swiss 3T3 fibroblasts (Rozengurt et al., 1983b; O'Neill et al., 1985) whereas an antiproliferative

effect was observed in normal fibroblasts (Hollenberg & Cuatrecasas, 1975) and B-lymphocytes (Muraguchi et al., 1984). A recent study indicates that forskolin, which stimulates adenylate cyclase (Seaman & Daly, 1981) inhibits the growth- promoting effect of PDGF in cultures of human foreskin fibroblasts (Heldin et al., 1989).

1.10.2. Role of cyclic GMP in cellular proliferation

In contrast to cyclic AMP described above, cultured 3T3 fibroblasts made quiescent by serum deprivation contained low levels of cyclic GMP which rose drammatically when the cells were released from quiescence (Moens et al., 1975; Rudland et al., 1974). More recently, atrial natruretic factor which elevates cyclic GMP content has been observed to have inhibit rat glomerular mesangial cell growth in culture (Johnson et al., 1988).

1.10.3. Cyclic nucleotide generation in vascular endothelial cells

In several studies, atrial natriuretic peptides and nitrovasodilators, which stimulate particulate and soluble guanylate cyclase, respectively, have been shown to elevate cyclic GMP content in endothelial cells cultured from bovine and pig aorta and human umbilical vein (Brotherton, 1986; Leitman & Murad, 1986; Schini et al., 1988; Martin et al., 1988b). The vasoactive agents histamine, angiotensin II, acetylcholine and phenylephrine have been shown to elevate cyclic GMP content in endothelial cells cultured from rabbit aorta (Buonassi & Venter, 1976).

Adenosine and its analogues, forskolin, β- adrenoceptor agonists

and vasoactive agents which are known to activate adenylate cyclase have been observed to elevate cyclic AMP content in endothelial cells cultured from bovine pulmonary artery , bovine, rabbit, and pig aorta (Buonassi & Venter, 1976; Goldman et al., 1983; Makarski , 1981; Brotherton & Hoak, 1982; Legrand et al., 1989; 1990; Martin et al., 1988b).

All these studies indicate that adenylate cyclase and particulate and soluble guanylate cyclase are present in vascular endothelial cells.

1.10.4. Role of cyclic nucleotides in endothelial cell proliferation

It has been reported that cyclic AMP has differing actions on the proliferation of endothelial cells from different sources in culture: adenosine, which is known to increase intracellular cyclic AMP content (Goldman et al., 1983), was found to stimulate proliferation of bovine aortic and coronary microvascular endothelial cells (Meininger et al., 1988; Meininger & Granger, 1990), foetal bovine aortic endothelial cells (Presta et al., 1989a), and human dermal microvascular endothelial cells (Davison & Karasek, 1981). In contrast, an inhibition of growth was reported for bovine aortic and rat cerebrovascular endothelial cells (Leitman et al., 1986; Kempski et al., 1987).

In contrast to cyclic AMP, the role of cyclic GMP in endothelial cell proliferation has not been extensively investigated. One study indicated that a slight inhibition of proliferation was observed when bovine aortic endothelial cells were treated with

membrane permeant analogues of cyclic GMP (Leitman et al., 1986).

1.10.5. Role of cyclic nucleotides in smooth muscle cell proliferation

There is accumulating evidence that cyclic AMP is the intracellular mediator of vasorelaxation induced by prostacyclin and adenosine, whereas, cyclic GMP is the mediator for endothelium- derived relaxing factor (EDRF), atrial natriuretic factor and sodium nitroprusside (Rapoport & Murad, 1983; Lincoln & Fisher-Simpson, 1984; Itoh et al., 1985; Kurtz, 1987; Grace et al., 1988).

Recent reports indicate that as well as possessing vasorelaxant properties, adenosine, atrial natriuretic factor and sodium nitroprusside inhibit the proliferation of smooth muscle cell in culture. Elevation of cyclic AMP content by various treatments (adenosine and its analogues, forskolin, cyclic AMP phosphodiesterase (PDE) inhibitors, β- adrenoceptor agonists and cyclic AMP analogues) has an inhibitory action on proliferation of smooth muscle cells from different sources in culture including rat cerebrovascular (Kempski et al., 1987), rat aortic, human aortic and rabbit aortic (Nilsson & Olsson, 1984; Jonzon et al., 1985; Tertov et al., 1984; Fukumoto et al., 1988; Nakaki et al., 1990).

Elevation of cyclic GMP content by various treatments (nitrovasodilators, atrial natriuretic factor and the cyclic GMP analogue, 8 bromo cyclic GMP) inhibits the proliferation of rat and rabbit aortic smooth muscle cells stimulated to grow by serum and PDGF (Abell et al., 1989; Kariya et al., 1989; Garg & Hassid, 1989). Garg & Hassid (1989) proposed that nitric oxide (NO), the

active principle generated by nitrovasodilators, is the ultimate effector of the inhibition of growth observed. They further proposed that EDRF, now identified as nitric oxide (Palmer et al., 1987), is an endogenous regulator of smooth muscle cell growth within the arterial wall.

1.11. ENDOTHELIUM- DERIVED RELAXING FACTOR (EDRF)

The vascular endothelium is important in controlling vascular homeostasis by secreting a variety of substances such as endothelin and prostacyclin. It also produces and secretes another powerful vasodilator, endothelium- derived relaxing factor (EDRF)(Furchgott & Zawadzki, 1980).

The actions of EDRF were first described by Furchgott & Zawadzki (1980) who reported that acetylcholine induced relaxation of rabbit aortic preparations only when the endothelium was present. Further studies showed that endothelial cells release this factor spontaneously or in response to a variety of stimuli including vasoactive agents or increased blood flow or shear stress (Pohl et al., 1986; Rubanyi et al., 1986; Buga et al., 1991). Recent research has shown that smooth muscle cells grown in culture synthesize a vasoactive substance(s) that interacts with the endothelium to stimulate the production of EDRF (Warren et al., 1990).

It was not until 1987 that Palmer et al. (1987) identified EDRF as nitric oxide (NO) by a chemiluminescence techniques. Nitric oxide is a small lipophilic molecule that can readily permeate biological membranes.

1.11.1. Biosynthesis of EDRF

In the last few years the biosynthetic pathway of EDRF has been elucidated. L- arginine was found to be the physiological precursor for basal and stimulated nitric oxide formation (Palmer et al., 1988b; Schmidt et al., 1988). Nitric oxide is formed from the terminal guanidino group(s) of L- arginine by the enzyme nitric oxide synthase. This reaction is stereoselective and specific since D- arginine and other basic amino acids cannot act as a substrate for this enzyme (Palmer et al., 1988a). Figure 2 illustrates the biosynthetic pathway of EDRF.

The nitric oxide synthesized by vascular endothelial cells rapidly diffuses out to the underlying smooth muscle cells or nearby platelets in the lumen of the blood vessel where its actions are mediated by stimulation of soluble guanylate cyclase. Stimulation of this enzyme results from the binding of nitric oxide to a ferrous haem moiety (Craven & De Rubertis, 1978). The associated rise in cellular cyclic GMP content mediates the actions of EDRF (Rapoport & Murad, 1983; Mülsch et al., 1987; Martin et al., 1988b).

Endothelial cells contain soluble guanylate cyclase and a consequence of this is that they are themselves sensitive to the EDRF they produce (Martin et al., 1988b). The effects of EDRF on endothelial function are, however, unknown. It has been shown recently that the endothelial cell is not the only cell with the capacity to produce and release nitric oxide (NO): macrophages, neutrophils, smooth muscle cells, central and peripheral neurons have all been shown to release this substance (Hibbs et al.,

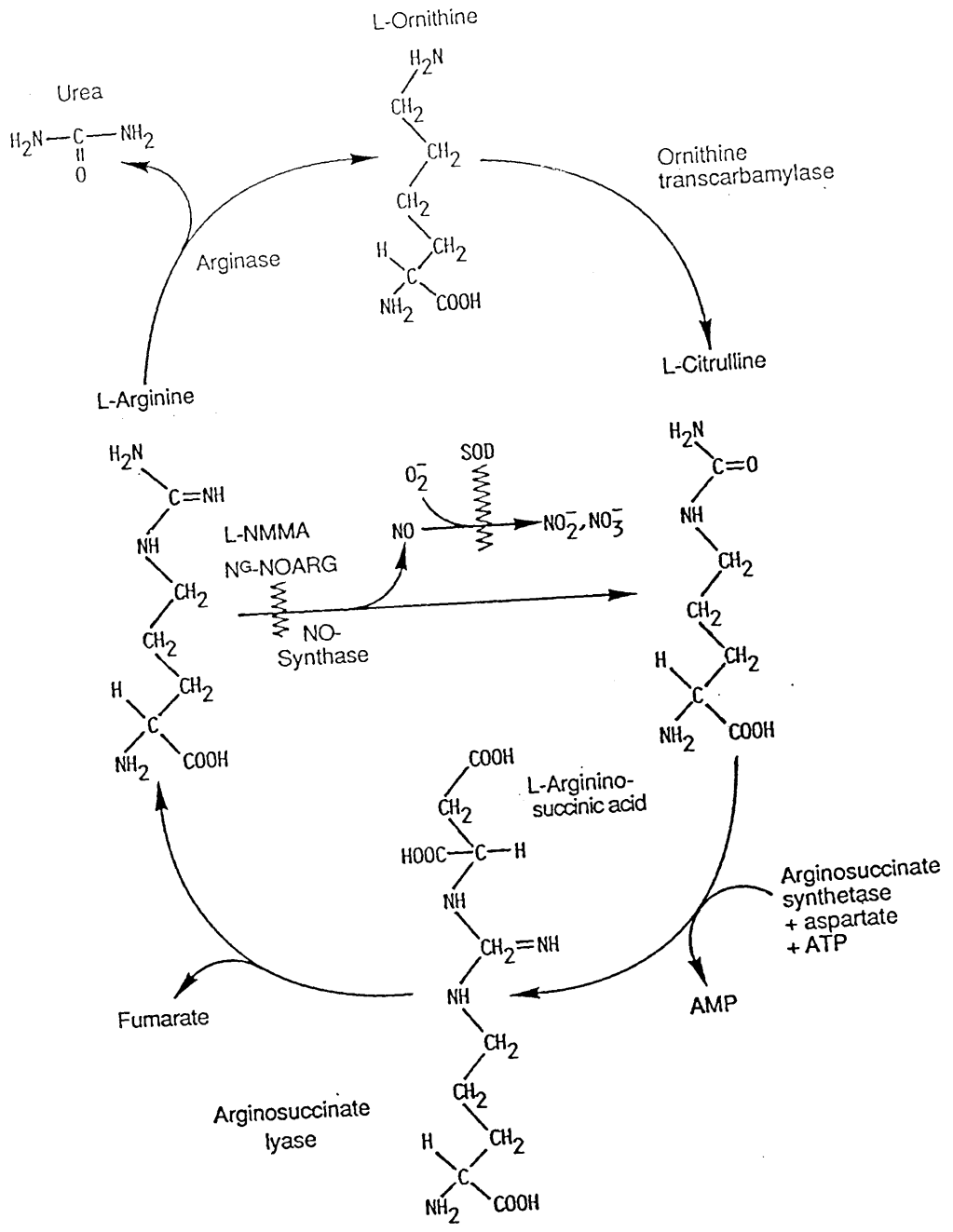


Figure 2: Schematic diagram of the biosynthesis of EDRF. EDRF, now identified as nitric oxide (NO)(Palmer et al., 1987), is formed from the terminal guanidino nitrogen(s) of L- arginine by the enzyme nitric oxide synthase (NO synthase). This reaction can be inhibited by N^G- monomethyl L- arginine (L-NMMA) or N^G- nitro L- arginine (N^G- NOARG). NO is readily degraded to nitrite (NO_2^-) and nitrate (NO_3^-) ions by superoxide radicals (O_2^-). Superoxide dismutase (SOD) inhibits this breakdown.

1987a; Gillespie et al., 1989; Mehta et al., 1990; Wood et al., 1990; Garthwaite, 1990). These findings suggest that nitric oxide is a widespread messenger molecule in intercellular communication.

1.11.2. Role of EDRF in atherosclerosis

The endothelial cell layer lining the vascular wall is normally confluent and possesses both anticoagulant and antithrombotic properties. It has been found that EDRF can influence platelet function; for example, it inhibits platelet aggregation, induces platelet disaggregation and inhibits platelet adhesion to the vascular endothelium (Radomski et al., 1987a; 1987b; 1987c; Hawkins et al., 1988; Sneddon & Vane, 1988).

Since EDRF has these antithrombotic properties, its loss might aggravate the atherosclerotic process by allowing the adhesion of platelets to the vessel wall. This could promote the localised release of platelet- derived mitogens such as PDGF.

In aortas from humans and animals with atherosclerosis, it has been found that endothelium- dependent vasodilation mediated through EDRF is impaired (Cox et al., 1989; Freiman et al., 1986; Guerra et al., 1989; Nabel et al., 1990). It was proposed that this early state of atherosclerosis may be associated with an impaired ability of the endothelial cells to produce EDRF. In experimentally induced atherosclerosis resulting from hypercholesterolemia, high levels of low density lipoproteins (LDL) are produced. This not only promotes attachment of monocytes to the endothelium (Alderson et al., 1986) but leads to

inactivation of EDRF after its release from the endothelial cells (Galle et al., 1991).

Furthermore, EDRF, now identified as nitric oxide, has been proposed to be an endogenous growth regulator for smooth muscle cells present within the arterial wall (Garg & Hassid, 1989). The impaired release of nitric oxide could, therefore, promote the abnormal proliferation of smooth muscle cells which is observed during the development of atherosclerosis.

1.12. Aim

The aim of this part of the study was to investigate the effects of cyclic nucleotides and the activation of protein kinase C on proliferation of pig aortic endothelial cells and rat aortic smooth muscle cells in culture.

2.1. CYCLIC NUCLEOTIDE PHOSPHODIESTERASES

The transduction signals in response to activation of cell-surface receptors by an extracellular stimulus involves a series of rapid intracellular events which translates the external signal into specific cellular responses. One exception is the activation of soluble guanylate cyclase where the receptor is not on the cell surface but on the soluble enzyme itself (Craven & De Rubertis, 1978). The extracellularly generated signals give rise to intracellularly generated second messengers such as calcium, diacyglycerol, cyclic AMP and cyclic GMP which then change cell function through phosphorylation of distinct target proteins (Lincoln & Corbin, 1983; Corbin et al., 1988; Tremblay et al., 1988).

Cyclic nucleotide activity is terminated by degradation and this was originally thought to occur by a single enzymatic activity. It was the observations of Thompson & Appleman (1971) which showed that degradation and therefore inactivation of cyclic AMP and cyclic GMP is catalysed by not one but a large group of different cyclic nucleotide- phosphodiesterases (PDEs). PDEs terminate the actions of cyclic nucleotides by catalysing their hydrolysis to the respective nucleoside 5'-monophosphate.

2.1.1. Nomenclature of PDE families

Many groups have defined different forms of PDE on the basis of the order of elution from a DEAE- Trisacyl chromatography column, utilising names such as peak I, II, III, or IV, often modified with regard to their substrate specificity (cyclic AMP or cyclic GMP), kinetic properties (K_M and V_{MAX}), sensitivity to calcium/ calmodulin and response to selective PDE inhibitors. Unfortunately many of these peaks of activity contain multiple activites and the order of elution of isozymes from DEAE varies with species, tissue, pH and eluting salt (reviewed in Beavo, 1988).

Beavo & Reifsnyder (1990) recently proposed a new classification of PDE isozymes based upon the primary protein and its cDNA sequence information. They identified at least five distinct but related families coding for cyclic nucleotide PDE in mammals. Table 3 indicates the nomenclature of the PDE isozymes.

Table 3 : Nomenclature of PDE- isozymes

	Family
I	Ca^{2+}- calmodulin- dependent family PDE
II	cGMP- stimulated family PDE
III	cGMP- inhibited family PDE
IV	cAMP- specific family PDE
V	cGMP- specific family PDE

Moreover most of these families contain two or more closely related subspecies. Members of one family share between 20 and 25% sequence homology with members of another. Much of this homology is found to occur in the C- terminal part of the PDE which is known to be part of the catalytic domain. The data currently available suggest that most of the individual subfamily members are encoded by different but highly homologous genes (70-90% sequence homology). Many of these subfamilies have multiple members that are likely to be products of alternative mRNA splicing.

2.1.2. Identification and localization of PDE isozymes

It is likely that identification of the cellular and subcellular distribution of each PDE isozyme will aid the development of selective inhibitors as therapeutic agents for specific diseases. From extensive studies, it is becoming apparent that there is a variation in the distribution and substrate specificity of PDE isozymes in numerous tissues and cells.

From the use of antibodies against the calcium/ calmodulin dependent PDE, it was found that this isozyme is present in high

concentrations in the dendrites of the Purkinje cells and in the cortical pyramidal cells in rat brains (Kincaid et al., 1987). The substrate specificity was higher for cyclic AMP than for cyclic GMP. Human blood platelets were found to contain three PDE activities: a calmodulin- independent PDE (I) which preferentially hydrolyses cyclic GMP, a cyclic GMP-stimulated PDE (II), and a cyclic AMP PDE (IV) (Hidaka & Asano, 1976; Weishaar et al., 1986). In contrast, human blood monocytes were demonstrated to contain only a soluble high affinity cyclic AMP PDE (IV) (Thompson et al., 1980). In adipocytes the predominant isozyme is the cyclic GMP- inhibited PDE (III) (Elks & Manganiello, 1984). The distribution of the cyclic GMP- specific PDE (V) is best characterized in the retina. The highest concentrations are found in the outer segments. Recent research has indicated that there are separate isozymes in the rod and cone photoreceptor outer segments (Hurwitz et al., 1985).

2.1.3. Selective inhibitors

The development of drugs which have the ability to inhibit selectively individual PDE isozymes could provide valuable tools with which to examine cyclic nucleotide regulated cellular processes. Butcher & Sutherland (1962) indicated that methylxanthines such as caffeine and theophyline inhibited cyclic AMP hydrolysis. Further research has led to the development of a number of selective inhibitors. These inhibitors have proven useful in characterizing the intracellular roles of the different molecular forms of PDE, but there is no inhibitor known to distinguish between members of the same isozyme family. The inhibitory profile of several of these selective PDE inhibitors as well as several non-

selective inhibitors is summarized in Table 4.

Table 4: Selective and non- selective inhibitors of the different PDE isozyme families.

INHIBITOR	PDE- ISOZYME FAMILY
Selective Inhibitors	
M & B 22948	Ca^{2+}/ CaM dependent PDE (I)
SK & F 94120	Cyclic GMP- inhibited PDE (III)
IBMX	Ca^{2+}/ CaM dependent PDE (I)
ROLIPRAM	Cyclic AMP- specific PDE (IV)
Non- Selective Inhibitors	
DIPYRIDAMOLE	Cyclic GMP- syimulated PDE (II)
	Cyclic AMP- specific PDE (IV)
TREQUINSIN	Cyclic GMP- stimulated PDE (II)
	Cyclic AMP- specific PDE (IV)

There is substantial interest in the use of selective PDE inhibitors as therapeutic agents in diseases. In one such study, selective PDE inhibitors were proposed to represent a new approach to the treatment of asthma. In this scheme bronchodilatation can be brought about through selective inhibitors of the PDE isozymes present in bronchial smooth muscle thus avoiding the side effects encountered with non- selective PDE inhibitors (Torphy, 1988).

2.2. AIM

The aim of this part of the project was to investigate the effects of selective and non- selective inhibitors of PDE isozymes on accumulation of cyclic AMP and cyclic GMP in intact pig aortic endothelial cells.

MATERIALS AND METHODS

3.1. ENDOTHELIAL CELLS

3.1.1. Endothelial cell culture

Pig aortae were obtained from a local abattoir. Less than 5 minutes after removal from animal, the aorta was flushed with sterile saline (0.9% w/v, Baxter, UK) containing benzyl penicillin (100U/ ml) and streptomycin (100µg/ ml) to remove any remaining blood. The aortae were then tied off at the larger thoracic end using string and cannulated at the smaller abdominal end with a 60 ml syringe containing the same saline solution. Saline was infused into the lumen before the vessels were transported to the laboratory, which took 30 minutes.

In a laminar flow hood (Flow), the intercostal arteries were cleared of remaining connective and fatty tissues and ligated ensuring that no fluid leakage occured. Any remaining saline was removed and 10-15 ml of sterile collagenase solution (Type II, Sigma, 0.2% in Dulbecco's modified Eagle's Medium (DMEM)) was introduced into the lumen. The aorta was then incubated at 37°C for 25 minutes. After incubation, the collagenase solution now containing detached endothelial cells was removed and the aorta gently massaged to loosen any remaining endothelial cells from their basement membrane. The collagenase solution was then flushed in and out several times to dislodge these remaining cells from the vessel wall. The resultant cell suspension was placed in a sterile centrifuge tube (50 ml, Falcon). 20 ml of sterile saline was then introduced into the aorta to remove any remaining cells and this suspension was combined with the first.

The cell suspension was then centrifuged at 200 g for 4

minutes at 10°C (IEC Centra 8R centrifuge), the supernatant was discarded and the cell pellet resuspended in 20 ml of DMEM supplemented with foetal calf serum (10%), newborn calf serum (10%), glutamine (4 mM), benzyl penicillin (100U/ ml) and streptomycin (100µg/ ml): this is subsequently referred to as normal serum-supplemented DMEM in the text. The cell suspension was centrifuged as before, the supernatant was discarded and the cell pellet resuspended in 5 ml of normal serum- supplemented DMEM. Cells were seeded initially into 80 cm^2 tissue culture flasks (Nunc) and normal serum- supplemented DMEM was added to give a final volume of 20 ml.

The cells were then grown at 37°C under an atmosphere of 5% CO_2 in air in an incubator (Flow CO_2 incubator model 220). The normal serum- supplemented DMEM was aspirated off every 2 or 3 days, the cells were then washed with 2 x 20 ml of sterile saline, and fresh culture medium was added. Cells normally grew to confluence within 6-8 days. Figure 3 shows the morphology of freshly isolated and confluent pig aortic endothelial cells in culture.

3.1.2. Characterization of pig aortic endothelial cells

Cells were characterized as endothelial cells by their growth as a strict monolayer with a typical cobblestone morphology.

Furthermore, we have previously reported their ability to secrete prostacyclin and endothelium-derived relaxing factor (Martin et al., 1988b), and fluoresce when incubated with the selective marker, acetylated low- density lipoprotein labelled with 1,1' dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchorate

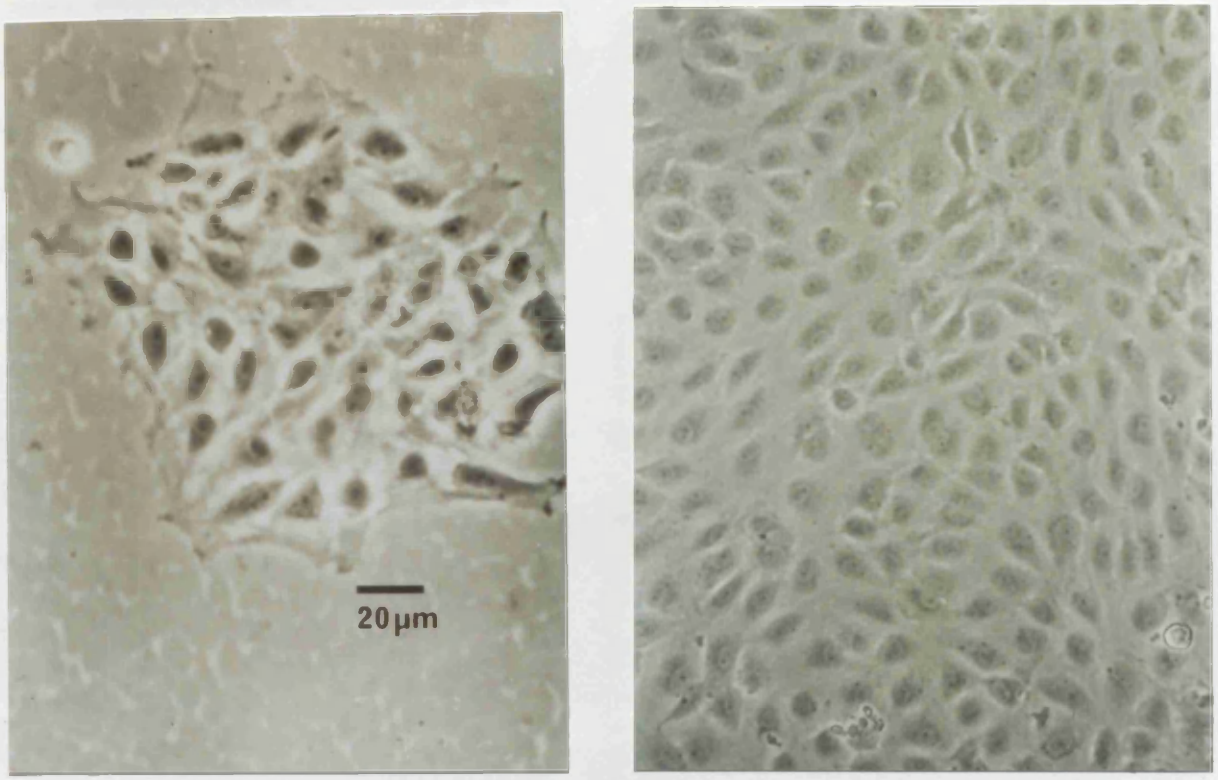


Figure 3: Phase contrast micrographs of pig aortic endothelial cells. 24 hours and 4 days after isolation from pig aorta by collagenase treatment. Endothelial cells were isolated as small clumps, but divided and multiplied to form a strict monolayer of cobblestone morphology.

(Voyta et al., 1984).

3.2 PROLIFERATION STUDIES

3.2.1. Haemocytometric Studies

Once the pig aortic endothelial cells (PAEC) reached confluence, the normal serum- supplemented DMEM was removed by aspiration and the cells washed with 2 x 10 ml of sterile saline. 10 ml trypsin (0.05%)/ EDTA (0.02%) solution (Flow) was then added, and the flask incubated at 37°C until the cells became detached (usually around 5 minutes). 2-3 ml of newborn calf serum was added to inactivate the trypsin/ EDTA solution. The resultant cell suspension was placed in a sterile centrifuge tube (50 ml, Falcon) and centrifuged at 200 g for 4 minutes at 10°C (IEC Centra 8R centrifuge). The supernatant was discarded and the cell pellet resuspended in 20 ml of normal serum- supplemented DMEM. The cell suspension was then centrifuged as before, but this time the cell pellet was resuspended in 10 ml of normal serum- supplemented DMEM. A 1 ml aliquot of the cell suspension was removed and cell density determined by the use of a haemocytometer. The cell suspension was subsequently diluted to a final density of 4.5 - 6.0 x 10^5 cells/ ml with normal serum- supplemented DMEM.

For proliferation studies , PAEC were seeded at a density of approximately 1 - 1.5 x 10^4 cells / cm^2 in 2 ml of normal serum-

supplemented DMEM in six- well plates (9.6 cm^2,Nunc). The effects of various drug treatments on proliferation were examined with time. All drugs were added twice daily with the exception of methylene blue which was added once daily. The normal serum-supplemented DMEM was removed by aspiration every 2 or 3 days, the cells were washed with 2 x 2 ml of sterile saline, and fresh culture medium added.

At various time points as indicated in the Results, the culture medium was removed by aspiration and cells washed with 2 x 2 ml of sterile saline. 1 ml of trypsin (0.05%)/ EDTA (0.02%) solution (Flow) was added. Cells were incubated at 37°C until they became detached (usually around 5 minutes). 0.5 ml of newborn calf serum was added to inactivate the trypsin/ EDTA solution. The resultant cell suspension was transferred to eppendorf tubes and the cell density determined by haemocytometry .

3.2.2. [^{3}H]-Thymidine Incorporation Studies

These were conducted in some experiments to hopefully obtain a more sensitive index of proliferation than could be obtained by haemocytometry .

Once the primary cultures of PAEC had grown to confluence in a 80 cm^2 flask, the normal serum- supplemented DMEM was removed by aspiration and the cells washed with 2 x 10 ml of sterile saline. 10 ml of trypsin (0.05%)/ EDTA (0.02%) solution (Flow) was added. The flask was then incubated at 37°C until the cells became detached (usually around 5 minutes). 2-3 ml of newborn calf serum was added to inactivate the trypsin/ EDTA solution and the re-

sultant cell suspension was transferred to a sterile centrifuge tube (50 ml, Falcon) and centrifuged at 200 g for 4 minutes at 10°C (IEC Centra 8R centrifuge). The supernatant was discarded and the cell pellet resuspended in 20 ml of normal serum- supplemented DMEM. The cell suspension was then centrifuged as before, the supernatant was discarded and the cell pellet resuspended in 10 ml of normal serum- supplemented DMEM. A 1 ml aliquot was removed and cell density determined by haemocytometry. The cell suspension was diluted to a final density of 6-8 x 10^5 cells/ ml with normal serum- supplemented DMEM.

PAEC were seeded at a density of approximately 1.5-2.0 x 10^4 cells/ cm^2 in six- well plates (9.6 cm^2, Nunc). The cells were grown in normal serum- supplemented DMEM for 24 hours. The normal serum- supplemented DMEM was removed by aspiration, the cells washed with 2 x 2 ml of sterile saline, and 2 ml of serum- free DMEM supplemented with glutamine (4 mM), benzyl penicillin (100U/ ml) and streptomycin (100µg/ ml) was added: this is subsequently referred to as serum- free DMEM in the text. The cells were incubated for a further 24 hours and at the end of this period, the serum- free DMEM was removed by aspiration. Cells were then challenged with drugs in DMEM supplemented with various concentrations of serum and then pulsed with a mixture of [^{3}H]-thymidine (2µCi/ well) and unlabelled thymidine (1µM) at 37°C for various times as indicated in the Results.

At the end of the incubation period, the cells were washed with 3 x 2 ml of 5% ice-cold trichloroacetic acid and solubilized in 0.5 ml of 0.25M ice-cold NaOH. The cells were then scraped off the

multiwell plates and harvested into scintillation vials. Any remaining cells were recovered by the addition of a second volume of 0.5 ml of NaOH and this extract was combined with the first. 5 ml of Ecoscint (Natural Diagnostics) was added to each vial followed by vortex mixing. The radioactivity in each vial was counted for 5 minutes using a Liquid Scintillation Counter (Packard 2000CA). The results were converted from c.p.m. to d.p.m. using an external standard.

3.3. SMOOTH MUSCLE CELLS

3.3.1. Smooth muscle cell culture

Vascular smooth muscle cells were harvested from the enzymatically dissociated aortae of Sprague- Dawley rats using a modification of the method described by Chamley- Campbell et al. (1979).

Briefly, Sprague-Dawley rats (200-300g) were killed, and under sterile conditions, the aorta was dissected out and placed in a petri dish containing 2-3 ml of DMEM. The aorta was cleared of fat, adventitial tissue and any remaining blood. The aorta was incubated for 5 minutes at 37°C in a sterile tube (15 ml, Falcon) containing 5 ml of DMEM containing collagenase (Type II, Sigma, 0.125%) and elastase (Type II, Sigma, 0.025%). The aorta was then transferred to fresh DMEM and any remaining fat and adventitia was removed. The aorta was then cut into 1-2 mm sections with scissors and incubated at 37°C in fresh DMEM-enzyme solution for 2-3 hours. The mixture was drawn through a sterile glass pipette to aid dispersal of cells.

The resultant cell suspension of rat aortic smooth muscle cells

was then centrifuged at 200 g for 4 minutes at 10°C (IEC Centra 8R centrifuge). The supernatant was discarded and the cell pellet resuspended in 10 ml of normal serum- supplemented DMEM. The cell suspension was centrifuged as before, the supernatant discarded and the cell pellet resuspended in 10 ml of normal serum- supplemented DMEM. The rat aortic smooth muscle cells (Rat ASMC) were seeded initially into a 25 cm^2 tissue culture flasks (Nunc) and grown at 37°C under an atmosphere of 5% CO_2 in air. The normal serum- supplemented DMEM was removed by aspiration every 2 or 3 days. The cells were then washed with 2 x 10 ml of sterile saline and fresh culture medium added.

When primary cultures of rat ASMC attained confluence (10-14 days), the culture medium was aspirated off, the cells were washed with 2 x 2 ml of sterile saline and 5 ml of trypsin (0.05%)/ EDTA (0.02%) solution (Flow) was added. The flask of rat ASMC was incubated at 37°C until the cells became detached (usually around 5 minutes). 1 ml of newborn calf serum was added to inactivate the trypsin/ EDTA solution and the resultant cell suspension was placed in a sterile centrifuge tube (15 ml, Falcon). The cell suspension was then centrifuged at 200 g for 4 minutes at 10°C (IEC Centra 8R Centrifuge), the supernatant was discarded and the cell pellet resuspended in 10 ml of normal serum- supplemented DMEM. The cell suspension was centrifuged as before, the supernatant was discarded, and the cell pellet resuspended once again in 10 ml of nomal serum- supplemented DMEM. The cell suspension was seeded into 80 cm^2 tissue culture flasks (Nunc). Normal serum- supplemented DMEM was added to give a final volume of 20 ml per flask. Figure 4 shows the morphology of

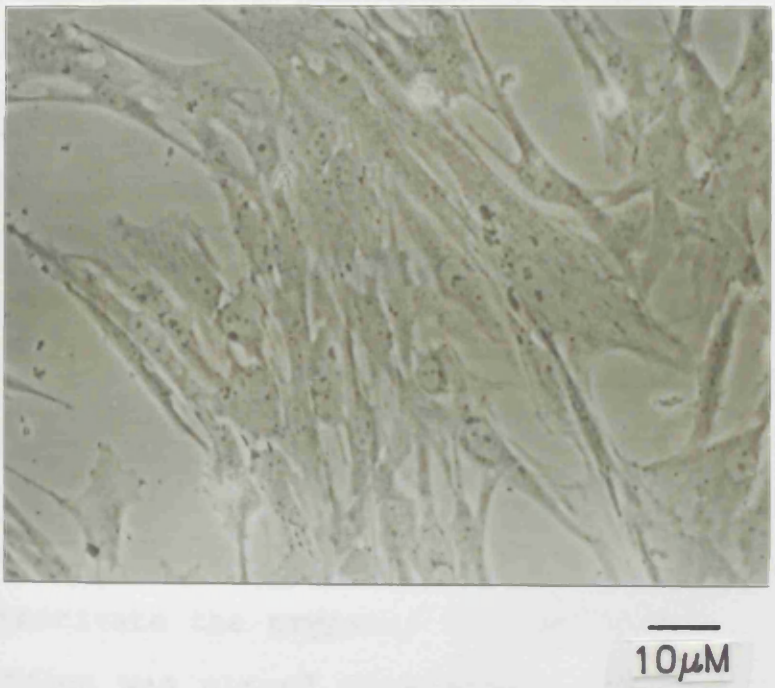


Figure 4: Phase contrast micrograph of subcultured rat aortic smooth muscle cells.

subconfluent secondary cultures of ASMC in culture.

3.3.2. Characterization of rat aortic smooth muscle cells

Characterization of the cells as smooth muscle cells was established by the following criteria: the cells grew in the typical 'hill and valley' pattern and were shown to fluoresce with anti-smooth muscle actin antibodies (Chamley et al., 1977).

3.4. PROLIFERATION STUDIES

3.4.1. Haemocytometeric Studies

When secondary cultures of rat ASMC grew to confluence in 80 cm^2 flasks, the normal serum- supplemented DMEM was removed by aspiration and the cells washed with 2 x 20 ml of sterile saline. 10 ml of trypsin (0.05%)/ EDTA (0.02%) solution (Flow) was added and the flasks were incubated at 37°C until the cells became detached (usually around 5 minutes). 2-3 ml of newborn calf serum was added to inactivate the trypsin/ EDTA solution and the resultant cell suspension was placed in a sterile centrifuge tube (50 ml, Falcon). The cell suspension was then centrifuged at 200 g for 4 minutes at 10°C (IEC Centra 8R Centrifuge), the supernatant discarded and the cell pellet resuspended in 20 ml of normal serum- supplemented DMEM. The cell suspension was then centrifuged as before, the supernatant was discarded and the cell pellet resuspended once again in 10 ml of normal serum- supplemented DMEM. A 1 ml aliquot of cell suspension was removed and cell density counted by haemocytometery. The cell suspension was diluted to a final density of 4.5-6 x 10^5 cells/ ml with normal serum- supplemented DMEM.

For proliferation studies, rat ASMC were seeded at a density of approximately 1.25-1.5 x 10^4 cells/ cm^2 in six- well plates (9.6 cm^2, Nunc). The rat ASMC were either grown in normal serum-supplemented DMEM or DMEM supplemented with foetal calf serum (5%), newborn calf serum (5%), glutamine (4 mM), benzyl penicillin (100U/ ml) and streptomycin (100µg/ ml): this is subsequently referred to as 10% serum- supplemented DMEM in the text. The effects of various drug treatments on proliferation were examined with time. All drugs were added twice daily after an initial period of 24 hours to allow the rat ASMC to plate down. The normal serum- supplemented DMEM, or the 10% serum- supplemented DMEM was removed by aspiration every 2 or 3 days. The cells were then washed with 2 x 2 ml of sterile saline, and fresh culture medium added.

At the various time points indicated in the Results, the culture medium was removed by aspiration, cells were washed with 2 x 2 ml of sterile saline and 1 ml of trypsin (0.05%)/ EDTA (0.02%) solution (Flow) was added. Cells were incubated at 37°C until the cells became detached (usually around 5 minutes). 0.5 ml of newborn calf serum was added to inactivate the trypsin/ EDTA solution. The resultant cell suspension was transferred to eppendorf tubes. A 10µl aliquot of cell suspension was removed and counted by haemocytometry. Rat ASMC between the 3rd and 15th passage were used in experiments described in the Results.

3.5. MEASUREMENT OF ENDOTHELIAL CELL CYCLIC NUCLEOTIDE CONTENT

3.5.1. Preparation of endothelial cell monolayers

Primary cultures of PAEC were isolated as described in 3.1.1.

except that all the cells harvested from each aorta were resuspended in 30 ml of normal serum- supplemented DMEM. 1 ml of cell suspension was then added into 5 multiwell dishes, each containing 6 wells (9.6 cm^2, Nunc). A further 1 ml of normal serum-supplemented DMEM was added to give a final volume of 2 ml per well.

Cells were then grown at 37°C under an atmosphere of 5% CO_2 in air in an incubator (Flow CO_2 incubator model 220). The culture medium was removed every 2-3 days by aspiration, the cells were washed with 2 x 2 ml of sterile saline and then fresh culture medium added. Cells were used for experimentation when confluent (attained within 7 days).

3.5.2. Experimental procedure

Following removal of the tissue culture medium by aspiration, the endothelial cells were washed with 2 x 2 ml of warmed (37°C) and gassed Kreb's solution containing (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24 and glucose 11; and then incubated in 2 ml of Kreb's solution at 37°C under an atmosphere of 5% CO_2 in air for at least 60 minutes.

At the appropriate time, drugs were added, the Kreb's bathing solution was quickly removed, and the cells were immediately extracted with 0.5 ml of ice cold 6% trichloroacetic acid (TCA). The cells were then scraped off the multiwell plates and harvested. Any remaining cells were recovered by the addition of a second volume of 0.5 ml TCA, and this extract was combined with the first. The extract was centrifuged at 10000 g

(MicroCentaur MSE) for 2 minutes and the pellet and supernatant were separated.

The supernatant, and in some experiments when indicated the Kreb's bathing solution, were stored for subsequent measurement of cyclic nucleotide content. The DNA content of the pellet was determined so that the cyclic nucleotide content could be expressed in pmol or fmol μg DNA^{-1}.

3.5.3. Preparation of trichloroacetic acid extracts for radioimmunoassay

The TCA extracts were neutralised to pH 5.5-6.0 by adding 2 ml of 0.5M tri-n-octylamine in freon (1,1,2,trichlorotrifluroethane) and vortex mixing for 90 seconds. The upper aqueous layer was removed using a glass Pasteur pipette ensuring that no contamination from the lower layer occured. The pH of the aqueous layer was checked with Whatman pH paper. The cyclic AMP and cyclic GMP content of the aqueous layer was determined by radioimmunoassay using New England Nuclear kits (Dupont).

3.6. RADIOIMMUNOASSAY

3.6.1. Principles of the cyclic AMP and cyclic GMP radioimmunoassay

The radioimmunoassays (RIA) for cyclic AMP and cyclic GMP were based on the basic principle of RIA first described by Yalow and Berson (1960): this consists of competition between radioactive and non-radioactive antigen for a fixed number of antibody binding sites. This interaction is represented schematically in Figure 5.

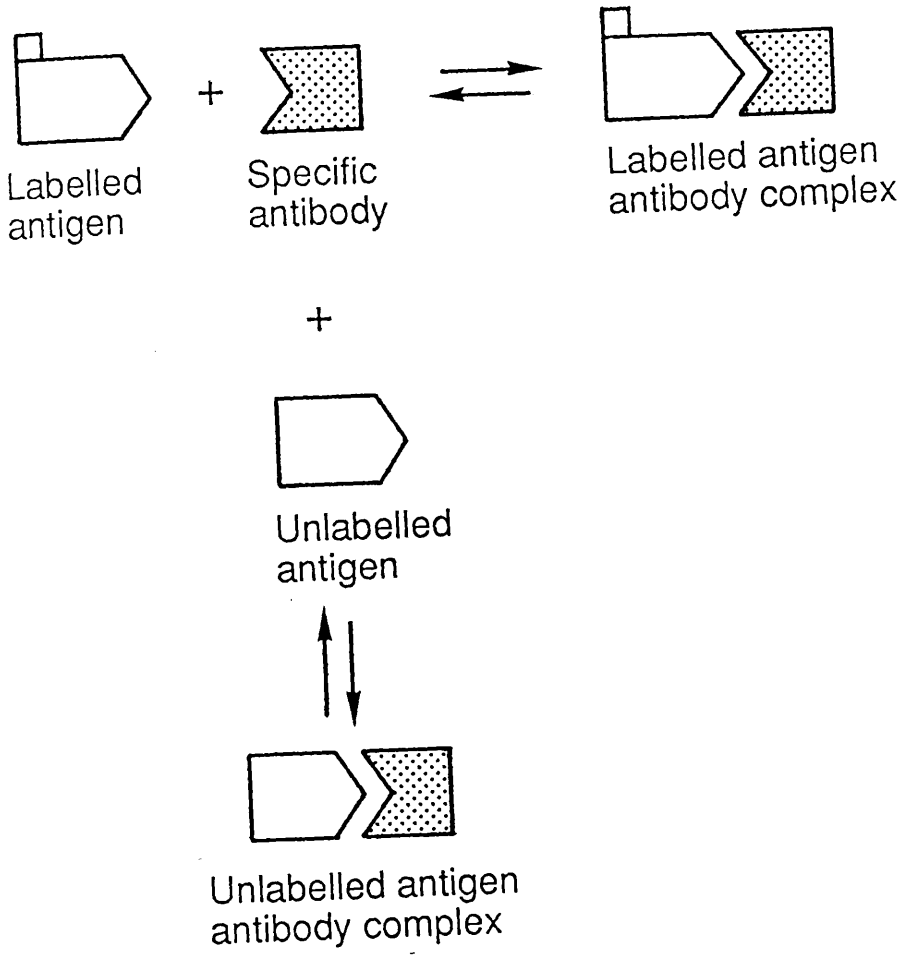


Figure 5: Schematic representation of the principle of radioimmunoassay (RIA). RIA is based on the competition between radiolabelled and unlabelled antigen for a fixed number of antibody binding sites. Increasing amounts of unlabelled antigen in the presence of fixed amounts of antibody and radiolabelled antigen, produce a decreasing amount of radiolabelled antigen bound to antibody. This relationship can be expressed as a standard curve after seperation of bound from free radiolabelled antigen, and amounts of unlabelled antigen determined by interpolation from the curve.

If increasing amounts of unlabelled antigen (i.e. in standards or unknown samples) and a fixed amount of labelled antigen (i.e. tracer) are allowed to react with a constant amount of antibody, a decreasing amount of labelled antigen is bound to the antibody. This relationship can be expressed as a standard curve and the amount of unlabelled antigen in a sample can be determined by interpolation from this curve.

3.6.2. RIA for cyclic GMP

Determination of cyclic GMP was adapted from the procedure of Steiner et al. (1972), using New England Nuclear RIA kits (Dupont). Steiner et al. (1972) reported that cyclic nucleotides substituted at the 2'-σ-position had a higher affinity for the antibody and thus displaced the [^{125}I]-labelled derivative better than the unsubstituted cyclic nucleotide. Thus standards and samples were acetylated with acetic anhydride to give 2'-σ-acetyl cyclic GMP thereby increasing the sensitivity of the assay (Harper & Brooker, 1975). The labelled antigen was a succinyl tyrosine-[^{125}I]-methyl ester derivative of cyclic GMP.

separation of bound from free antigen was achieved by the use of a pre-reacted primary and secondary antibody complex. The primary antibody was prepared in rabbits against a succinyl cyclic GMP albumin conjugate, while the second antibody was prepared in sheep against rabbit globulin. With this pre-reacted system, pipetting and incubation times were reduced compared to the usual sequential double antibody assay and no second incubation was required. After a single overnight incubation at 4°C, 1 ml of propan-1-ol was added to aid precipitation, the tubes were centi-

fuged, the supernatants discarded, and the radioactivity in the precipitates counted.

3.6.3. Chemicals for RIA

3.6.3.1. Cyclic GMP antiserum complex

One vial of lyophilized pre-reacted ,first and second antibody was supplied. It was reconstituted with 21 ml of distilled water. The resulting solution, in 0.1M sodium phosphate buffer with 0.05% thimerosal, pH 6.2, contained sufficient antibody to bind approximately 50-60% of the labelled antigen in the absence of unlabelled antigen when used as directed later. The reconstituted antiserum complex was stable for at least two months when stored at 2-8°C.

3.6.3.2. Succinyl cyclic GMP tyrosine methyl ester-[^{125}I] (ScGMP-TME-[^{125}I])

Two vials of concentrated tracer were supplied. Each vial contained approximately 28KBq (0.75µCi) on the calibration date in 1 ml of propan-1-ol:water solution (1:1). The concentrate was stable for at least 2 months when stored at 2-8°C.

3.6.3.3. Normal rabbit serum

The normal rabbit serum was used to measure the non-specific binding obtained using non-immune serum. Two vials of lyphilized normal rabbit serum were supplied. 5 ml of distilled water was added to one vial of ScGMP-TME-[^{125}I] concentrate, and to one vial of lyophilised normal rabbit serum. The entire contents of the reconstituted normal rabbit serum vial was added to the diluted ScGMP-TME-[^{125}I] vial and the resulting solution mixed

well. The resulting solution (approximately 11 ml) contained ScGMP-TME-[^{125}I] (0.068µCi/ ml), 1.0% normal rabbit serum, and 0.05M sodium acetate buffer, pH6.2.

3.6.3.4. Acetic anhydride and triethylamine

A mixture of these two solutions was used to acetylate cyclic GMP in the samples in order to increase the sensitivity of the assay. One vial of acetic anhydride and triethylamine was supplied. They were allowed to equilibrate to room temperature before use. When protected from moisture these chemicals were stable for at least two months. Immediately prior to use, one volume of acetic anhydride was mixed with two volumes of triethylamine; the exact volume of each was dependent upon the number of samples to be acetylated.

3.6.3.5. Cyclic GMP standard

This was the unlabelled antigen. One vial of lyophilized standard was supplied. It was reconstituted with exactly 20 ml of distilled water and contained 2000 pmol/ ml in 0.05M sodium acetate buffer, pH 6.2. The cyclic GMP standard had been calibrated spectrophotometrically by the manufacturer using the molar absorption coefficient for GMP, ϵ=13.7 x10^3/mol/cm at 252 nm, pH 7.0. The reconstituted standard was stable for at least two months when stored at 2-8°C.

3.6.4. Preparation of standard curve and assay of neutralized cell extracts

A series of 3.5 cm plastic tubes (Sarstedt) was numbered for identification and the standard cyclic GMP stock solution (2000

pmol/ ml) diluted with sodium acetate buffer, pH 6.2 to a concentration of 100 pmol/ ml. Further serial dilutions with sodium acetate buffer were made to prepare the following cyclic GMP standards for assay: 0.025. 0.05, 0.1, 0.25, 0.5, 1.0, 2.5 and 5.0 pmol/ ml. All standards were run in duplicate.

Tubes 1 and 2 were used to measure "total counts", tubes 3 and 4 were "blanks" and tubes 5 and 6 were used to measure the " '0' standard". 50µl of each standard solution (in duplicate) or sample was added to the appropriately labelled tube. All tubes except for "total counts" and "blank" received 5µl of the acetylation mixture ensuring that the reagents were added directly to the solution and were immediately vortex mixed for 2 seconds. All tubes received 50µl of [^{125}I] tracer solution, and 50µl of antiserum complex was added to all tubes except for "total counts" and "blanks". All tubes were vortex mixed for 5 seconds, covered with aluminium foil and equilibrated overnight (16-18 hours) at 4°C.

Following overnight incubation at 4°C, 1 ml of ice cold propan-1-ol was added to each tube except for "total counts". This aided the precipitation of the antigen-antibody complex. The tubes were vortex mixed and spun at 2000 g for 30 minutes at 4°C (Damon IEC Centrifuge). The supernatants were removed by aspiration (except for the "total counts"). All tubes were counted for 1 minute in a gamma counter (Packard Cobra Auto gamma).

3.6.5. Analysis of radioactivity

Analysis of the radioactivity in tubes (counting and background

reduction) was performed by an IBM-compatible computer with a defined RIA protocol. This allowed the user to define the nature of the sample (i.e.total counts, blanks, standards, unknowns and the number of replicates) and the count time. The standard curve was plotted with the charcteristics of a Bound Fraction RIA curve with a negative slope (spline fitted). The concentration for each unknown sample was calculated by interpolation from the standard curve and the value printed. Table 5 and Figure 6 represents a typical standard curve obtained for an acetylated cyclic GMP RIA.

3.6.6. RIA for cyclic AMP

Determination of cyclic AMP, like that of cyclic GMP, was adapted from the procedure of Steiner et al. (1972), using New England Nuclear RIA kits (Dupont). The standards and samples were not acetylated since high concentrations of cyclic AMP are found in endothelial cells and enhanced sensitivity is not required. The labelled antigen was a succinyl tyrosine-[^{125}I]-methyl ester derivative of cyclic AMP. Separation of bound from free antigen was achieved by the use of a pre-reacted primary and secondary antibody complex. The primary antibody was prepared in rabbits against a succinyl cyclic AMP albumin conjugate, while the second antibody was prepared in sheep against rabbit globulin.

3.6.7. Chemicals for RIA

3.6.7.1. Cyclic AMP antiserum complex

One vial of lyophilized pre-reacted, first and second antibody was supplied. It was reconstituted with 21 ml of distilled water. The resulting solution, in 0.1M sodium phosphate buffer with 0.05% thimerosal, pH 6.2, contained sufficient antibody to bind

	CPM	AVERAGE CPM
TOTAL COUNTS	6267	
	5761	6014
BLANK	98	
	107	103
'0' STANDARD	3594	
	3316	3455
0.025 pmol/ ml	2951	
	3219	3085
0.05 pmol/ ml	2762	
	2727	2745
0.1 pmol/ ml	2340	
	2223	2282
0.25 pmol/ ml	1485	
	1488	1487
0.5 pmol/ ml	1012	
	1098	1055
1.0 pmol/ ml	757	
	741	749
2.5 pmol/ ml	469	
	514	492
5.0 pmol/ ml	325	
	328	327

Table 5: Typical standard curve for acetylated RIA of cyclic GMP, showing decreased binding of [^{125}I]-ScGMP TME tracer to the antibody and therefore decreasing counts as the concentration of unlabelled cyclic GMP standard increases. "Total counts" measures total activity of [^{125}I]-ScGMP TME tracer, "Blank" measures non-specific binding, and "0 standard" measures total binding of [^{125}I]-ScGMP TME tracer to antibody.

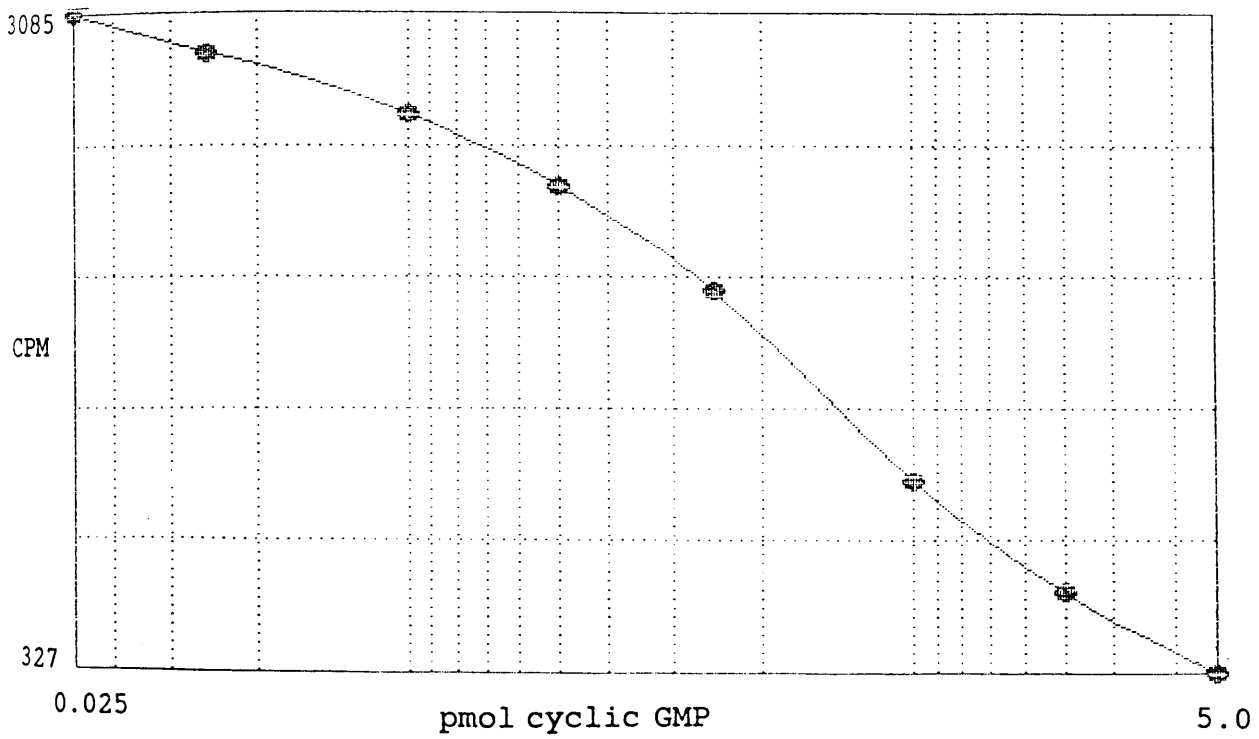


Figure 6: Typical computer printout of an acetylated cyclic GMP RIA standard curve. Unknown cyclic GMP concentrations were calculated from this curve by the computer.

approximately 50-60% of the labelled antigen in the absence of unlabelled antigen when used as directed later. The reconstituted antiserum complex was stable for at least two months when stored at 2-8°C.

3.6.7.2. Succinyl cyclic AMP tyrosine methyl ester-[^{125}I] (ScAMP-TME-[^{125}I])

Two vials of concentrated tracer were supplied. Each vial contained approximately 1.5µCi on the calibration date in 1 ml of propanol: water solution (1:1). The concentrate was stable for at least two months when stored at 2-8°C.

3.6.7.3. Cyclic AMP carrier serum

Two vials of lyophilized carrier serum were supplied. 5 ml of distilled water was added to one vial of ScAMP-TME-[^{125}I] concentrate, and to one vial of lyophilized cyclic AMP carrier serum. The entire contents of the reconstituted cyclic AMP carrier serum was added to the diluted ScAMP-TME-[^{125}I] and the resulting solution mixed well. The resulting solution (approximately 11 ml) contained ScAMP-TME-[^{125}I] (0.14µCi/ ml), 1% carrier serum, and 0.1% sodium azide in sodium acetate buffer, pH 6.2.

3.6.7.4. Cyclic AMP standard

This is the unlabelled antigen. One vial of lyophilized standard was supplied. It was reconstituted with exactly 2 ml of distilled water and contained 5000 pmol/ ml in 0.05M sodium acetate buffer, pH 6.2 and 0.1% sodium azide. The cyclic AMP standard had been calibrated spectrophotometrically by the manufacturer using the molar absorption coefficient for AMP, ϵ=14.6 x10^3/mol/cm at 259

nm, pH 6.9. The reconstituted standard was stable for at least two months when stored at 2-8°C.

3.6.8. Preparation of standard curve and assay of neutralized cell extracts

A series of 3.5 cm plastic tubes (Sarstedt) was numbered for identification and the standard cyclic AMP stock solution (5000 pmol/ ml) diluted with sodium acetate buffer, pH 6.2 to obtain the following cyclic AMP standards for assay: 0.5, 1.0, 2.5, 5.0, 10.0, 25.0 and 50.0 pmol/ ml. All standards were run in duplicate.

Tubes 1 and 2 were used to measure "total counts", tubes 3 and 4 were "blanks" and tubes 5 and 6 were used to measure the" '0' standard". 50µl of each standard solution (in duplicate) or sample was added to the appropriately labelled tube. All tubes received 50µl of [^{125}I] tracer solution and 50µl of antiserum complex except for "total counts" and "blanks". All tubes were vortex mixed for 5 seconds, covered with aluminium foil and equilibrated overnight (16-18 hours) at 4°C.

Following overnight incubation at 4°C, 1 ml of ice cold propan-1-ol was added to each tube except for "total counts". This aided the precipitation of the antigen-antibody complex. The tubes were vortex mixed and spun at 2000 g for 30 minutes at 4°C (Damon IEC Centrifuge). The supernatants were removed by aspiration (except for the "total counts"). All tubes were counted for 5 minutes in a gamma counter (Packard Cobra Auto gamma).

3.6.9. Analysis of radioactivity

Analysis of the radioactivity in tubes (counting and background reduction) was performed by an IBM-compatible computer with a defined RIA protocol. This allowed the user to define the nature of the sample (i.e. total counts, blanks, standards, unknowns and the number of replicates) and the count time. The standard curve was plotted with the characteristics of a Bound Fraction RIA curve with a negative slope (spline fitted). The concentration for each unknown was calculated by interpolation from the standard curve and the value printed. Table 6 and Figure 7 represents a typical standard curve obtained for a cyclic AMP RIA.

3.7. MEASUREMENT OF DNA CONTENT OF SAMPLES

3.7.1 Principles of DNA Assay

The DNA content of the samples was measured by the fluorescence technique as previously described by Kissane and Robins (1958). Briefly, the DNA content of the samples can be quantified fluorometrically by the reaction of 3,5, diaminobenzoic acid (DABA, Sigma) with the deoxyribose liberated from DNA.

3.7.2. Preparation of 3,5,diaminobenzoic acid

A 2M solution was prepared by dissolving 12g of DABA in 40 ml of 4M HCl acid. The resulting dark brown solution was decolourized by extraction with activated charcoal. 100 mg of activated charcol (Sigma) was added, the solution was vortex mixed then spun at 2000 g for 5 minutes at 4°C (IEC Centra 8R Centrifuge). The supernatant was decanted and re-extracted with a further 100 mg of activated charcoal with vortex mixing and centrifugation as before. This procedure was repeated until the resulting solution

	CPM	AVERAGE CPM
TOTAL COUNTS	13180	
	11931	12556
BLANK	180	
	191	186
'0' STANDARD	6809	
	7489	7154
0.5 pmol/ ml	6247	
	6295	6271
1.0 pmol/ ml	6118	
	6085	6102
2.5 pmol/ ml	5290	
	5182	5236
5.0 pmol/ ml	4197	
	4168	4183
10.0 pmol/ ml	2928	
	3422	3175
25.0 pmol/ ml	2061	
	2022	2042
50.0 pmol/ ml	1425	
	1367	1396

Table 6: Typical curve for RIA of cyclic AMP, showing decreased binding of [^{125}I]-ScGMP TME tracer to the antibody and therefore decreasing counts as the concentration of unlabelled cyclic GMP standard increases. "Total counts" measures total activity of [^{125}I]-ScGMP TME tracer, "Blank" measures non- specific binding, and "0 standard" measures total binding of [^{125}I]-ScGMP TME tracer to antibody.

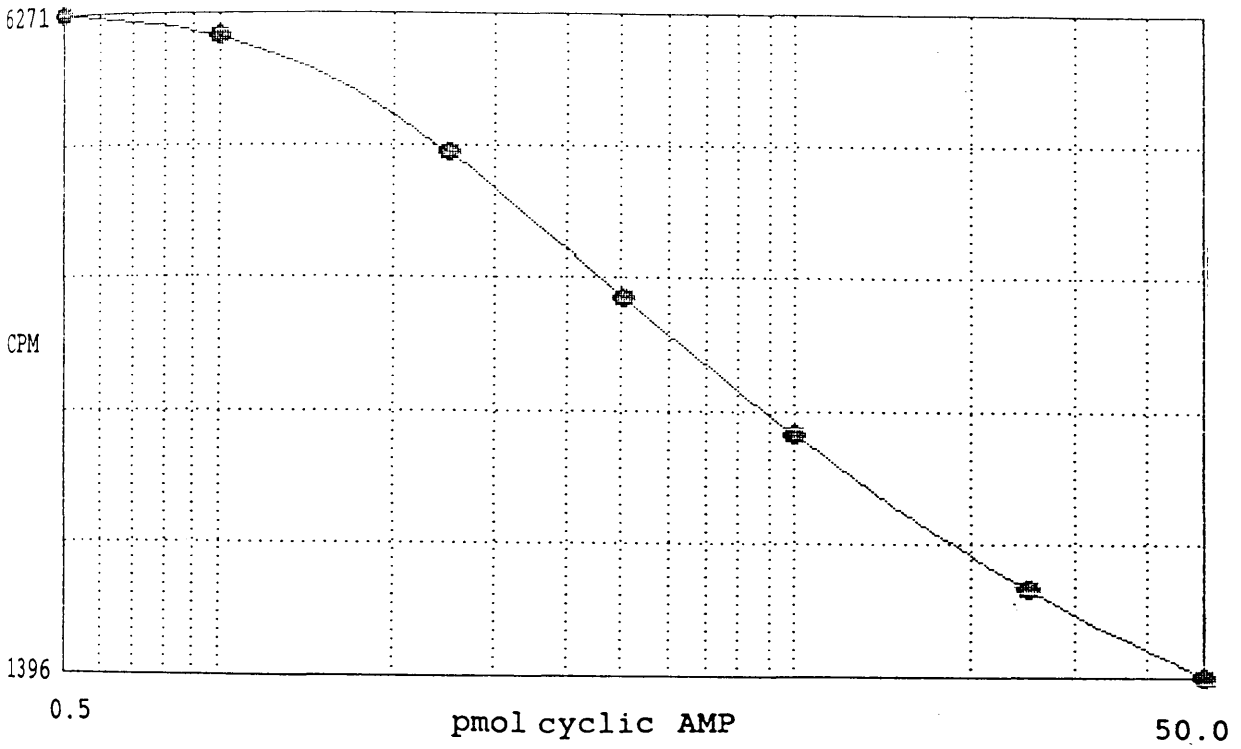


Figure 7: Typical computer printout of a cyclic AMP RIA standard curve. Unknown cyclic AMP concentrations were calculated from this curve by the computer.

was a pale straw colour. The solution was used immediately or stored at -20°C in 1 ml aliquots.

3.7.3 Preparation of DNA standards

A 500 µg/ ml solution of DNA was prepared by dissolving 5 mg DNA (Salmon-testes, Sigma) in 10 ml of 1M NH_4OH. An aliquot of the solution was diluted further with distilled water to prepare another stock solution of 50µg DNA/ ml. The DNA standards for preparation of a standard curve were 1.0, 2.5, 5.0, 10.0 and 20.0µg DNA/ ml prepared in duplicate from the stock DNA solutions. Duplicate blanks were also prepared by adding 20µl of 1M NH_4OH to two eppendorf tubes. The DNA standards and blanks were evaporated to dryness in drying oven.

3.7.4. Preparation of cell pellets for analysis

Before the DNA content of the cell pellets could be determined, an extraction procedure had to be carried out to remove other materials, for example lipids, which yield fluorescent products with DABA, and therefore interfere with the accuracy of the DNA measurement. This was as follows: 200µl of 0.1M potassium acetate was added to the cell pellets. The samples were vortex mixed and then centrifuged at 10000 g for 5 minutes at room temperature. The supernatants were removed by aspiration and discarded. 200µl of ethanol was added to the pellets followed by an incubation at 60°C for 30 minutes. The samples were left to cool then centifuged at 10000 g for 5 minutes and the supernatants discarded. A further 200µl of ethanol was added to the pellets, followed by vortex mixing, centrifugation and removal of the supernatants as before. The extracted pellets were evaporated to

dryness overnight.

After allowing the extracted pellets and DNA standards to evaporate to dryness, 120µl of 2M DABA was added and the samples incubated at 60°C for 30 minutes. In this step, the purine deoxynucleotides are hydrolysed by the strongly acidic DABA, and a fluoroscent product is formed between DABA and the liberated deoxyribose. After cooling, 720µl of 0.6M perchloric acid was added to each sample followed by centrifugation at 10000 g for 5 minutes at room temperature.

The Fluorimeter (Aminco-Bowman) was calibrated using the DNA standards and blanks. Excitation was at a wavelength of 406 nm and fluorescence was measured at 520 nm. The fluorescence of each sample was measured and the DNA content determined from the standard curve. The fluorescence obtained was linearly proportional to the amount of DNA present in the sample. Figure 8 shows a typical standard curve.

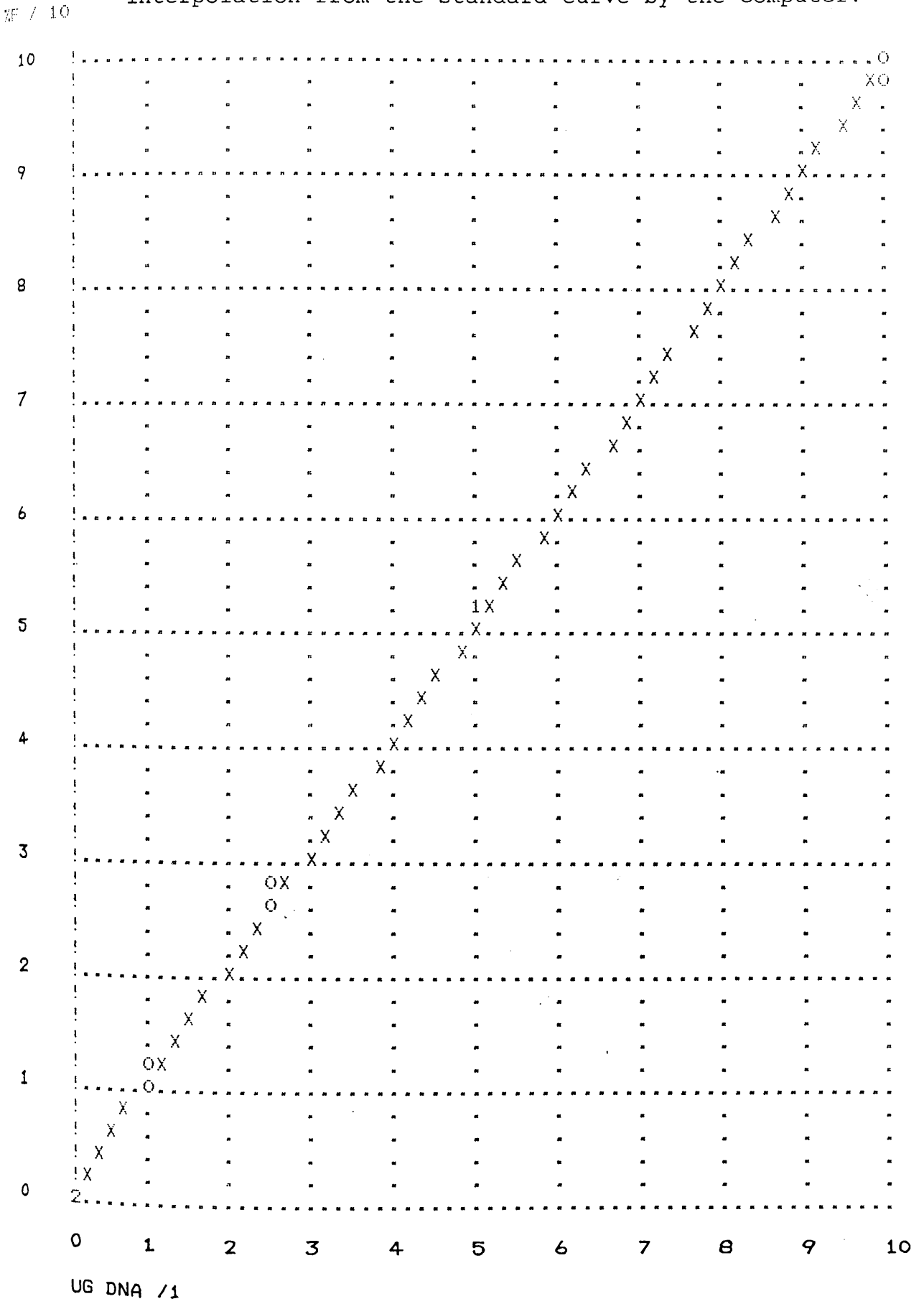
3.8. DRUGS AND REAGENTS

3.8.1. Tissue culture

Dulbecco's modified Eagle's medium (DMEM), benzyl penicillin, streptomycin, glutamine, foetal calf serum and newborn calf serum were purchased from Gibco Ltd (Paisley, Scotland). Trypsin/ EDTA was purchased from Flow Laboratories (Irvine, Scotland).

Tissue culture flasks (25 cm^2 and 80 cm^2), six-well multidishes (9.6 cm^2) were supplied from Nunc (Denmark) and sterile centifuge tubes (15 ml and 50 ml) were supplied by Falcon (UK). Sterile

Figure 8: Typical standard curve for DNA determination. The DNA content of the unknown samples were obtained by interpolation from the standard curve by the computer.



normal saline (0.9% w/v) was purchased from Baxter (UK).

3.8.2. Reagents

Freon (1,1,2,trichlorotrifluorethane), NaOH pellets, trisodium citrate and trichloroacetic acid were supplied from AnalaR (UK). Potassium acetate and sodium dithionite were obtained from BDH (Glasgow, Scotland). Perchloric acid was obtained from Searle Company Ltd (UK). Methyl [^{3}H]-thymidine was supplied from Amersham (UK). Ecoscint was supplied from National Diagnostics (UK).

3.8.3. Drugs

Atriopeptin II, Brij- 35 solution (polyoxyethylene 23 lauryl ether), 8 bromo guanosine 3':5'-cyclic monophosphate, butylated hydroxytoluene, L- canavanine, clonidine, collagenase (Type II), catalase (bovine liver), diaminobenzoic acid, dimethyl sulphoxide, deoxyribonucleic acid (DNA, salmon testes), dipyridamole, dibutyryl adenosine 3':5'-cyclic monophosphate, drabkins reagent, elastase (Type I, porcine pancreas), haemoglobin (bovine erythrocytes), haemoglobin (human), histamine diphosphate, (±)isoprenaline hydrochloride, , methylene blue, L-N^G- nitro arginine, phorbol 12- myristate 13-acetate, 4α- phorbol 12,13- didecanoate, paraquat (1,1'- dimethyl-4,4'-bipyridinium dichloride), L-phenylephrine hydrchloride, potassium ferricyanide, salbutamol, sodium nitroprusside, superoxide dismutase (bovine erythrocytes), thymidine, tri-n-octylamine, and vitamin E (DL-α-tocopherol acetate) were purchased from the Sigma Chemical Company Ltd (Poole,UK).

Dideoxyforskolin, forskolin and staurosporine were purchased from

Calbiochem (Nottingham, UK). Dobutamine was obtained from Eli Lilly Company Ltd (UK), glyceryl trinitrate (10% w/w lactose) was obtained from Napp Laboratories (UK) and cimetidine was obtained from SmithKline Beecham (UK).

N^G-monomethyl L- arginine and N^G-monomethyl D- arginine were a generous gift from Dr.R.M.J.Palmer of Wellcome Laboratories (UK).

M & B 22948 (2-0-propoxyphenyl-8-azapurin-6-one), rolipram (4-[3-cyclopentyloxy-4-methoxyphenyl]-2-pyrrolidone) and trequinsin (9,10-dimethoxy-2-mesitylimino-3-methyl-3,4,6,7,-tetrahydro-2H-pyrimido[6,1-a]isoquinolin-4-one) were a generous gift from Dr.J.E.Scuncess of Rhone-Poulenc Ltd (UK).

All drugs in an aqueous solution were sterilised by filtration through a Millipore filter (0.2µM pore size). Some drugs did not require sterilisation i.e. butylated hydroxytoluene, phorbol 12-myristate 13-acetate, 4α-phorbol 12,12-didecanoate, trequinsin and vitamin E since they were dissolved in 100% ethanol and dideoxyforskolin, forskolin and rolipram since they were dissolved in DMSO. At the dilutions used, the maximum concentrations of ethanol (0.1% v/v) and DMSO (0.1% v/v) had no effect on proliferation of endothelial or smooth muscle cells (data not shown).

3.8.4. Kreb's solution

The Kreb's solution was made from a 10x concentrate which contained the following (mM): NaCl 1180, KCl 48, $MgSO_4$ 12, KH_2PO_4 12 and $CaCl_2$ 25. The solution was diluted 1:10 with distilled water

when required and glucose and $NaHCO_3$ added to achieve final concentrations 11 mM and 24 mM, respectively. The working Kreb's solution was incubated at 37°C and gassed with 5% CO_2 in air for an hour before use.

3.8.5. Preparation of oxyhaemoglobin

Bovine haemoglobin type 1 (Sigma) contains a mixture of oxyhaemoglobin and its oxidized form, methaemoglobin. Pure reduced haemoglobin (oxyhaemoglobin) was prepared by adding to a 1 mM solution of Sigma haemoglobin in distilled water, a 20- fold molar excess of the reducing agent, sodium dithionite. The sodium dithionite was then removed by dialysis against 100 volumes of distilled water for 2-3 hours at 4°C. The resulting solution of oxyhaemoglobin was used immediately or stored frozen in aliquots at -20°C for up to 14 days.

3.8.6. Preparation of methaemoglobin

50 ml of whole blood was removed from a volunteer and placed in two sterile centifuge tubes (50 ml, Falcon). Tri sodium citrate (3.8%) was added to prevent the blood from clotting. The blood was then centrifuged at 200 g for 5 minutes at room temperature (IEC Centra 8R Centrifuge). The clear supernatant was discarded and the red blood cells were resuspended with 10 ml of sterile saline (0.9% w/v, Baxter, UK), centrifuged as before, and then, discard clear supernatant and the cells were resuspended with 10 ml of sterile saline. This procedure was repeated 3 times. 5 ml of sterile distilled water was added to lyse the red blood cells. This solution was then centrifuged at 1100 g. for 15 minutes to remove cellular debris. Pure oxidised haemogloblin

(methaemoglobin) was prepared by adding 2 ml of the oxidant, potassium ferricyanide (0.06M). The potassium ferricyanide was then removed by dialysis against 100 volumes of sterile distilled water for 2 to 3 hours at room temperature.

3.8.6.1. Quantification of methaemoglobin

3.8.6.2. Principles of assay

Determination of the concentration of methaemoglobin is based on the principle first proposed by Stadie (1920) with several modifications (Drabkin & Austin, 1935). Briefly, the methaemoglobin content in a sample can be quantified spectrophotometrically following the reaction between methaemoglobin and potassium cyanide. The product cyanmethaemoglobin has an absorption maximum at 540 nm. The colour intensity at this wavelength is proportional to total methaemoglobin concentration.

3.8.6.3. Chemicals for assay

3.8.6.3.1. Drabkin's Solution

One vial of dry mixture consisting of sodium bicarbonate, potassium ferricyanide and potassium cyanide (100: 20: 5) was supplied. This was reconstituted with 1000 ml of distilled water. The resulting solution was mixed with 0.5 ml of 30% Brij- 35 solution (polyoxyethylene 23 lauryl ether). When protected from light, this solution was stable for at least 6 months.

3.8.6.3.2. Cyanmethaemoglobin Standard

The cyanmethaemoglobin standard was prepared as follows: one vial of lyophilized haemoglobulin (human) was added to 50 ml of Drabkin's solution. The resulting solution is mixed well and allowed

to stand for at least 30 minutes before use. The cyanmethaemoglobin standard had been calibrated spectrophotometrically by the manufacturer at 540 nm and the molecular weight of haemoglobin was taken as 64458 daltons. The cyanmethaemoglobin solution gives an absorbance equivalent to that of a whole blood sample containing 18 grams haemoglobin per 100 ml. This solution was stable for at least 6 months when stored at 0-5°C.

3.8.6.3.3. Preparation of standard curve and assay of samples

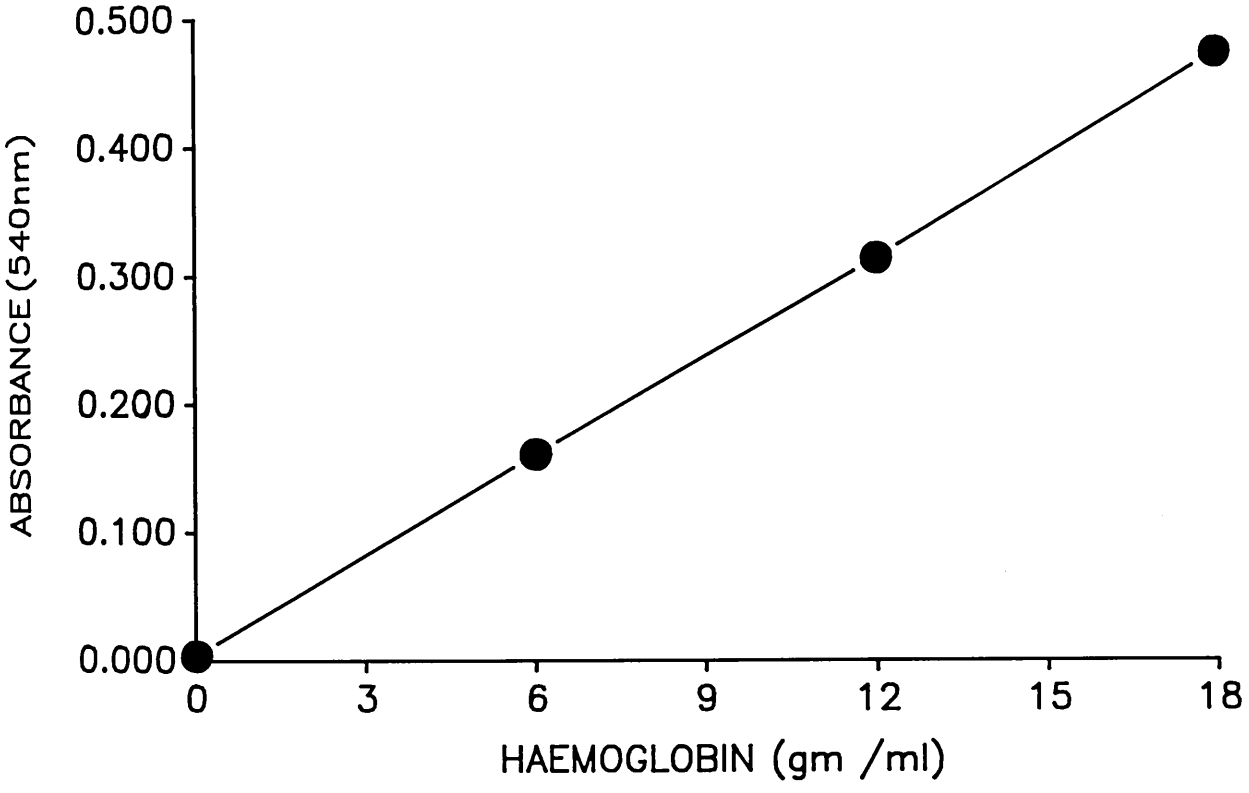
A series of tubes was numbered for identification. The cyanmethaemoglobin standard solution was diluted with Drabkin's solution to prepare the following standards: 0, 6, 12, 18 grams haemoglobin per 100 ml. All standards were run in duplicate. The tubes containing the "0 standard" are referred to as the "reference". For measurement of unknowns, 20µl of sample was added to 5 ml of Drabkin's solution. Allow to stand for 15 minutes at room temperature. The absorbance of the standards and samples was determined against the "reference" at a wavelength at 540 nm in an UV- visible recording spectrophotometer (UV- 240, Shimadzu). The same cuvette was used for each recording.

3.8.6.3.4. Analysis of methaemoglobin content

Analysis of methaemoglobin content was determined by plotting a standard curve, this was found to be linear and passed through the origin. The concentration for each sample (grams of methaemoglobin per 100 ml) was calculated by interpolation from the standard curve. Figure 9 represents a typical standard curve obtained for a methaemoglobin assay.

Figure 9: Typical standard curve for methaemoglobin determination.

The methaemglobin concentration of the samples were obtained by interpolation from the standard curve by the spectrophotometer (UV- 240, Shimadzu).



3.8.7 Statistical analysis

Results are expressed as the mean ± s.e. mean and comparisons were made by the use of Student's t-test or by the non-parametric Mann Whitney test when there was unequal variance between samples. A probability of 0.05 or less was considered to be significant. In the Results, n represents the number of observations.

RESULTS

4.1. PIG AORTIC ENDOTHELIAL PHOSPHODIESTERASES

Little is known about the processes in endothelial cells responsible for the catabolism of cyclic AMP and cyclic GMP. In this study, we attempted to evaluate the role of the phosphodiesterase (PDE) enzymes present in endothelial cells by isolating the various subtypes and studying the effects of selective inhibitors on cyclic AMP and cyclic GMP accumulation.

4.1.1. Isolation of PDE isozymes from PAEC

This part of the project was carried out in collaboration with Dr. J. E. Souness, Rhone- Poulenc Ltd., in his Dagenham laboratory.

Two peaks of cytosolic PDE activity were resolved by DEAE-Trisacryl chromatography (Figure 10). The first peak, eluted at 0.12M NaCl, exhibited activity against both cyclic AMP and cyclic GMP, and the addition of 1μM cyclic GMP stimulated cyclic AMP hydrolysis 2- fold. The ability of this first enzyme to hydrolyse cyclic GMP was not augmented in the presence of calcium (2 mM) and calmodulin (0.5 unit/ml). The second peak of activity was eluted with 0.19M NaCl and selectively hydrolysed cyclic AMP, displaying little or no activity against cyclic GMP (Figure 10, Souness et al., 1990).

The method for purification of the PDE isozymes is described in Appendix I.

These results indicate the presence of two PDE isozymes in pig aortic endothelial cells, a cyclic GMP- stimulated PDE (Type II) and a cyclic AMP PDE (Type IV). It was found that equal hydrolytic activity was associated with the cytosolic and particulate fraction of pig aortic endothelial cell homogenates.

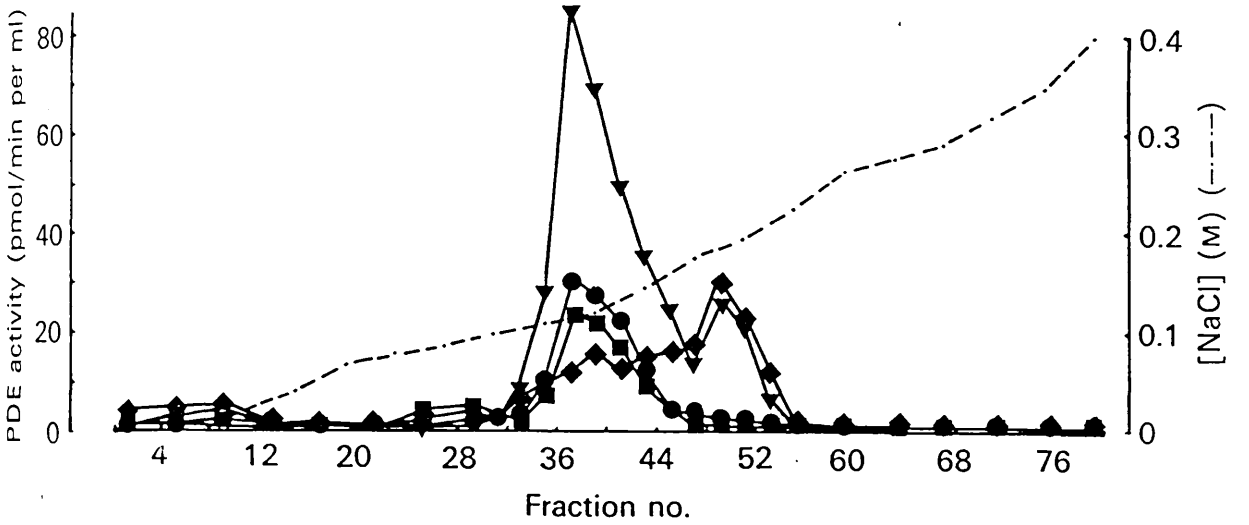


Figure 10: DEAE- Trisacryl chromatography of PDE activity of the 100000g supernatant fraction from the pig aortic endothelial cells. Preparation of a cytosolic fraction from the pig aortic endothelial cells and its chromatography on DEAE- Trisacryl is described in Souness et al. (1990). Fractions were assayed for cyclic AMP PDE activity (2µM substrate) in the absence (◆) and presence (▼) of 1µM- cyclic GMP, and for cyclic GMP PDE activity (1µM substrate) in the absence (■) and presence (●) of 2mM- $CaCl_2$ plus 0.5 unit of calmodulin. All assays were performed in the presence of 200µM- EGTA.

4.1.2. Selective inhibitors

The activity of selective cyclic nucleotide PDE inhibitors against endothelial cell cyclic GMP- stimulated and cyclic AMP PDEs are shown in Table 7 (Souness et al., 1990).

The method for this is described in Appendix II.

M & B 22948, a selective inhibitor of cyclic GMP PDEs (Type I) in smooth muscle was only weakly effective in inhibiting the two PDE- isozymes present in PAEC. SK & F 94120, a selective inhibitor of cyclic GMP- inhibited PDE (Type III) also exhibited weak activity against the two PDE isozymes. In contrast, dipyridamole and trequinsin, two non- selective PDE inhibitors, potently inhibit both isozymes, whereas rolipram, a selective cyclic AMP PDE (Type IV) inhibitor, selectively inhibited the cyclic AMP PDE present in PAEC.

We next examined the effects of dipyridamole, trequinsin and rolipram to determine whether the PDE isozymes found in cell homogenates had any role in regulating cyclic AMP and cyclic GMP levels in intact pig aortic endothelial cells. All these and subsequent experiments were carried out in Glasgow.

4.2. CYCLIC GMP CONTENT IN ENDOTHELIAL CELLS

4.2.1. Effect of spontaneously- released EDRF on cyclic GMP content in pig aortic endothelial cells

It has been suggested that spontaneously- released EDRF is responsible for the resting level of cyclic GMP in endothelial cells through the activation of soluble guanylate cyclase (Martin et al., 1988b).

Inhibitor	Cyclic GMP-stimulated PDE			Cyclic AMP PDE
	Cyclic AMP	Cyclic AMP (+ cyclic GMP)	Cyclic GMP	
M & B 22948	708	45	50	93
Trequinsin	4	0.6	0.6	0.2
Dipyridamole	17	5	3	6
SK & F 94120	643	485	558	>1000
Rolipram	435	448	417	3

Table 7: Inhibition of seperate PDE activities by various compounds. IC_{50} values (expressed as µM) were determined on cyclic GMP- stimulated PDE with 2µM- cyclic AMP as substrate in the presence or absence of 1µM- cyclic GMP, or with 1µM- cyclic GMP as substrate. Cyclic AMP PDE was assayed with 1µM- cyclic AMP as substrate. The results represent the means of duplicate determinations performed on two different batches of the enzymes.

When primary cultures of PAEC were pretreated with haemoglobin (10μM) which binds to and inactivates EDRF, the resting level of cyclic GMP was reduced from 67 ± 6 to 9.9 ± 2.8 fmol μg DNA^{-1} (n=6). Bradykinin (0.1μM) induced an increase in intracellular cyclic GMP content after a 1.5 minute exposure, from 67 ± 6 to 650.8 ± 92.2 fmol μg DNA^{-1} (n=6), a 9.7- fold increase. This increase was blocked by pretreatment with haemoglobin (10μM, Figure 11).

4.2.2. Effects of L- NMMA and L- canavanine

EDRF has been identified as nitric oxide and endothelial cells are known to synthesize nitric oxide from L- arginine. There is general agreement that the converting enzyme, NO- synthase can be inhibited by N^G- monomethyl L- arginine (L- NMMA) and some but not all reports suggest that L- canavanine can also inhibit this enzyme in endothelial cells (Palmer et al., 1988b; Schmidt et al., 1988; Rees et al., 1988; 1990).

Untreated cells had a resting intracellular cyclic GMP content of 77 ± 17 fmol μg DNA^{-1} (n=6). Following pretreatment for 20 minutes, L- NMMA (300μM) reduced the intracellular cyclic GMP content to 17 ± 2.5 fmol μg DNA^{-1} (n=6), a 4.5- fold decrease, whereas L- canavanine (300μM) had no effect (Figure 12). Bradykinin (0.1μM), a stimulant of endothelial EDRF production, induced an increase in intracellular cyclic GMP content after a 1.5 minute exposure to 342.9 ± 74.6 fmol μg DNA^{-1} (n=6), a 4.5-fold increase. This increase was blocked following 20 minutes pretreatment of PAEC with L- NMMA (300μM) but not with L-canavanine (300μM, Figure 12).

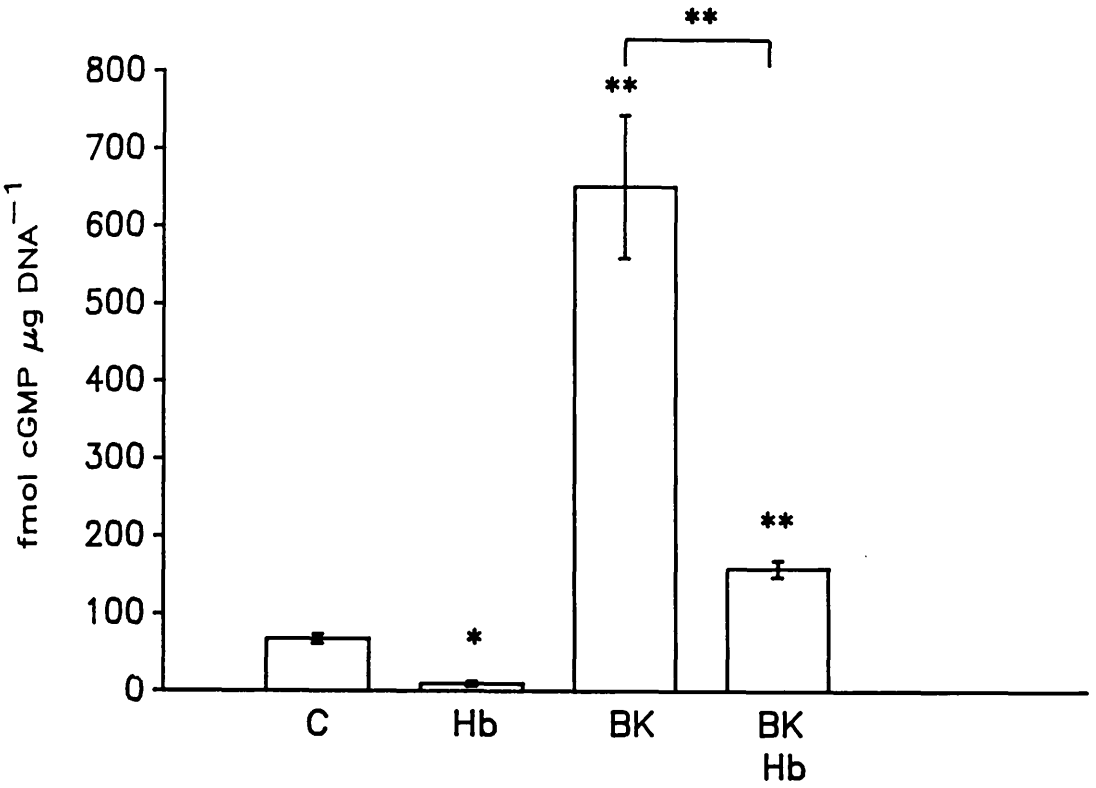


Figure 11: Effects of haemoglobin on the cyclic GMP content of PAEC in the presence and absence of bradykinin. Cells were exposed either to no drugs (C) or haemoglobin (10µM, Hb) for 18.5 minutes before being incubated with or without bradykinin (0.1µM, BK) for a further 1.5 minutes. The incubation was terminated by removal of Krebs and addition of 6% trichloroacetic acid (TCA) and cyclic GMP quantified by radioimmunoassay. Bars represent mean ± s.e. mean content of cyclic GMP (fmol µg DNA^{-1}, n=6). * P< 0.05; ** P< 0.005; denotes a difference from untreated cells, or between two groups joined with a bracket.

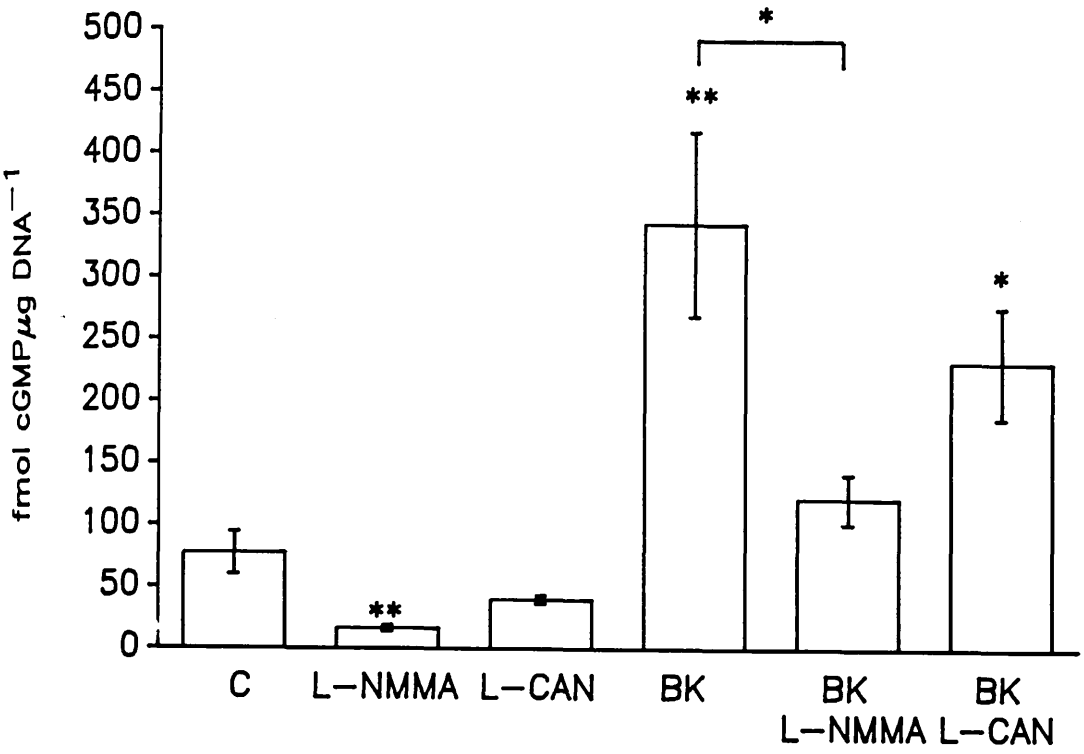


Figure 12: Effects of N^G- monomethyl L- arginine (L- NMMA) and L-canavanine on cyclic GMP content of PAEC in the presence and absence of bradykinin. Cells were exposed to either no drugs (C), L- NMMA (300µM) or L- canavanine (300µM) for 18.5 minutes before being incubated with or without bradykinin (0.1µM, BK) for a further 1.5 minutes. The incubation was terminated by removal of Krebs and addition of 6% TCA and cyclic GMP quantified by radioimmunoassay. Bars represent mean ± s.e. mean content of cyclic GMP (fmol µg DNA^{-1}, n=6). * $P< 0.05$; ** $P< 0.005$; denotes a difference from untreated cells, or, between two groups joined with a bracket.

4.2.3. Effects of dipyridamole

Although dipyridamole is a potent inhibitor of both the cyclic GMP- stimulated PDE and the cyclic AMP PDE present in homogenates of PAEC, only the former hydrolyses cyclic GMP (Table 7, Figure 10). The effects of dipyridamole were investigated on the cyclic GMP content to determine if this enzyme played a role in regulating the cellular content of this cyclic nucleotide.

The intracellular cyclic GMP content in untreated cells remained constant over a period of 60 minutes (Figure 13A). Dipyridamole (25μM) induced an increase in intracellular cyclic GMP content which peaked at 5 minutes: it rose from 40.1 ± 4.2 to 379.5 ± 80.1 fmol μg DNA^{-1} (n=6-18), a 9.5- fold increase, then declined rapidly to a level 2- 3 fold above the original resting level and subsequently remained constant for up to 60 minutes. This increase was blocked completely following pretreatment with haemoglobin (10μM, Figure 13A). At this point it was not certain that the elevation of cyclic GMP induced by dipyridamole resulted from inhibition of the cyclic GMP- stimulated PDE isozyme. It is known that dipyridamole blocks the nucleoside transporter protein in several cell types (Pearson et al., 1978; Cabral et al., 1984; Plagermann & Woffendin, 1988). The nucleoside transporter is a simple carrier with a broad substrate specificity which includes cyclic nucleotides. Therefore, dipyridamole may have elevated cyclic GMP content by inhibiting the efflux of this molecule from the cell. This possibility was investigated by measuring the leakage of cyclic GMP into the Kreb's bathing medium (Figure 13B).

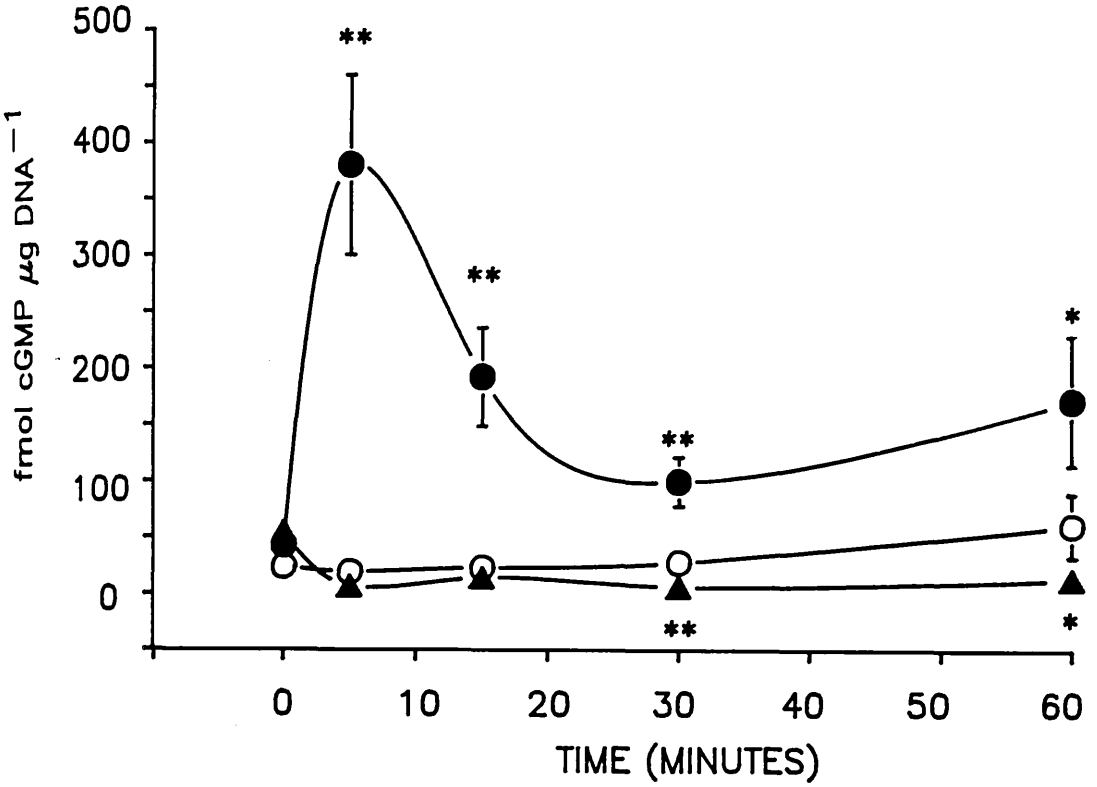


Figure 13A: Time course of the effects of dipyridamole on the intracellular cyclic GMP content of PAEC. Cells were exposed either to no drugs (o), dipyridamole (25µM, ●), or a combination of dipyridamole (25µM) and haemoglobin (10µM, ▲). The cells were then incubated for 60 minutes at 37°C. At the time points indicated the incubation was terminated by removal of the krebs and addition of 6% TCA and the intracellular cyclic GMP content quantified by radioimmunoassay. Points represent mean ± s.e. mean content of cyclic GMP (fmol µg DNA^{-1}, n=6). When error bars are not seen they are contained within the symbols. * $P<0.05$; ** $P<0.005$; denotes a significant difference from untreated cells.

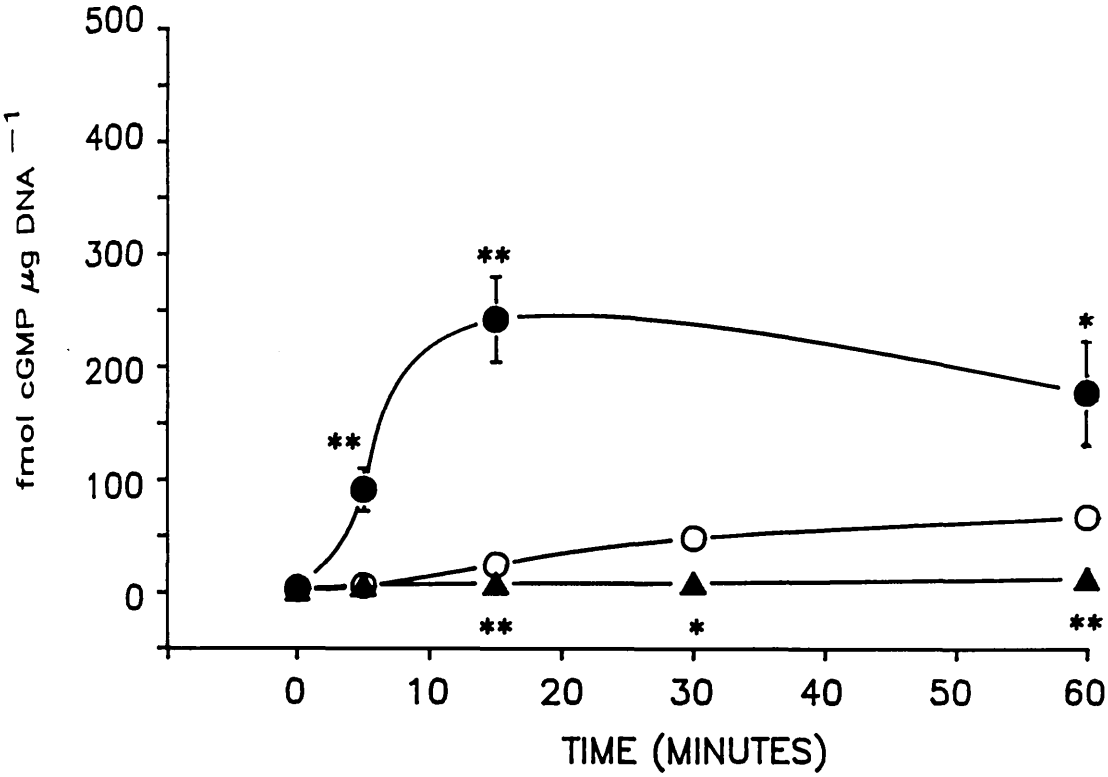


Figure 13B: Time course of the effects of dipyridamole on the extracellular accumulation of cyclic GMP. These data were obtained from the same experiment shown in Figure 13A. Cells were exposed either to no drugs (o), dipyridamole (25µM, ●) or a combination of dipyridamole (25µM) and haemoglobin (10µM, ▲). The cells were then incubated for 60 minutes at 37°C. At the time points indicated the Krebs was removed and the extracellular content of cyclic GMP quantified by radioimmunoassay. Points represent mean ± s.e. mean content of cyclic GMP (fmol µg DNA^{-1}, n=6). When error bars are not seen, they are contained within the symbols. * $P< 0.05$; ** $P< 0.005$; denotes a significant difference from untreated cells.

The cyclic GMP content of the Kreb's bathing untreated cells increased during the 60 minutes incubation, indicating leakage does occur: it rose from 2.8 ± 0.3 to 67.4 ± 11.0 fmol µg DNA^{-1} (n=18) at 60 minutes (Figure 13B). Dipyridamole (25 µM) increased the leakage of cyclic GMP into the Kreb's bathing medium: it reached a plateau after 15 minutes of 241.7 ± 38.0 fmol µg DNA^{-1} (n=6), a 9.5- fold increase, and then remained relatively constant for the remainder of the 60 minutes incubation period. This increased accumulation of cyclic GMP stimulated by dipyridamole was blocked following pretreatment with haemoglobin (10µM, Figure 13B).

From these results it is likely that dipyridamole (25µM) elevates intracellular cyclic GMP content, not by blocking the efflux of this molecule from the cell, but by inhibiting the cyclic GMP-stimulated PDE isozyme.

4.2.4. Effects of trequinsin

Trequinsin is a potent inhibitor of both the cyclic GMP-stimulated PDE and the cyclic AMP PDE (Table 7). The effects of trequinsin on the cyclic GMP content of PAEC were therefore examined.

The intracellular cyclic GMP content of untreated cells remained relatively constant during 60 minute incubation period (Figure 14). Trequinsin (10µM) induced an increase in intracellular cyclic GMP content which peaked at 30 minutes exposure: it rose from 98.0 ± 14.9 to 454.4 ± 69.7 fmol µg DNA^{-1} (n=6-7), a 4.5-fold increase, and then remained relatively constant during the

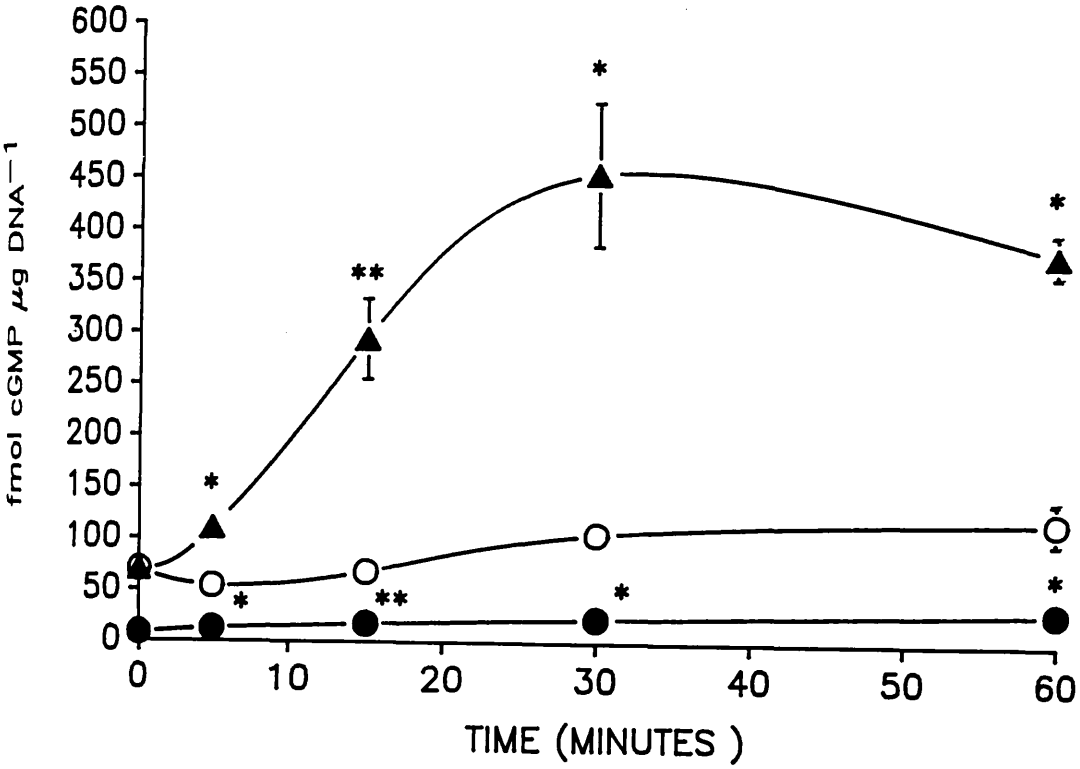


Figure 14: Time course of the effect of trequinsin on intracellular cyclic GMP content in PAEC. Cells were preincubated for 15 minutes in the absence or presence of haemoglobin (10µM, o). Then received no drugs (o), or trequinsin (10µM) in the presence (●) or absence (▲) of haemoglobin and were incubated at 37°C for a further 60 minutes. At the time points indicated, the incubation was terminated by the removal of the Krebs and addition of 6% TCA and cyclic GMP content quantified by radioimmunoassay. Points represent mean ± s.e. mean content of cyclic GMP (fmol µg DNA^{-1}, n=4-7). When error bars are not seen they are contained within symbols. * $P< 0.05$; ** $P< 0.005$; denotes a significant difference from untreated cells.

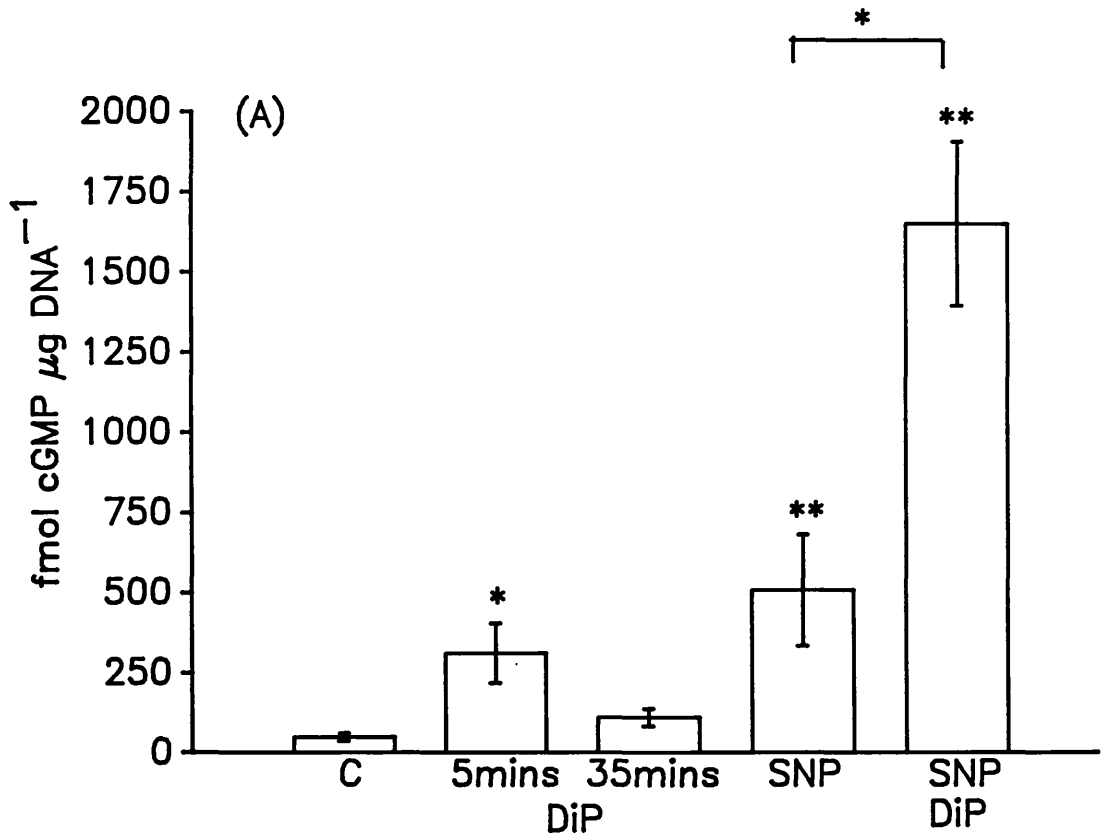
60 minute incubation. The increased accumulation of cyclic GMP was blocked following pretreatment with haemoglobin (10μM, Figure 14).

4.2.5. Effects of sodium nitroprusside

Having established that inhibitors of the cyclic GMP- stimulated PDE could elevate the cyclic GMP content of PAEC by inhibiting the hydrolysis of cyclic GMP formed following stimulation of soluble guanylate cyclase by spontaneously produced EDRF, the ability of dipyridamole to potentiate the actions of another stimulant of soluble guanylate cyclase, sodium nitroprusside was examined.

Untreated cells had a resting intracellular cyclic GMP content of 48.7 ± 11.7 fmol μg DNA^{-1} (n=6, Figure 15A). Dipyridamole (25μM) induced a rapid rise in intracellular cyclic GMP content after 5 minutes exposure: the maximum increase was 6.4- fold and then diminished to 2.3- fold above control after 35 minutes (Figure 15A). Sodium nitroprusside (1μM) was added for 2 minutes to untreated cells or to cells pretreated with dipyridamole (25μM) for 33 minutes. In untreated cells, sodium nitroprusside (1μM) increased the intracellular cyclic GMP content from 48.7 ± 11.7 to 504.5 ± 172.7 fmol μg DNA^{-1} (n=5-6), a 10.9- fold increase (Figure 15A). In dipyridamole pretreated cells, the sodium nitroprusside- stimulated increase in cyclic GMP content was enhanced 3.25- fold (Figure 15A).

The leakage of cyclic GMP into the Kreb's bathing medium was also examined. The cyclic GMP content of the Kreb's bathing untreated


(A)
fmol cGMP μg DNA⁻¹
2000
1750
1500
1250
1000
750
500
250
0
*
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*
C
5mins DiP
35mins
SNP
SNP DiP

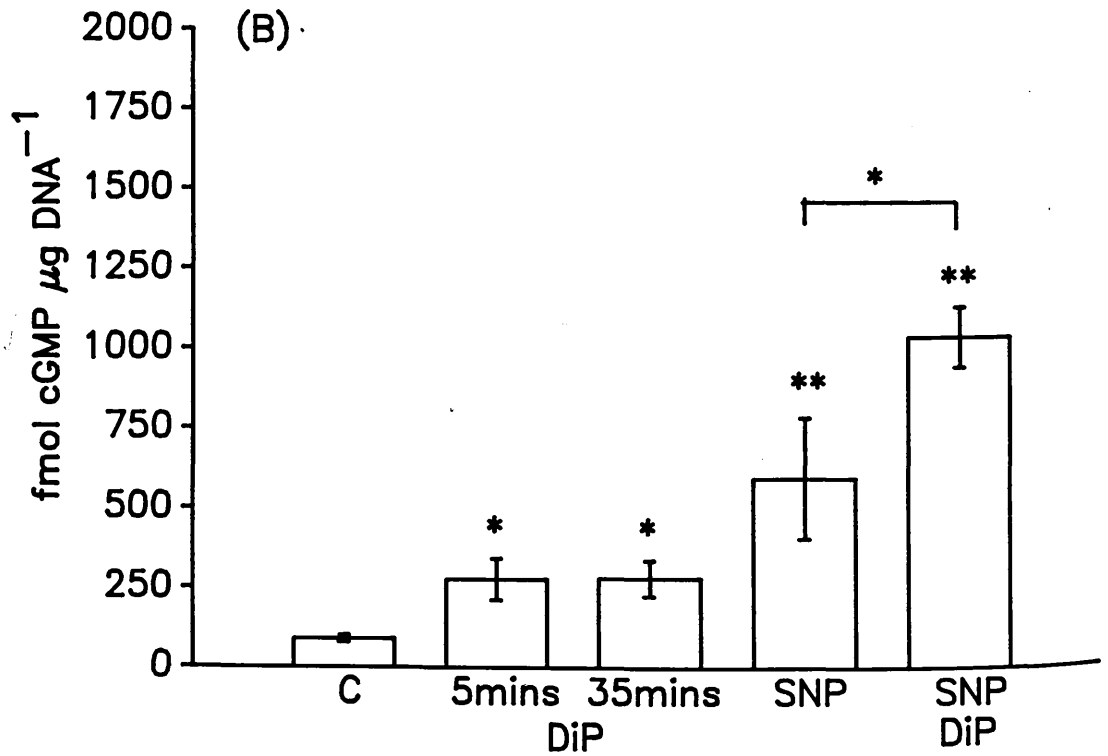

(B)
fmol cGMP μg DNA⁻¹
2000
1750
1500
1250
1000
750
500
250
0
*
**
*
*
C
5mins DiP
35mins
SNP
SNP DiP

Figure 15: Effects of dipyridamole and sodium nitroprusside on intracellular and extracellular accumulation of cyclic GMP. PAEC were incubated for 35 minutes with or without dipyridamole (25μM, DiP) during which time the rapid rise in intracellular cyclic GMP content had diminished from the peak value obtained at 5 minutes to the value indicated after 35 minutes. Untreated cells or cells pretreated with dipyridamole (25μM, DiP) for 33 minutes were then exposed to sodium nitroprusside (1μM, SNP) for a further 2 minutes. The incubation was terminated by removal of the Krebs bathing solution and addition of 6% TCA. The cyclic GMP content of the cells and the Krebs bathing solution was then measured by radioimmunoassay. The cyclic GMP content of the cells is shown on the top panel (A) and that of the Krebs bathing solution is shown in the bottom panel (B). Bars represent mean ± s.e. mean content of cyclic GMP (fmol μg DNA^{-1}, n=5-6). * $P< 0.05$; ** $P< 0.005$; denotes a significant difference from untreated cells, or, between two groups joined by a bracket.

cells after 35 minutes incubation was 89.9 ± 11.1 fmol µg DNA^{-1} (n=6). Dipyridamole (25µM) induced a rise in the cyclic GMP content of the Kreb's: after 5 minutes it rose to 277.4 ± 64.6 fmol µg DNA^{-1} (n=6), a 3- fold increase and remained constant for the remainder of the 35 minute incubation. When added to untreated cells, sodium nitroprusside (1µM) induced a marked increase in the cyclic GMP content of the Kreb's after 2 minutes exposure to 594.3 ± 189.0 fmol µg DNA^{-1} (n=6), a 6.6- fold increase. In cells pretreated with dipyridamole (25µM) for 35 minutes, the sodium nitroprusside (1µM)- induced accumulation of cyclic GMP in the kreb's was enhanced by 1.7- fold (Figure 15B).

4.2.6. Effects of atriopeptin II

Having established that inhibition of the cyclic GMP- stimulated PDE could potentiate the increase in cyclic GMP content induced by two stimulants of soluble guanylate cyclase, namely EDRF and sodium nitroprusside, the ability of PDE inhibitors to potentiate the actions of atriopeptin II, a stimulant of particulate guanylate cyclase, was investigated. In these experiments, the cells were pretreated for 20 minutes with haemoglobin (10µM). This abolished any activation of soluble guanylate cyclase by spontaneously- released EDRF, thereby allowing a clearer investigation of the effects of activating particulate guanylate cyclase.

When cells were exposed to haemoglobin (10µM) the resting content of cyclic GMP was 10.5 ± 1.6 fmol µg DNA^{-1} (n=12). In these cells, atriopeptin II (10nM) induced an increase in intracellular cyclic GMP content which peaked after 5 minutes exposure, rising

to 53.8 ± 8.5 fmol µg DNA^{-1} (n=6), a 5.1- fold increase. It subsequently stayed relatively constant for the remainder of the 30 minute incubation (Figure 16). The ability of atriopeptin II to induce increases in intracellular cyclic GMP content in haemoglobulin (10µM) pretreated cells was observed over the concentration range of 0.1 to 100 nM and the maximum obtained was 485.9 ± 44.0 fmol µg DNA^{-1} (n=8, Figure 17).

4.2.7. Effect of dipyridamole, trequinsin and rolipram on the atriopeptin II- induced elevation of cyclic GMP

In these experiments all cells were pretreated with haemoglobin (10µM) to inhibit stimulation of soluble guanylate cyclase, and under these conditions the resting level of cyclic GMP was 14.8 ± 1.8 fmol µg DNA^{-1} (n=29). Subsequent treatment with atriopeptin II (10nM) for 15 minutes induced an increase in intracellular cyclic GMP content to 92.8 ± 8.2 fmol µg DNA^{-1} (n=12), a 6.3-fold increase (Figure 18). Treatment for 35 minutes with dipyridamole (25µM) had no effect on the resting level of cyclic GMP but trequinsin (25µM), induced a 5.3- fold increase (Figure 18). The selective inhibitor of the cyclic AMP PDE isozyme, rolipram (25µM) had no effect on the resting level of cyclic GMP following a 35 minute incubation. Furthermore, pretreatment with dipyridamole (25µM) and trequinsin (25µM) each enhanced the elevation of cyclic GMP content induced by atriopeptin II (10nM, Figure 18). In contrast, rolipram (25µM) had no effect on the atriopeptin II (10nM)- induced increase in cyclic GMP content (Figure 18).

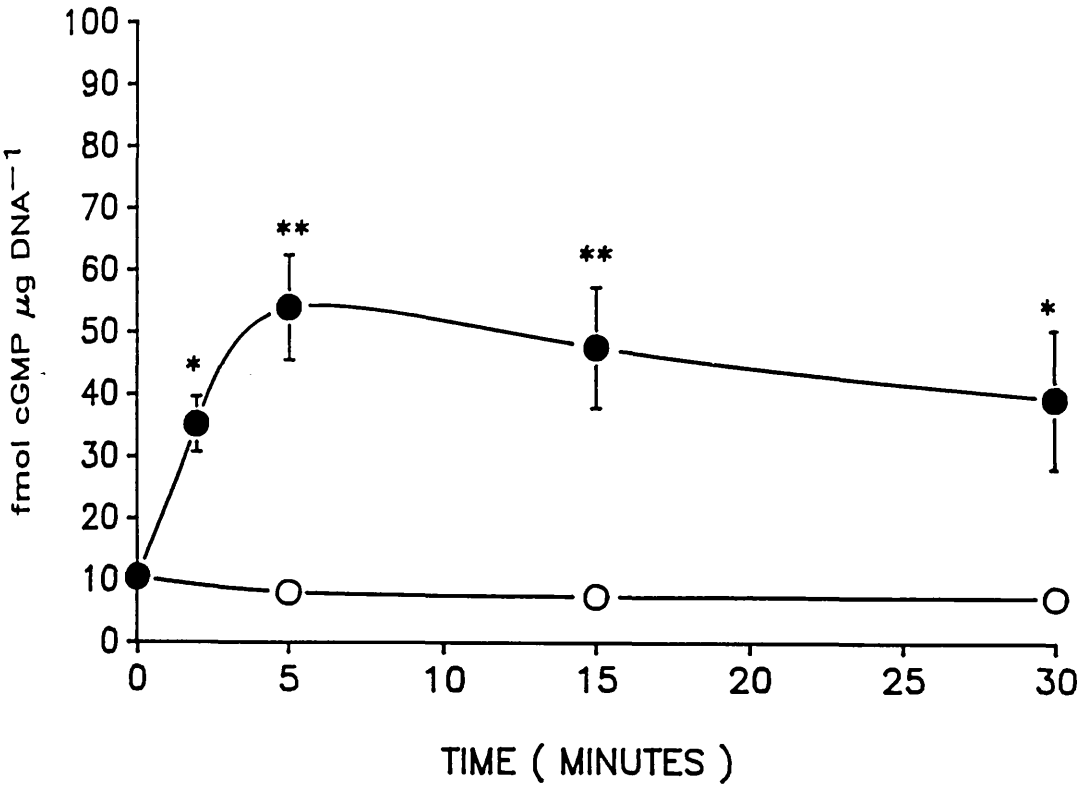


Figure 16: Effects of atriopeptin II on the cyclic GMP content of PAEC in the presence of haemoglobin. All cells were pretreated for 20 minutes with haemoglobin (10μM) to inhibit stimulation of soluble guanylate cyclase before being incubated with (●) or without (o) atriopeptin II (10nM). At the time points indicated the incubation was terminated by removal of Krebs and addition of 6% TCA and the cyclic GMP content measured by radioimmunoassay. Points represent mean ± s.e. mean content of cyclic GMP (fmol μg DNA^{-1}, n=6-12). When error bars are not seen they are contained within symbols. * $P< 0.05$; ** $P< 0.005$; denotes a significant difference from cells not receiving atriopeptin II.

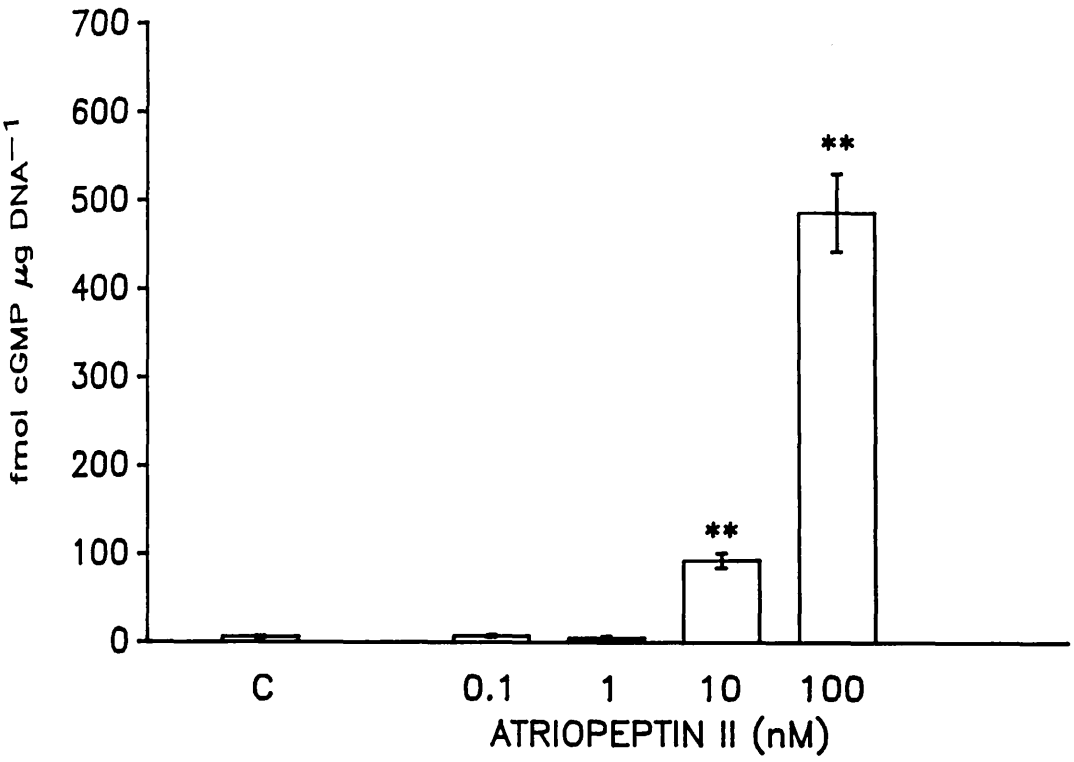


Figure 17: Concentration- effect relationship showing the ability of atriopeptin II to elevate the cyclic GMP content of PAEC in the presence of haemoglobin. All cells were pretreated for 20 minutes with haemoglobin (10µM) to block any stimulation of soluble guanylate cyclase. Cells then received either no drugs (C) or were treated for a further 15 minutes with atriopeptin II (0.1- 100nM). The incubation was then terminated by removal of Krebs and addition of 6% TCA and the cyclic GMP content quantified by radioimmunoassay. Bars represent mean ± s.e. mean content of cyclic GMP (fmol µg DNA^{-1}, n=6-29). ** $P< 0.0005$ denotes a significant difference from cells which did not receive atriopeptin II.

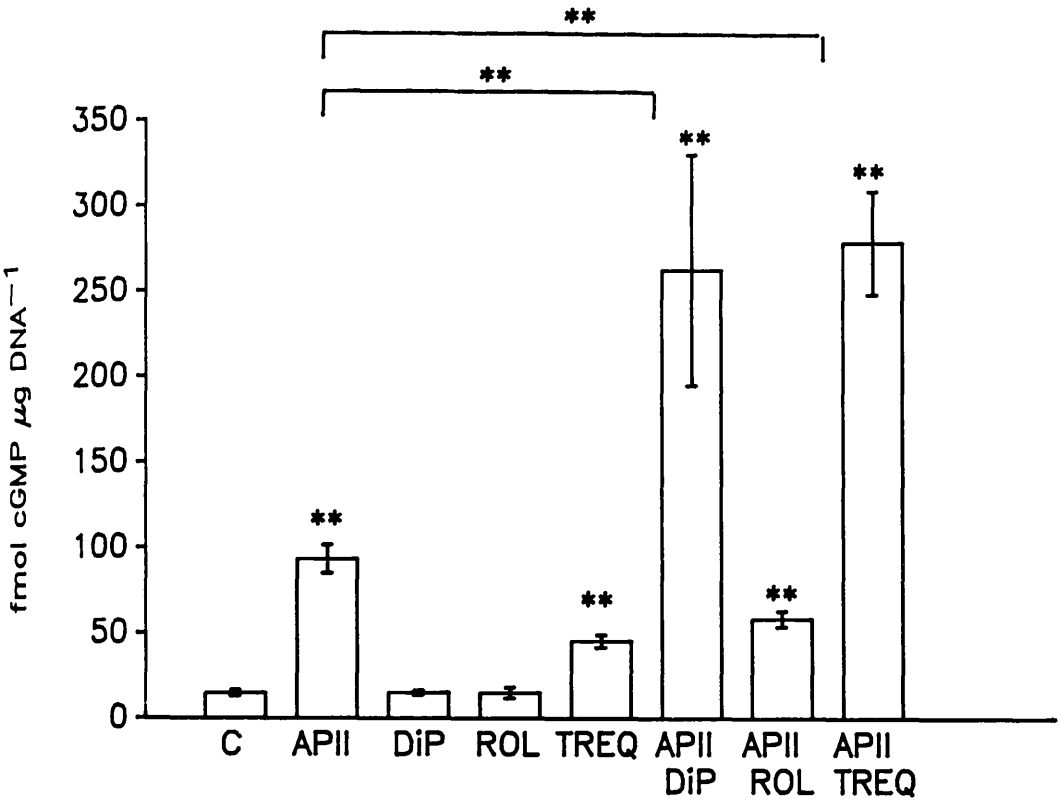


Figure 18: Effect of dipyridamole, rolipram and trequinsin on the atriopeptin II- induced increase in cyclic GMP content in PAEC. All cells were pretreated for 20 minutes with haemoglobin (10μM). Where indicated cells received either no drugs (C), or received dipyridamole (25μM, DiP), rolipram (25μM, ROL). or trequinsin (10μM, TREQ) during the 20 minute pre- incubated period. The cells were then incubated with or without atriopeptin II (10nM, AP II) for a further 15 minutes. The incubation was terminated by removal of Krebs and addition of 6% TCA and cyclic GMP content was quantified by radioimmunoassay. Bars represent mean ± s.e. mean cyclic GMP content (fmol μg DNA^{-1}, n=6-29).

** P< 0.0005; denotes a significant difference from untreated cells, or, between groups joined by a bracket.

4.3. CYCLIC AMP CONTENT IN ENDOTHELIAL CELLS

It was clear from the preceding section (4.2) that drugs which had been shown to inhibit the cyclic GMP- stimulated PDE in endothelial cell homogenates had the ability to elevate cyclic GMP levels in intact cells. It is likely therefore that the cyclic GMP- stimulated PDE has an important role in regulating the cyclic GMP content of endothelial cells. In this section an attempt was made to determine if the cyclic AMP PDE found in endothelial cell homogenates had a role in regulating the intracellular content of cyclic AMP in intact cells by examining the effects of inhibitors of this enzyme.

4.3.1. Effects of dipyridamole and isoprenaline

The resting level of cyclic AMP in primary cultures of PAEC was 256.7 ± 39.5 fmol µg DNA^{-1} (n=4, Figure 19). Addition of isoprenaline (10µM), a β- adrenoceptor agonist, for 5 minutes had no effect on the resting content of cyclic AMP (Figure 19). The non- selective inhibitor, dipyridamole (25µM), had no effect on the resting level of cyclic AMP after a 35 minute incubation period, but enhanced the ability of isoprenaline (10µM) to increase cyclic AMP content: in the combined presence of isoprenaline and dipyridamole the cyclic AMP content rose 1.7-fold (Figure 19).

4.3.2. Effects of dipyridamole and forskolin

Untreated cells had a resting cyclic AMP content of 1080.0 ± 130.0 fmol µg DNA^{-1} (n=10). Forskolin (10µM), a direct activator of the catalytic subunit of adenylate cyclase (Seamon & Daly, 1981), had no effect upon the resting level of cyclic AMP

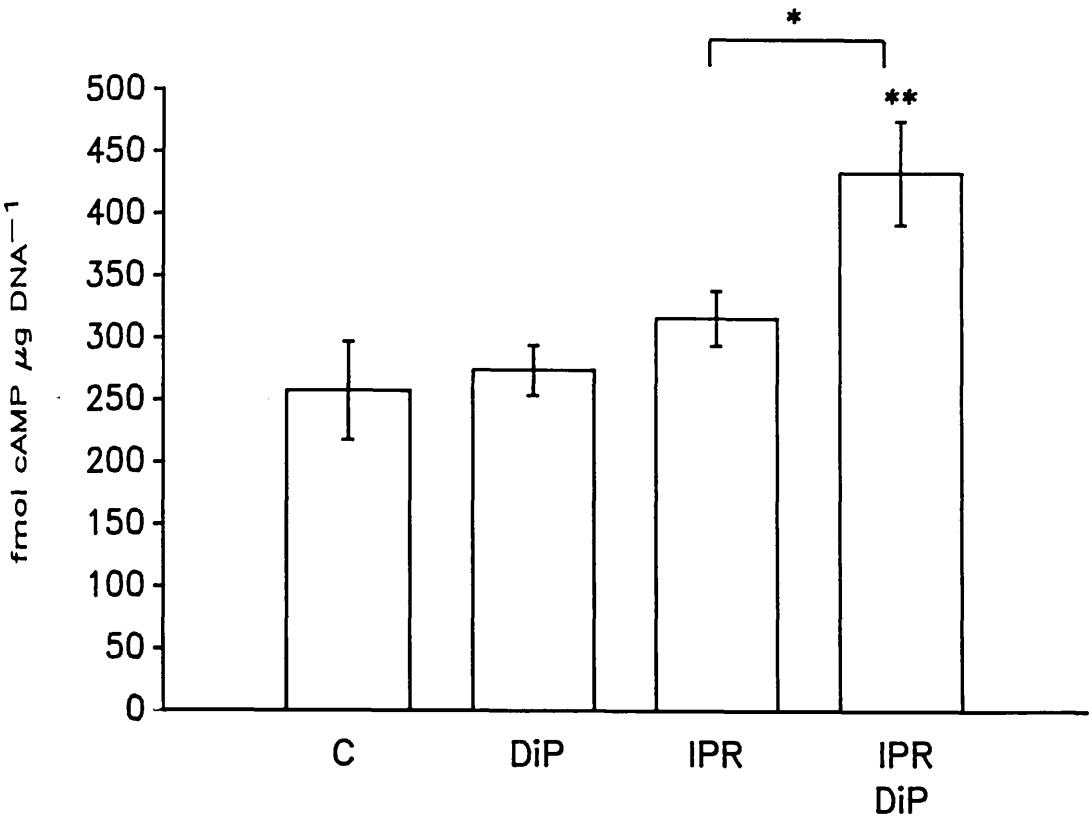


Figure 19: Effects of dipyridamole and isoprenaline on the cyclic AMP content of PAEC. Cells were treated with either no drugs (C), or with dipyridamole (25µM, DiP) for 30 minutes before being incubated with or without isoprenaline (10µM. IPR) for a further 5 minutes. The incubation was then terminated by the removal of Krebs and addition of 6% TCA and cyclic AMP content quantified by radioimmunoassay. Bars represent mean ± s.e. mean content of cyclic AMP (fmol µg DNA^{-1}, n=6). * $P< 0.05$; ** $P< 0.005$; denotes a significant difference from untreated cells, or, between two groups joined by a bracket.

following a 2 minute exposure (Figure 20). Pretreatment for 10 minutes with dipyridamole (100μM) induced an increase in cyclic AMP content from 1080.0 ± 130.0 to 1640.0 ± 200.0 fmol μg DNA^{-1} (n=10). When forskolin (10μM) was added subsequently, the cyclic AMP content rose to 3880.0 ± 850.0 fmol μg DNA^{-1} (n=5), a 2.8-fold rise (Figure 20).

4.3.3 Effects of rolipram and forskolin

Untreated cells had a resting cyclic AMP content of 230.5 ± 10.3 fmol μg DNA^{-1} (n=7). Treatment with forskolin (30μM) for 5 minutes induced an increase in the cyclic AMP content: it rose to 811.9 ± 84.6 fmol μg DNA^{-1} (n=7), a 3.5- fold increase (Figure 21). Pretreatment for 30 minutes with the selective inhibitor of the cyclic AMP PDE, rolipram (25μM), induced an increase in cyclic AMP content: it rose to 296.4 ± 10.7 fmol μg DNA^{-1} (n=6), a 1.3- fold increase, and enhanced the forskolin (30μM)- induced increase in cyclic AMP content to 2232.0 ± 386.0 fmol μg DNA^{-1} (n=7), a 2.7- fold enhancement (Figure 21).

From this section it is clear that the cyclic AMP PDE found in endothelial homogenates has a role in regulating the cyclic AMP content in intact cells.

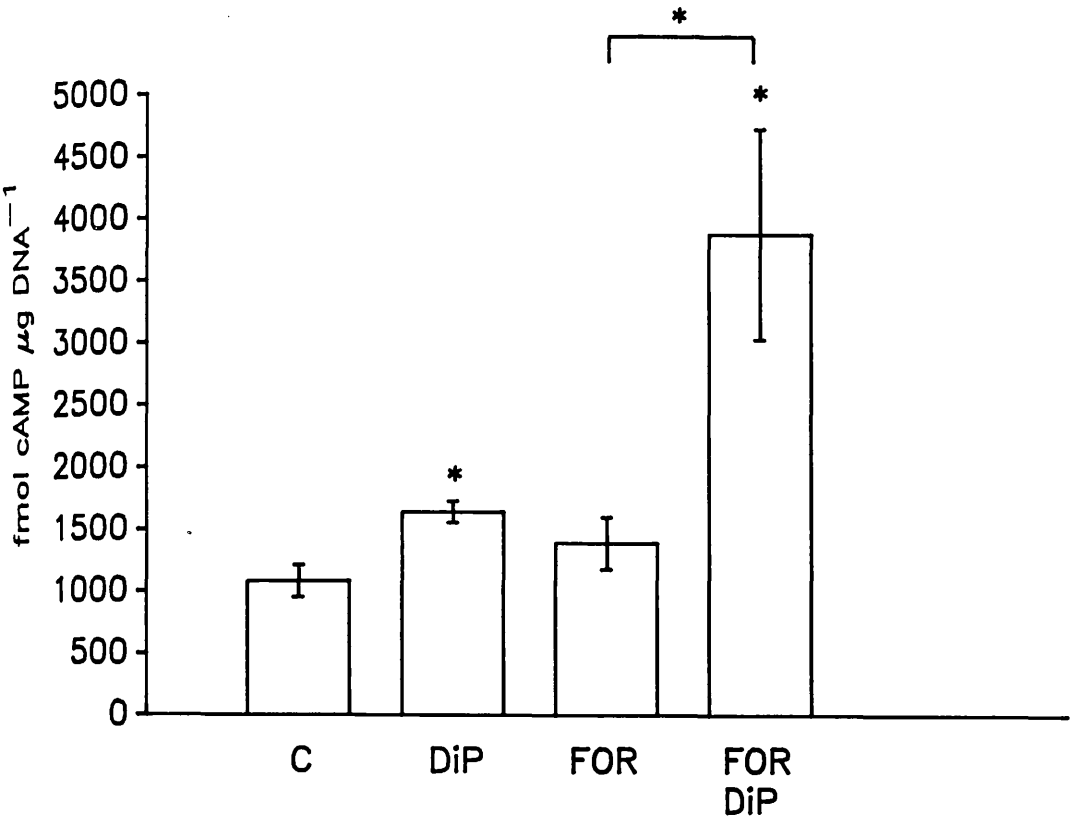


Figure 20: Effects of dipyridamole and forskolin on the cyclic AMP content of PAEC. Cells were treated with either no drugs (C), or with dipyridamole (100µM, DiP) for 10 minutes before being incubated with or without forskolin (10µM, FOR) for a further 2 minutes. The incubation was terminated by removal of Krebs and addition of 6% TCA and cyclic AMP content quantified by radioimmunoassay. Bars represent mean ± s.e. mean content of cyclic AMP (fmol µg DNA^{-1}, n=6). * $P< 0.05$; denotes a significant difference from untreated cells, or, between two groups joined by a bracket.

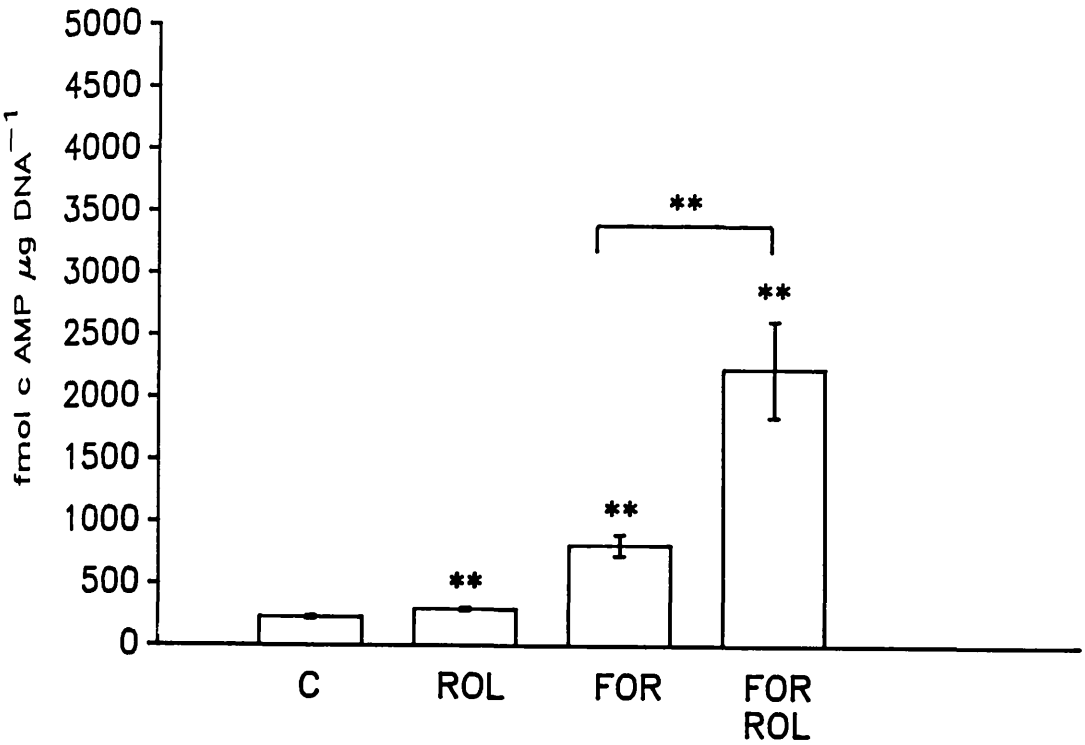


Figure 21: Effects of rolipram and forskolin on the cyclic AMP content of PAEC. Cells were treated with either no drugs (C), or with rolipram (25µM, ROL) for 30 minutes before being incubated with or without forskolin (30µM, FOR) for a further 5 minutes. The incubation was terminated by removal of Krebs and addition of 6% TCA and cyclic AMP content quantified by radioimmunoassay. Bars represent mean ± s.e. mean content of cyclic AMP (fmol µg DNA^{-1}, n=6-7). ** P< 0.005 denotes a significant difference from untreated cells, or, between two groups joined by a bracket.

5.1. PROLIFERATION OF PIG AORTIC ENDOTHELIAL CELLS

5.1.1. Effects of phorbol 12-myristate 13-acetate on proliferation of PAEC

To determine whether stimulation of protein kinase C (PKC) modulates the ability of pig aortic endothelial cells (PAEC) to proliferate in normal serum-supplemented DMEM, the effects of an activator of PKC, phorbol 12-myristate 13-acetate (PMA)(Ashendel, 1985), were examined.

PAEC seeded at a density of 10^4 cells/ cm^2 in normal serum-supplemented DMEM (10% foetal calf and 10% newborn calf serum) grew to confluence within 6-8 days (Figure 22). PMA (0.3µM), when added twice daily, produced a marked reduction in cell numbers compared with untreated cells: 86 ± 2% (n=6) reduction was observed at day 8 (Figure 22). The ability of PMA to reduce cell numbers was observed over the concentration range of 0.1nM to 1µM: the maximum reduction obtained after 4 days growth was 63 ± 2% (n=6, Figure 23). The ability of PMA (0.3µM) to reduce cell numbers was not associated with the accumulation of the vital stain, trypan blue, but a small increase in cell detachment was observed (Figure 24).

The inactive phorbol ester, 4α- phorbol 12,13- didecanoate (0.3µM), lacked the ability of PMA (0.3µM) to reduce cell numbers when assessed throughout an 8 day period (Figure 22). It is likely therefore that the ability of PMA to reduce cell numbers results from the activation of PKC.

5.1.2. Effects of staurosporine

The effects of staurosporine, a microbial alkaloid known to

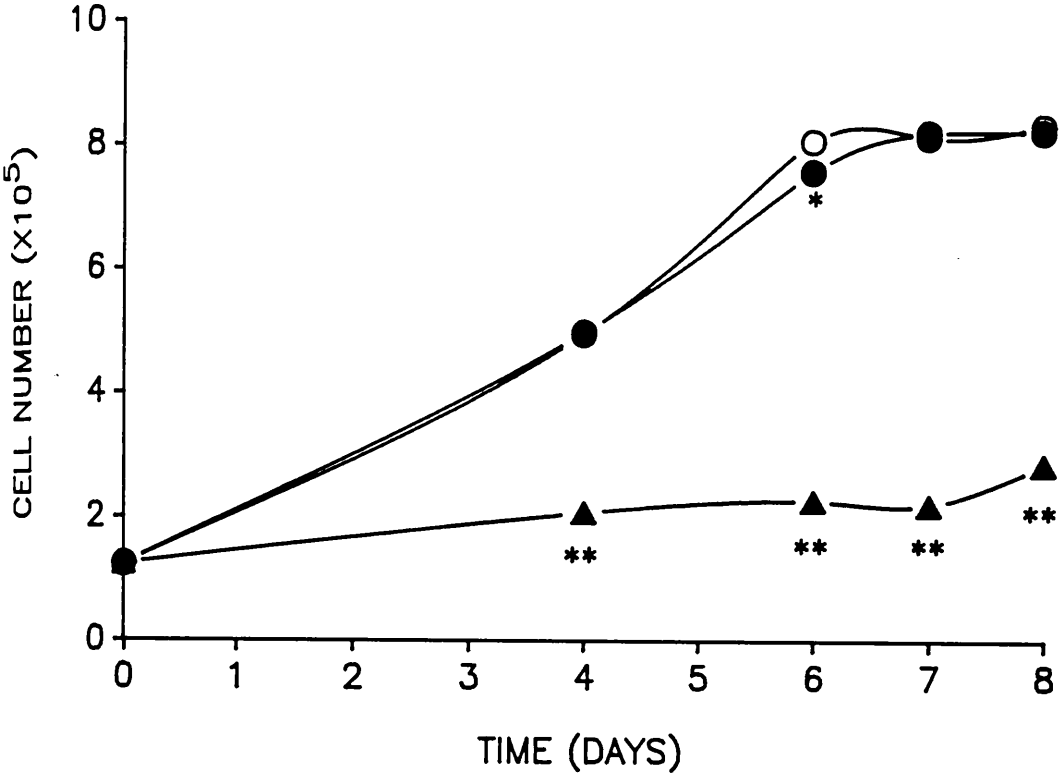


Figure 22: Effects of the active phorbol ester, phorbol 12-myristate 13- acetate (PMA) and the inactive phorbol ester, 4α-phorbol didecanoate (4α- PDD), on the proliferation of PAEC. PAEC were seeded at a density of 10^4 cells/ cm^2 in normal serum-supplemented DMEM and received either no drug (o), PMA (0.3μM,▲) or 4α- PDD (0.3μM, ●) twice daily. At the points indicated, cells were counted by haemocytometry. Points show mean cell numbers (n=6); all s.e. means are contained within the symbols.
* $P< 0.05$; ** $P< 0.0005$; denotes a significant difference from untreated cells on that day.

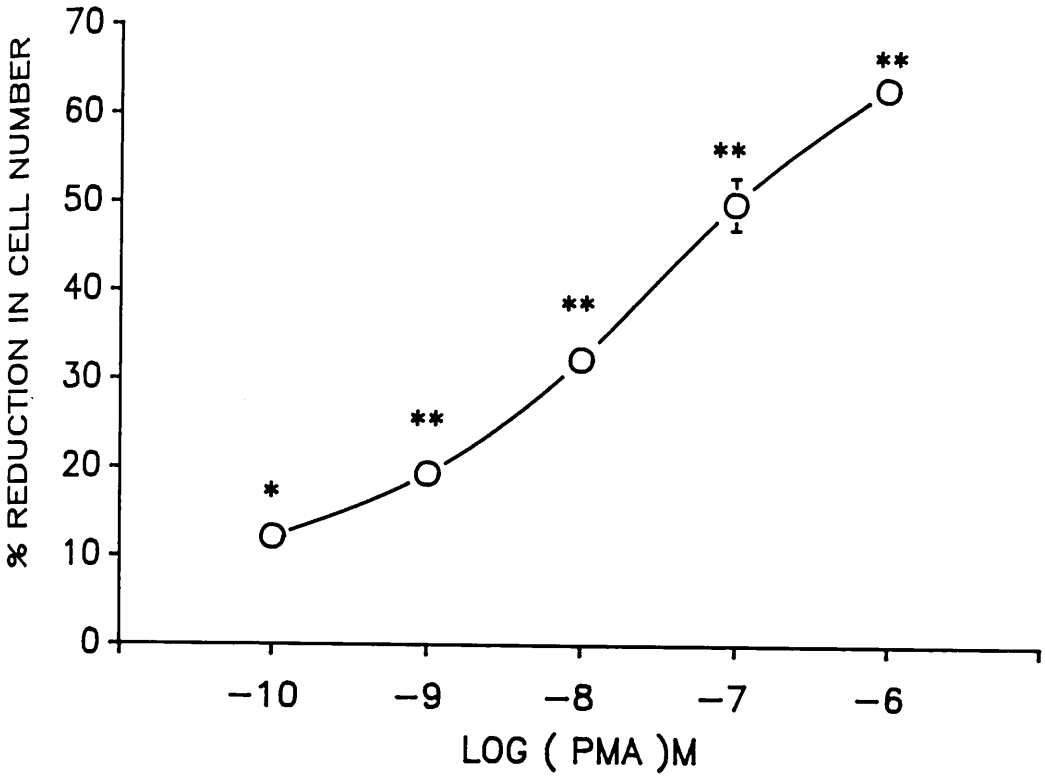
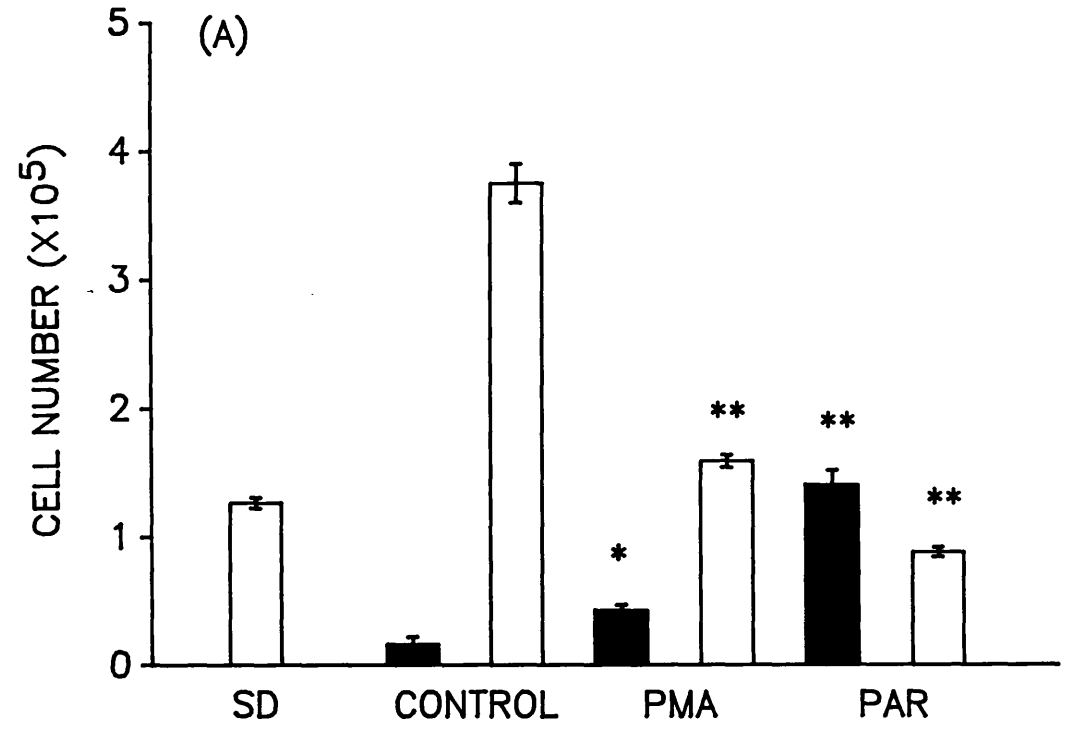


Figure 23: Concentration- effect curve showing the ability of PMA to inhibit proliferation of PAEC. PAEC were seeded at a density of 10^4 cells/ cm^2 in normal serum- supplemented DMEM. PMA (0.1nM-1μM) was added twice daily. The cells were allowed to grow for 4 days and then counted by haemocytometry. The results are expressed as the mean ± s.e. mean reduction (%) of cell number when compared with untreated cells (n=6); when error bars are not seen they are contained within symbols. * P< 0.005; ** P< 0.0005; denotes a significant difference from untreated cells.


(A)
CELL NUMBER (X10^5)
5
4
3
2
1
0
*
**
**
**
SD
CONTROL
PMA
PAR

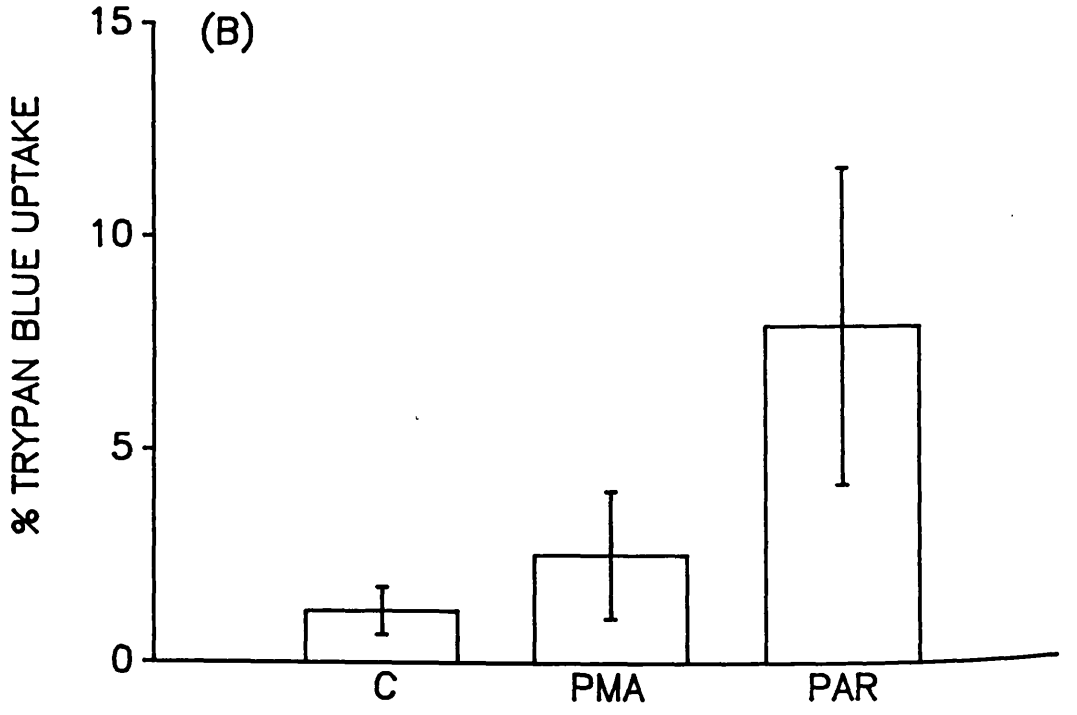

(B)
% TRYPAN BLUE UPTAKE
15
10
5
0
C
PMA
PAR

Figure 24: Effects of PMA and paraquat on proliferation, attachment and on trypan blue uptake by PAEC. PAEC were seeded at a density (SD) of 1.25 x 10^4 cells / cm^2 in normal serum- supplemented DMEM and received either no drug (CONTROL), PMA (0.3µM) or paraquat (1mM, PAR), added twice daily. The cells were allowed to grow for 4 days. (A) The cells attached (□) and cells floating in medium (■) were counted by haemocytometry. Bars show the mean cell number ± s.e. mean (n=6). (B) Cells were treated with trypan blue (0.1% v/v in 0.9% NaCl) for 30 minutes. Bars show mean ± s.e. mean percentage of cells taking up trypan blue (n=6). * P< 0.05; ** P< 0.005; denotes a significant difference from untreated cells.

inhibit the activation of PKC (Tamaoki et al., 1986), was therefore examined on PAEC grown in normal serum- supplemented DMEM in the absence and presence of PMA.

Staurosporine (10nM), when added twice daily, produced a slight inhibition of cell numbers by itself (15 ± 1% at day 8) but had no effect on the ability of PMA (0.3µM) to reduce cell numbers (Figure 25).

5.1.3. Effects of PMA assessed by [^{3}H]-thymidine incorporation

Phorbol esters have been reported to initially stimulate then inhibit proliferation of pig aortic endothelial cells through activation then down- regulation of PKC (Uratsuji & DiCorleto, 1988).

My studies counting cells by haemocytometery showed that PMA (0.3µM) induced only a reduction in cell numbers with no early stimulatory phase. By employing a more sensitive index of proliferation, i.e.[^{3}H]-thymidine incorporation, it was expected that any early stimulation of proliferation would be measured with ease.

PAEC were seeded at a density of 1.5 x 10^4 cells/ cm^2 in 6 well dishes and allowed to grow for 24 hours in normal serum- supplemented DMEM. The cells were incubated for a further 24 hours in serum- free DMEM in order to slow growth. The cells were then challenged with PMA (0.3µM) in DMEM containing a range of serum concentrations and pulsed with a mixture of [^{3}H]-thymidine (2µCi/ well) and thymidine (1µM).

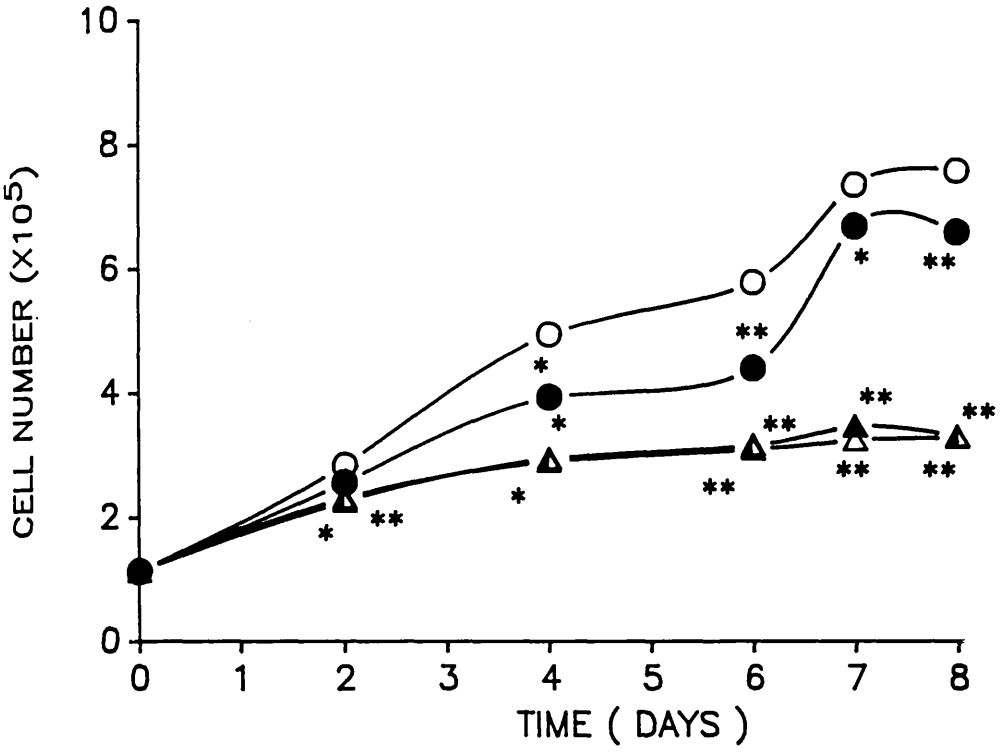


Figure 25: Effects of staurosporine on the antiproliferative action of PMA on PAEC. PAEC were seeded at a density of 1.3 x 10^4 cells / cm^2 in normal serum- supplemented DMEM and received either no drug (o), PMA (0.3µM, Δ), staurosporine (10nM, ●), or a combination of PMA and STAUR (▲), added twice daily. At the time points indicated, cells were counted by haemocytometry. Points show mean cell number ± s.e. mean (n=6); all s.e. means are contained within the symbols. * P< 0.01; ** P< 0.005; denotes a significant difference from untreated cells on that day.

The concentration dependence of serum in stimulating [^{3}H]-thymidine incorporation by PAEC in an 18 hour period was observed over the range 0 to 20%: the incorporation of [^{3}H]-thymidine observed with normal serum- supplemented DMEM (10% foetal calf and 10% newborn calf serum) was 3.1- fold greater that that obtained in serum- free DMEM (Figure 26). Treatment with PMA (0.3μM) was found to inhibit incorporation of [^{3}H]-thymidine by PAEC grown in 0% serum-, 4% serum-, and normal serum- supplemented DMEM during the 18 hour incubation (Figure 26): the inhibitions were 43.5 ± 3.5% (n=12) for 0% serum-, 42.1 ± 4% (n=12) for 4% serum- and 43.7 ± 4.0% (n=12) for normal serum-supplemented DMEM.

5.1.4. Time Course of action of PMA on [^{3}H]- thymidine incorporation

PAEC were seeded at a density of 1.5 x 10^4 cells/ cm^2 in 6 well dishes and allowed to grow for 24 hours in normal serum- supplemented DMEM and then for a further 24 hours in serum- free DMEM in order to slow growth. The cells were then challenged with either serum- free DMEM, 4% serum- supplemented DMEM, or, PMA (0.3μM) in 4% serum- supplemented DMEM. [^{3}H]- thymidine was added at time zero and incorporation measured after 4, 8, 12, and 24 hours.

Even PAEC incubated in serum- free DMEM continued to incorporate [^{3}H]-thymidine over the 24 hour pulse period (Figure 27). The ability of 4% serum to stimulate incorporation of [^{3}H]-thymidine was observed at 4, 8, 12, and 24 hours (Figure 27). As observed

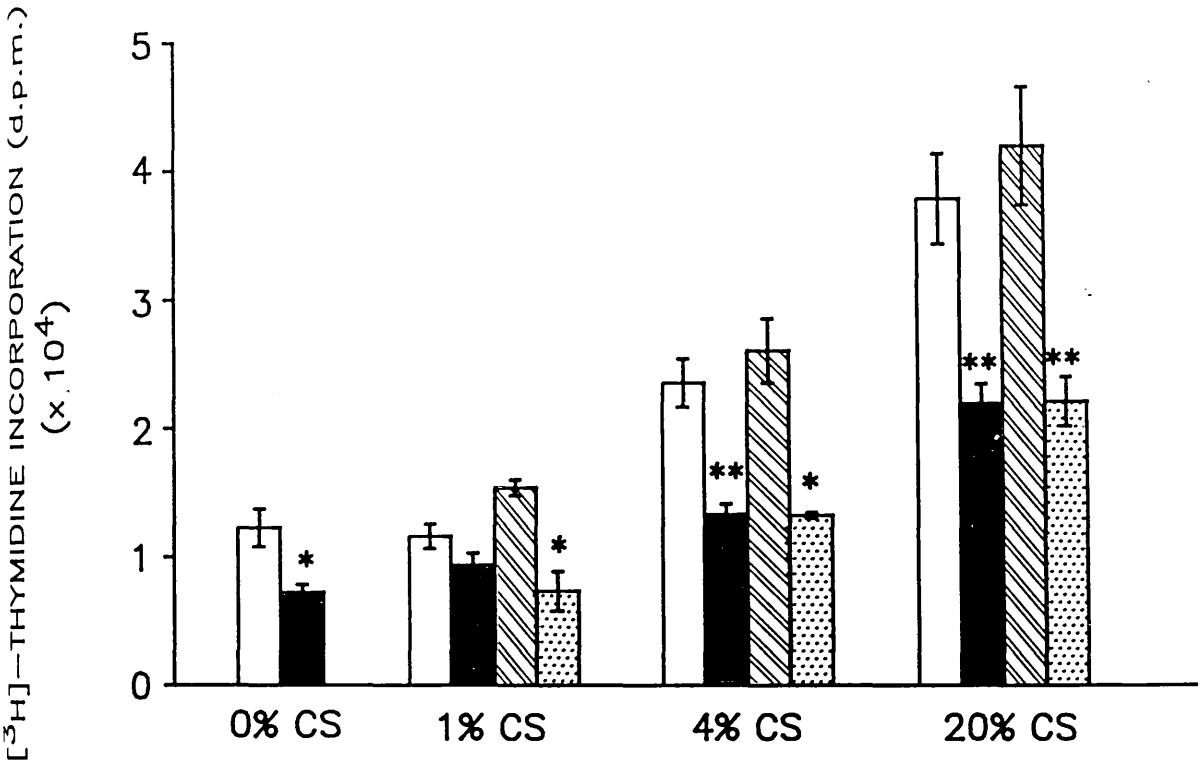


Figure 26: Effects of PMA and staurosporine on [^{3}H]-thymidine incorporation by PAEC grown in a range of serum concentrations. PAEC were seeded at a density of 1.5 x 10^4 cells/ cm^2 and allowed to grow for 24 hours in normal serum- supplemented DMEM. After a further 24 hours incubation in serum- free DMEM, the cells were challenged with either no drugs (□), PMA (0.3µM, ■), staurosporine (10nM, ▨), or a combination of PMA and staurosporine (⊠) in serum- free, 1%, 4% and 20% serum- supplemented DMEM and pulsed with a mixture of [^{3}H]-thymidine (2µCi/ well) and thymidine (1µM) for 18 hours. Bars show the mean ± s.e. mean [^{3}H]-thymidine incorporation (n=6-12). * $P < 0.05$; ** $P < 0.005$; denotes a significant difference from untreated cells at that particular serum concentration.

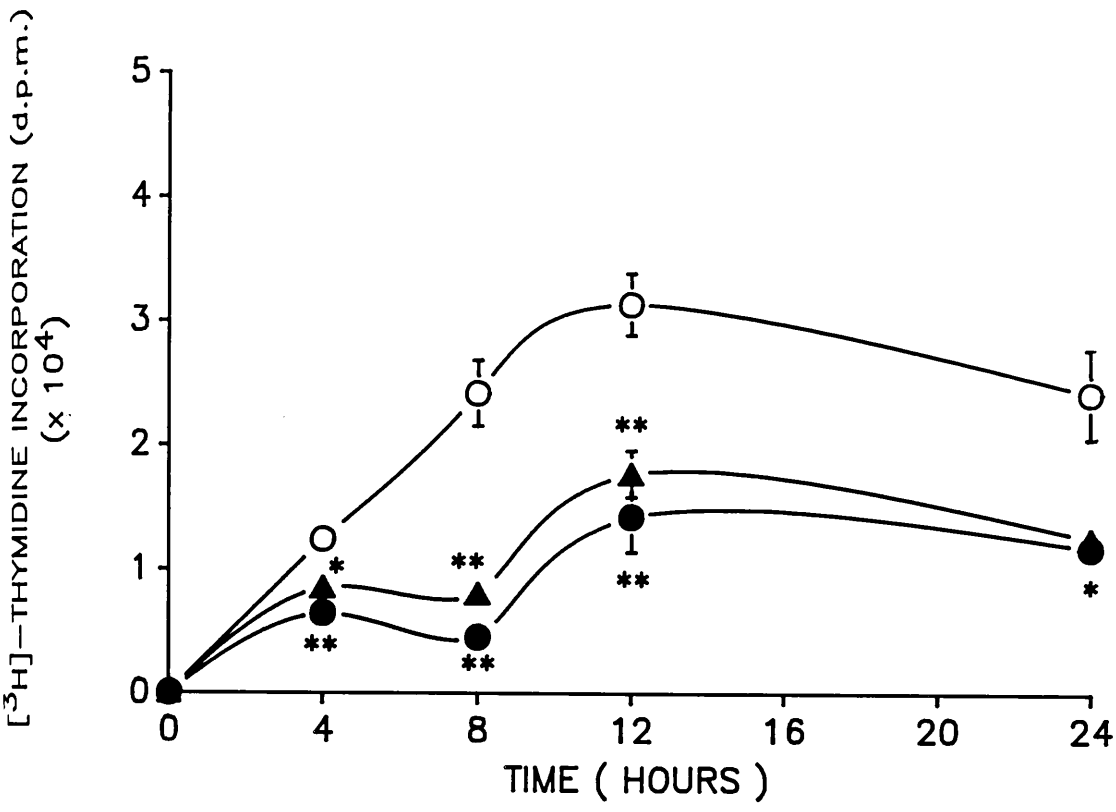


Figure 27: Effects of PMA on [^{3}H]-thymidine incorporation by PAEC. PAEC were seeded at a density of 1.5 x 10^4 cells/ cm^2 and allowed to grow for 24 hours in normal serum- supplemented DMEM. After a further 24 hour incubation in serum- free DMEM, the cells were challenged with no drugs in either 4% serum- supplemented DMEM (o), or serum- free DMEM (●), or PMA (0.3μM) in 4% serum-supplemented DMEM (▲) and [^{3}H]-thymidine incorporation measured at 4, 8, 12 and 24 hours. Points show the mean ± s.e. mean [^{3}H]-thymidine incorporation (n=6); when error bars are not seen they are contained within symbols. * P, 0.05; ** P< 0.005; denotes a significant difference from untreated cells grown in 4% serum-supplemented DMEM.

in experiments where proliferation was assessed by haemocytometery, PMA (0.3μM) inhibited proliferation as assessed by [^{3}H]-thymidine incorporation by cells grown in 4% serum-supplemented DMEM. Inhibition was observed at 4, 8, 12, and 24 hours with no stimulation at any time point (Figure 27).

The inhibitor of protein kinase C, staurosporine (10nM) had no effect upon the ability of serum (1-20%) to stimulate [^{3}H]-thymidine incorporation by PAEC (Figure 26). At a higher concentration of 100nM, however, staurosporine decreased [^{3}H]-thymidine incorporation by PAEC grown in 4% serum- supplemented DMEM by 34.1 ± 5.5% (n=6, Figure 28). At neither concentration (10 or 100nM) did staurosporine have any effect upon the ability of PMA (0.3μM) to decrease [^{3}H]-thymidine incorporation in response to serum (1-20%, Figures 26 and 28).

5.1.5. Role of Oxygen- Derived Free Radicals

It is known that phorbol esters stimulate the production of oxygen- derived free radicals in endothelial cells (Matsubara & Ziff, 1986). To determine whether the inhibitory actions of PMA were due to the extracellular actions of destructive oxygen-derived free radicals , the effects of the superoxide scavanger, superoxide dismutase (SOD) were examined. SOD (30U/ ml), when added twice daily, produced a slight inhibition of cell numbers by itself (29 ± 2%, n=6, at day 5, but no significant inhibition was observed at day 8), and had no effect on the ability of PMA (0.3μM) to inhibit cell numbers (Figure 29).

If PMA reduces cell numbers by generating free radicals intracel-

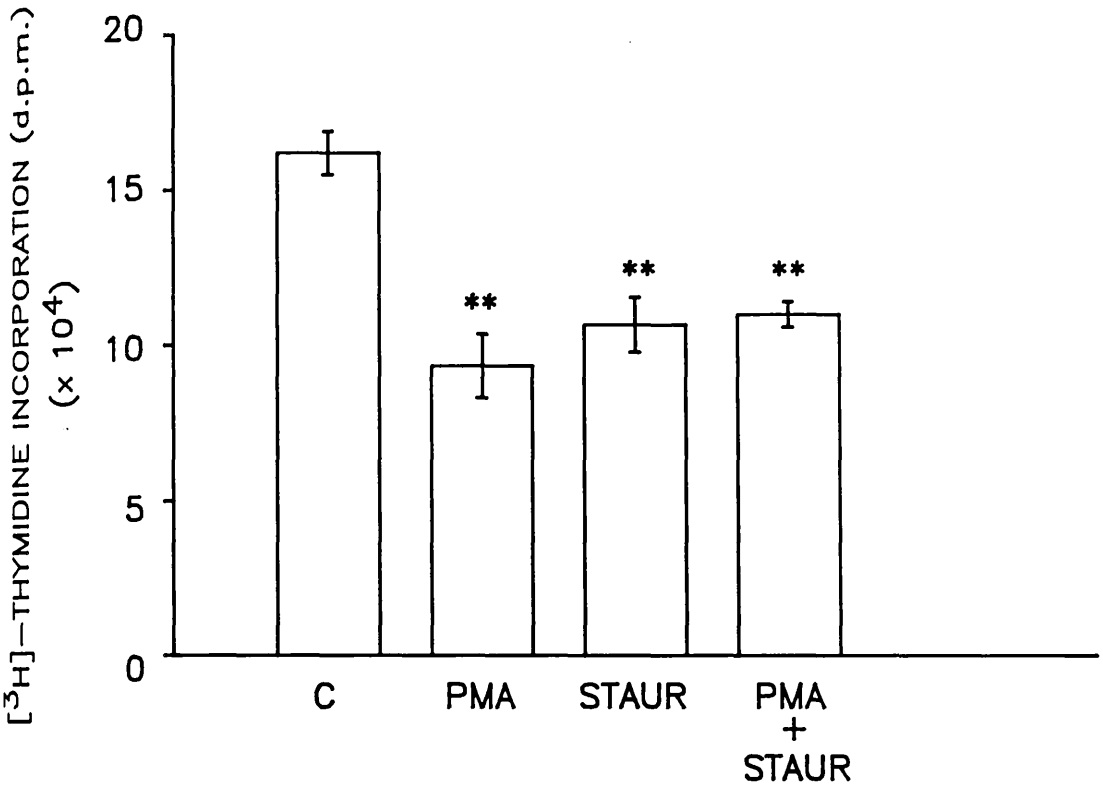


Figure 28: Effects of PMA and staurosporine on [^{3}H]-thymidine incorporation by PAEC. PAEC were seeded at a density of 1.5 x 10^4 cells/ cm^2 and allowed to grow for 24 hours in normal serum-supplemented DMEM. After a further incubation in serum- free DMEM, the cells were grown in 4% serum- supplemented DMEM, challenged with either no drugs (C), PMA (0.3µM), staurosporine (0.1µM, STAUR), or a combination of PMA and STAUR and pulsed with a mixture of [^{3}H]- thymidine (2µCi/ well) and thymidine (1µM) for 18 hours. Bars show the mean ± s.e. mean [^{3}H]-thymidine incorporation (n=6). ** P< 0.005; denotes a significant difference from untreated cells.

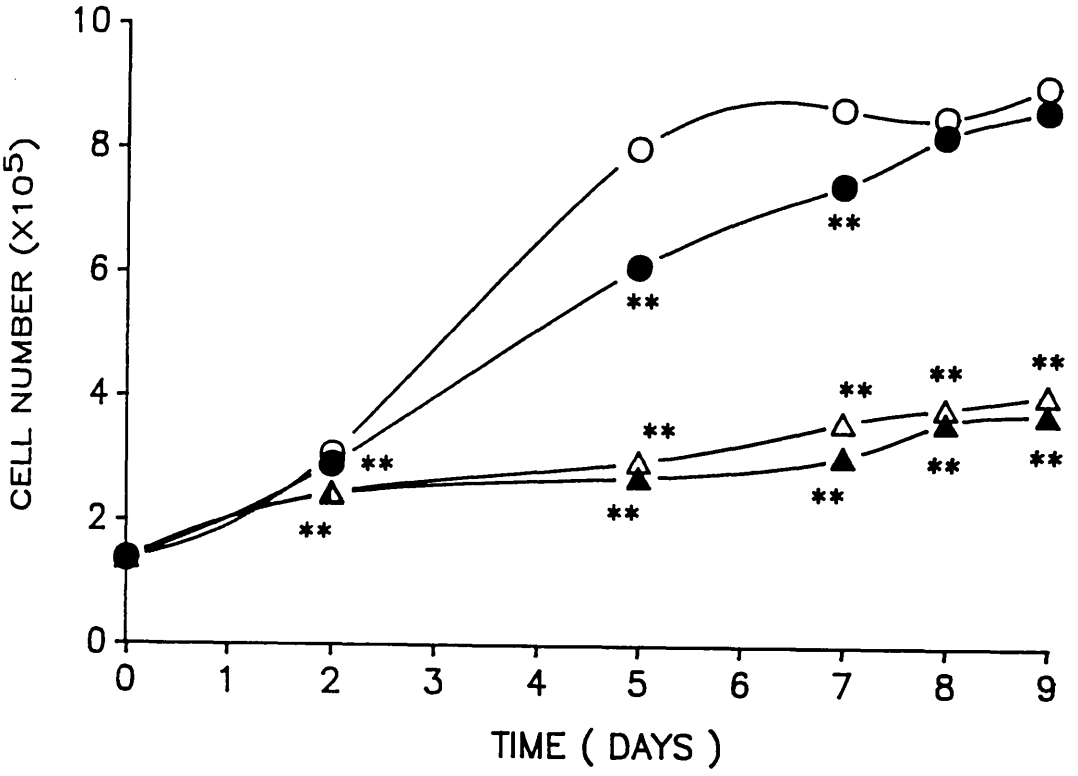


Figure 29: Effects of superoxide dismutase on the antiproliferative action of PMA on PAEC. PAEC were seeded at a density of 1.4 x 10^4 cells / cm^2 in normal serum- supplemented DMEM and received either no drug (o), PMA (0.3µM, Δ), superoxide dismutase (30U/ml, SOD, ●), or a combination of PMA and SOD (▲), added twice daily. At the time points indicated cells were counted by haemocytometry . Points show mean cell number ± s.e. mean (n=6); all s.e. means are contained within the symbols. * P< 0.005; ** P< 0.0005; denotes a significant difference from untreated cells on that day.

lularly it might be expected that paraquat, an agent known to generate oxygen derived free radicals intracellularly (Minakami et al., 1990) would share this property. Paraquat (1μM-1mM), when added twice daily, produced a concentration- dependent reduction in cell numbers (Figure 30): the maximum reduction obtained after 4 days growth was 91 ± 2% (n=6). This reduction in cell number, unlike that induced by PMA, was associated with accumulation of trypan blue and a significant increase in the detachment of cells (Figure 24).

Superoxide dismutase converts superoxide dismutase to hydrogen peroxide and this can be removed subsequently by catalase. The effects of combined treatment with SOD and catalase (CAT) on the ability of PMA and paraquat to reduce cell numbers were examined. When a combination of SOD (30U /ml) and CAT (30U /ml) was added twice daily, no reduction in cell numbers was observed in contrast to the inhibitory effects of SOD alone (Figure 31). Furthermore, this combination of SOD and CAT had no effect on the ability of PMA (0.3μM) or paraquat (10μM) to reduce cell numbers (Figure 31).

Vitamin E and butylated hydroxytoluene (BHT), two lipid soluble agents known to act as intracellular radical scavangers (Ruch & Klaunig, 1988; Hennig et al., 1990) were examined on the ability of PMA and paraquat to reduce cell numbers. Vitamin E (30μM) and BHT (30μM) when added twice daily, each had no effect on cell numbers by themselves, had no effect on the ability of PMA (0.3μM) to reduce cell numbers, but reversed the ability of paraquat (10μM) to reduce cell numbers (Figure 32). It is likely

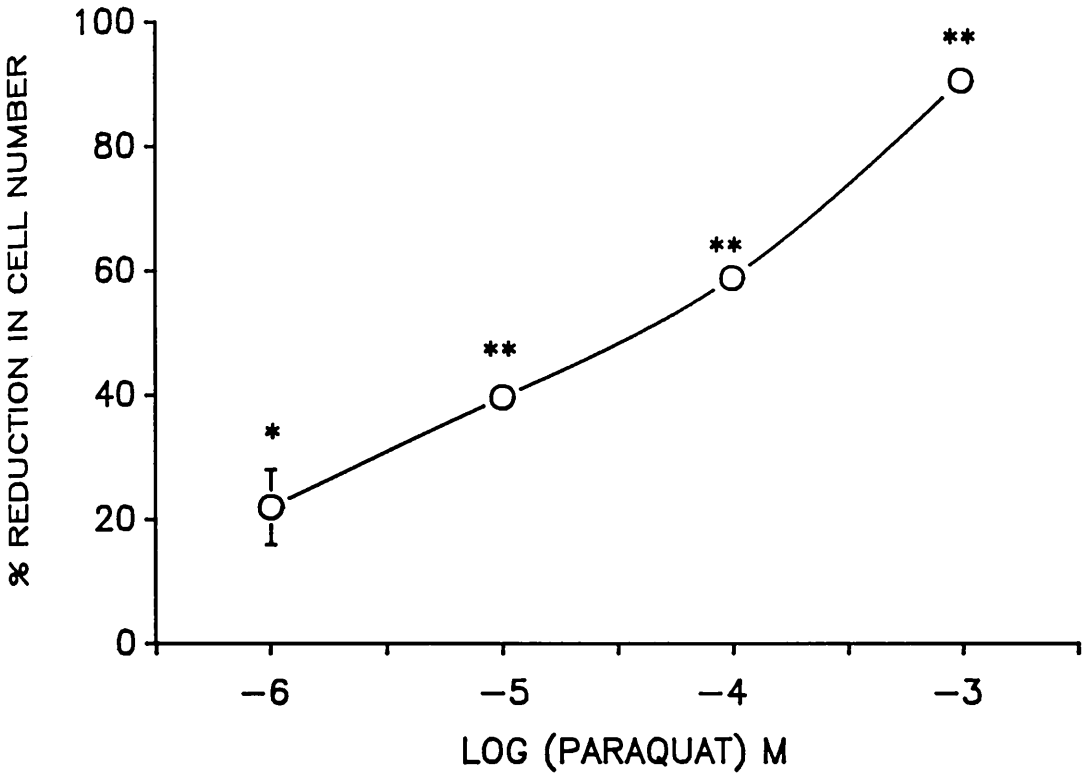
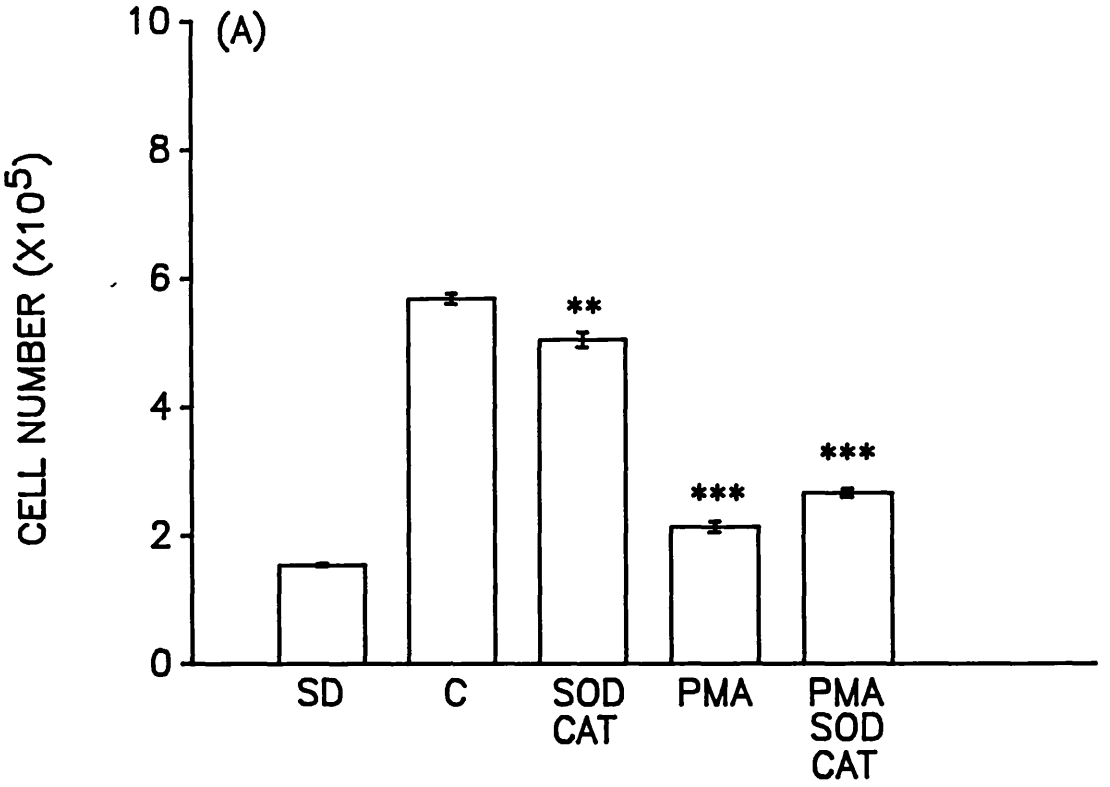


Figure 30: Concentration- effect curve showing the ability of paraquat to inhibit proliferation of PAEC. PAEC were seeded at a density of 1.5 x 10^4 cells / cm^2 in normal serum- supplemented DMEM. Paraquat (1µM- 1mM) was added twice daily. The cells were allowed to grow for 4 days and then counted by haemocytometry. The results are expressed as the mean ± s.e. mean reduction (%) of cell number when compared with untreated cells (n=6); when error bars are not seen they are contained within the symbols. * P< 0.05; ** P< 0.005; denotes a significant difference from untreated cells.


(A)
CELL NUMBER (X10^5)
10
8
6
4
2
0
**
***
***
SD
C
SOD CAT
PMA
PMA SOD CAT

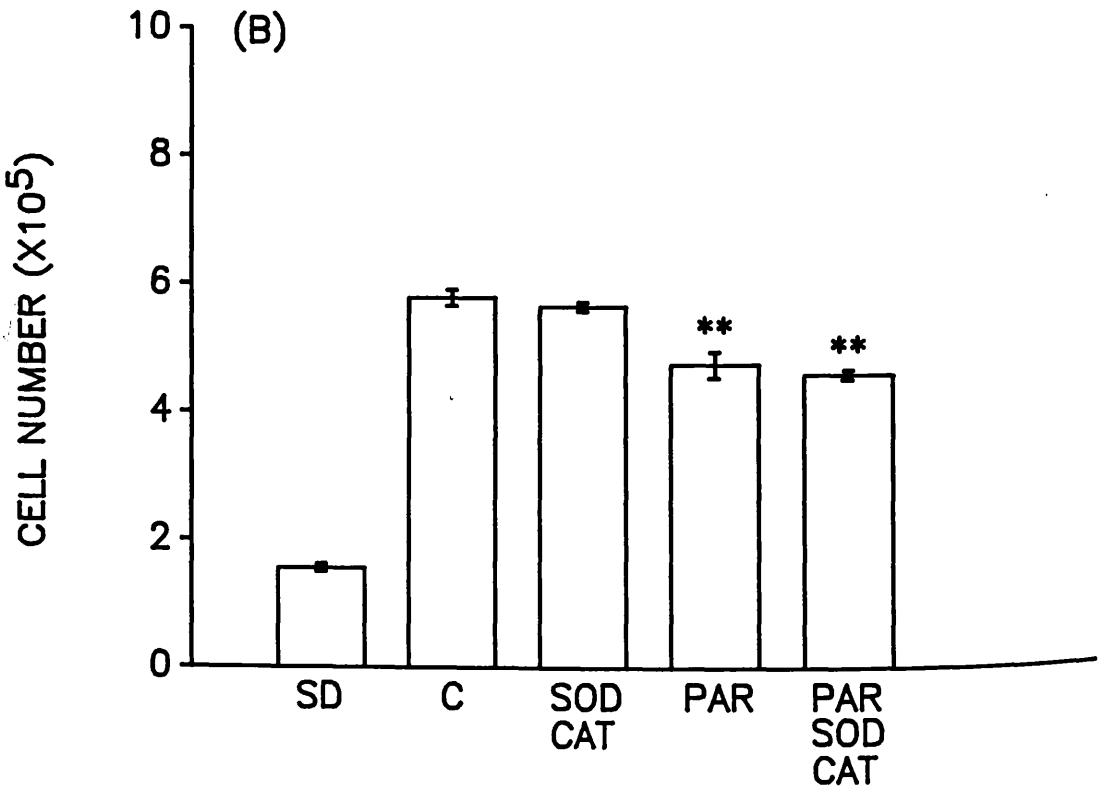
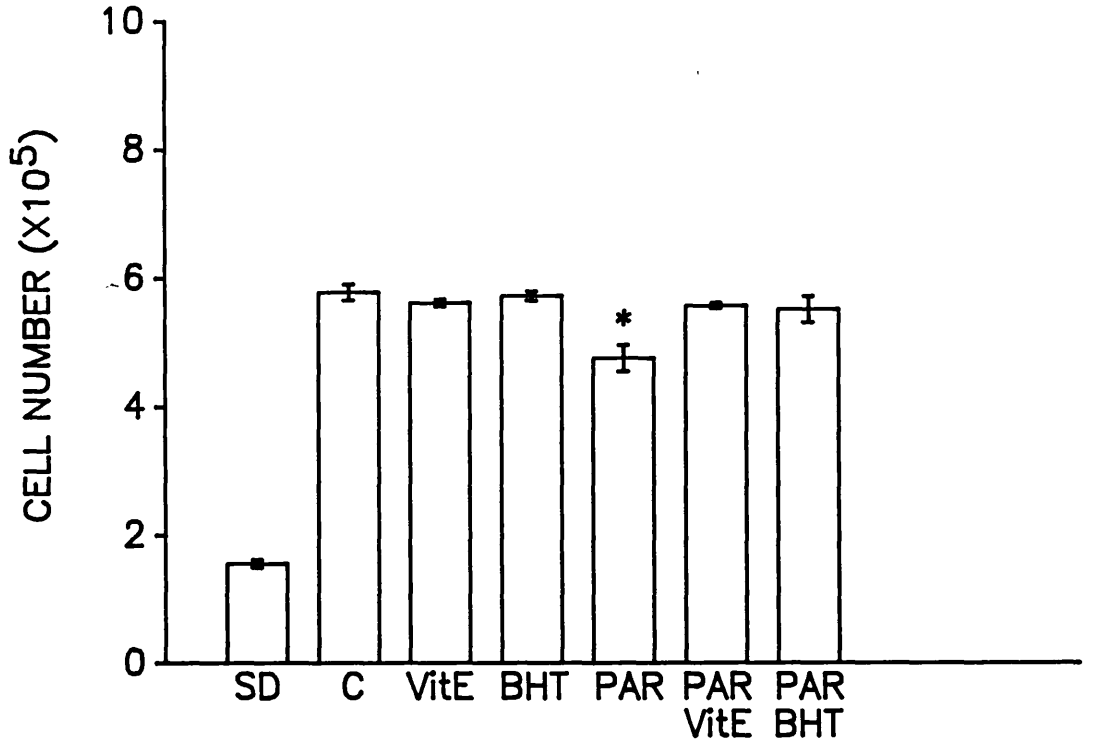

(B)
CELL NUMBER (X10^5)
10
8
6
4
2
0
**
**
SD
C
SOD CAT
PAR
PAR SOD CAT

Figure 31: Effects of combined treatment with superoxide dismutase and catalase on the antiproliferative actions of PMA and paraquat on PAEC. PAEC were seeded at a density (SD) of 1.25-1.5 x 10^4 cells/ cm^2 in normal serum- suppplemented DMEM.

(A) Cells received either no drug (C), PMA (0.3µM), a combination of superoxide dismutase (30U / ml, SOD) and catalase (30U / ml, CAT), or a combination of PMA, SOD and CAT.

(B) Cells received either no drug (C), paraquat (10µM, PAR), a combination of SOD (30U / ml) and CAT (30U / ml), or a combination of PAR, SOD and CAT.

Drugs were added twice daily. The cells were allowed to grow for 4 days and then counted by haemocytometry. Bars show the mean cell number ± s.e. mean (n=6). * P< 0.05; ** P< 0.005; *** P< 0.0005; denotes a significant difference from untreated cells.


CELL NUMBER (X10^5)
10
8
6
4
2
0
*
SD
C
VitE
BHT
PAR
PAR VitE
PAR BHT

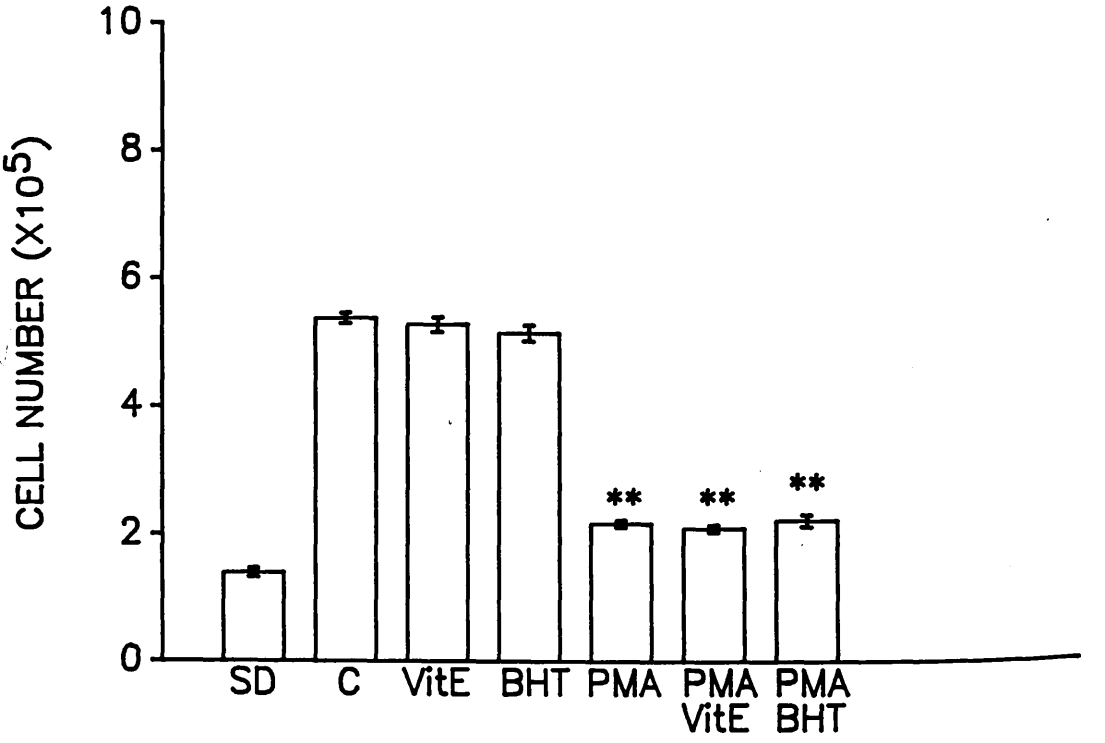

CELL NUMBER (X10^5)
10
8
6
4
2
0
**
**
**
SD
C
VitE
BHT
PMA
PMA VitE
PMA BHT

Figure 32: Effects of vitamin E and butylated hydroxytoluene on the antiproliferative actions of PMA and paraquat on PAEC. PAEC were seeded at a density of 1.3- 1.5 x 10^4 cells/ cm^2 in normal serum- supplemented DMEM.

(A) Cells received either no drug (C), paraquat (10µM, PAR), vitamin E (30µM, VitE), butylated hydroxytoluene (30µM, BHT), a combination of PAR and vitamin E, or a combination of PAR and BHT.

(B) Cells received either no drug (C), PMA (0.3µM), vitamin E (30µM, VitE), BHT (30µM), a combination of PMA and vitamin E, or a combination of PMA and BHT.

Drugs were added twice daily. The cells were allowed to grow for 4 days and then counted by haemocytometry. Bars show the mean cell number ± s.e. mean (n=6). * $P<0.005$; ** $P< 0.0005$; denotes a significant difference from untreated cells.

therefore that the reduction in cell numbers produced by superoxide dismutase, paraquat and PMA result from increased generation of hydrogen peroxide extracellularly, intracellular generation of radicals, and a mechanism unrelated to radical generation, respectively.

5.2. EFFECTS OF CYCLIC NUCLEOTIDES ON PROLIFERATION OF PAEC

Cyclic nucleotides have been shown to modulate the proliferation of many diverse cell types (Friedman, 1981). Furthermore, it has been reported that cyclic AMP has differing actions on proliferation of endothelial cells from different sources in culture: stimulation of proliferation in foetal bovine aortic, bovine aortic coronary, and human dermal microvascular endothelial cells (Davison & Karasek, 1981; Presta et al., 1989a; Meininger et al., 1988; Meininger & Granger, 1990) and inhibition of proliferation in bovine aortic and rat cerebrovascular endothelial cells (Leitman et al., 1986; Kempski et al., 1987).

In contrast to cyclic AMP, the effects of cyclic GMP on endothelial cell proliferation has not been extensively investigated. One report on bovine aortic endothelial cells showed that elevation of cyclic GMP content inhibited growth slightly (Leitman et al., 1986).

The objects of this part of the study were to determine the effects of cyclic AMP and cyclic GMP on proliferation of PAEC in culture and to investigate any possible interactions between PMA and the cyclic nucleotides on proliferation.

5.2.1. Effects of dibutyryl cyclic AMP

The membrane permeant analogue of cyclic AMP, dibutyryl cyclic AMP (30μM), when added twice daily to cells grown in normal serum- supplemented DMEM induced a reduction in cell numbers throughout an 8 day period: 35 ± 2% (n=6) reduction was observed at day 8. In addition dibutyryl cyclic AMP (30μM) had no effect on the ability of PMA (0.3μM) to reduce cell numbers at any time during the 8 day period (Figure 33).

The effects of dibutyryl cyclic AMP (30μM) were further studied on proliferation using an alternative index i.e. [^{3}H]-thymidine incorporation. PAEC were seeded at a density of 1.5 x 10^4 cells/ cm^2 in 6 well dishes and allowed to grow for 24 hours in normal serum- supplemented DMEM and then for a further 24 hours in serum- free DMEM in order to slow growth. The cells were then challenged with dibutyryl cyclic AMP (30μM) in DMEM containing a range of serum concentrations and pulsed with a mixture of [^{3}H]-thymidine (2μCi/well) and thymidine (1μM). Treatment with dibutyryl cyclic AMP (30μM) was found to have no effect on PAEC grown in 1% serum-, 4% serum- and 20% serum- supplemented DMEM during the 18 hour incubation (Figure 34). Dibutyryl cyclic AMP (30μM) was found to have no effect upon the ability of PMA (0.3μM) to decrease [^{3}H]-thymidine incorporation in response to serum (1-20%, Figure 34).

A possible explanation for the difference observed between [^{3}H]-thymidine incorporation and haemocytometery studies (no effect and inhibiton, respectively) might be attributed to the different

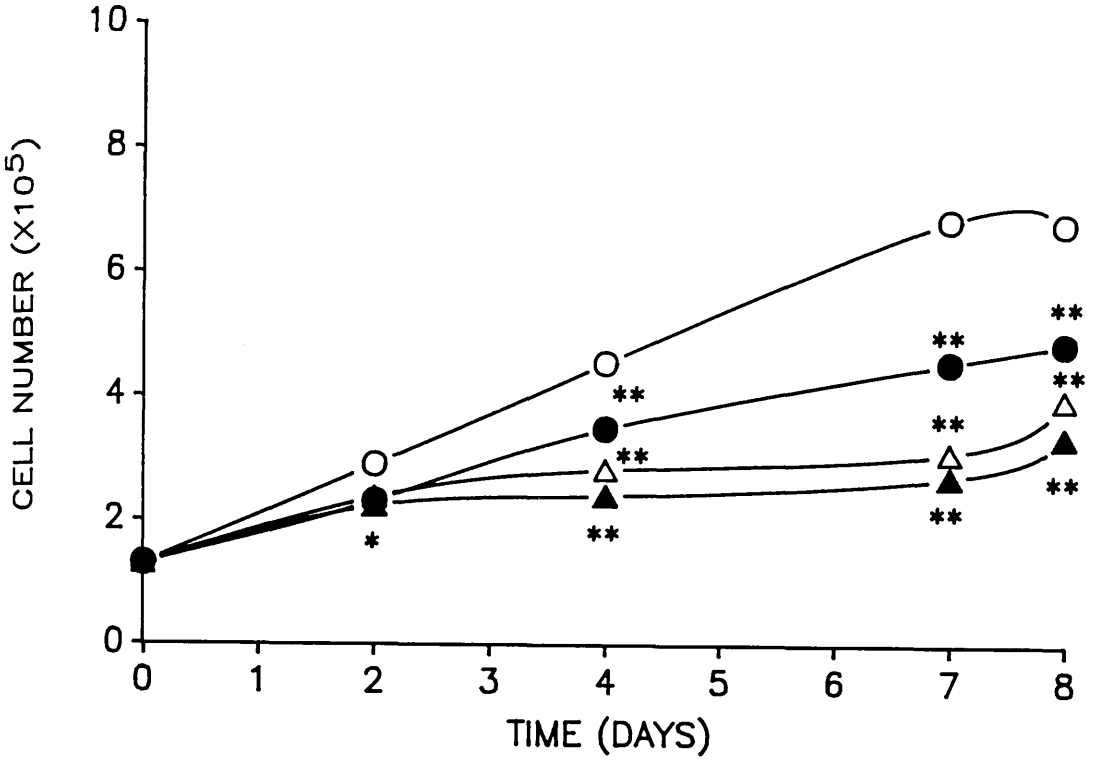


Figure 33: Effects of dibutyryl cyclic AMP on the antiproliferative action of PMA on PAEC. PAEC were seeded at a density of 1.3 x 10^4 cells/ cm^2 and grown in normal serum- supplemented DMEM and received either no drug (o), dibutyryl cyclic AMP (30µM, ●), PMA (0.3µM, Δ), or a combination of PMA and dibutyryl cyclic AMP (▲), added twice daily. At the points indicated the cells were counted by haemocytometry. Points show mean ± s.e. mean cell numbers (n=6); all s.e. means are contained within the symbols.

* P< 0.005; ** P< 0.0005; denotes a significant difference from untreated cells on that day.

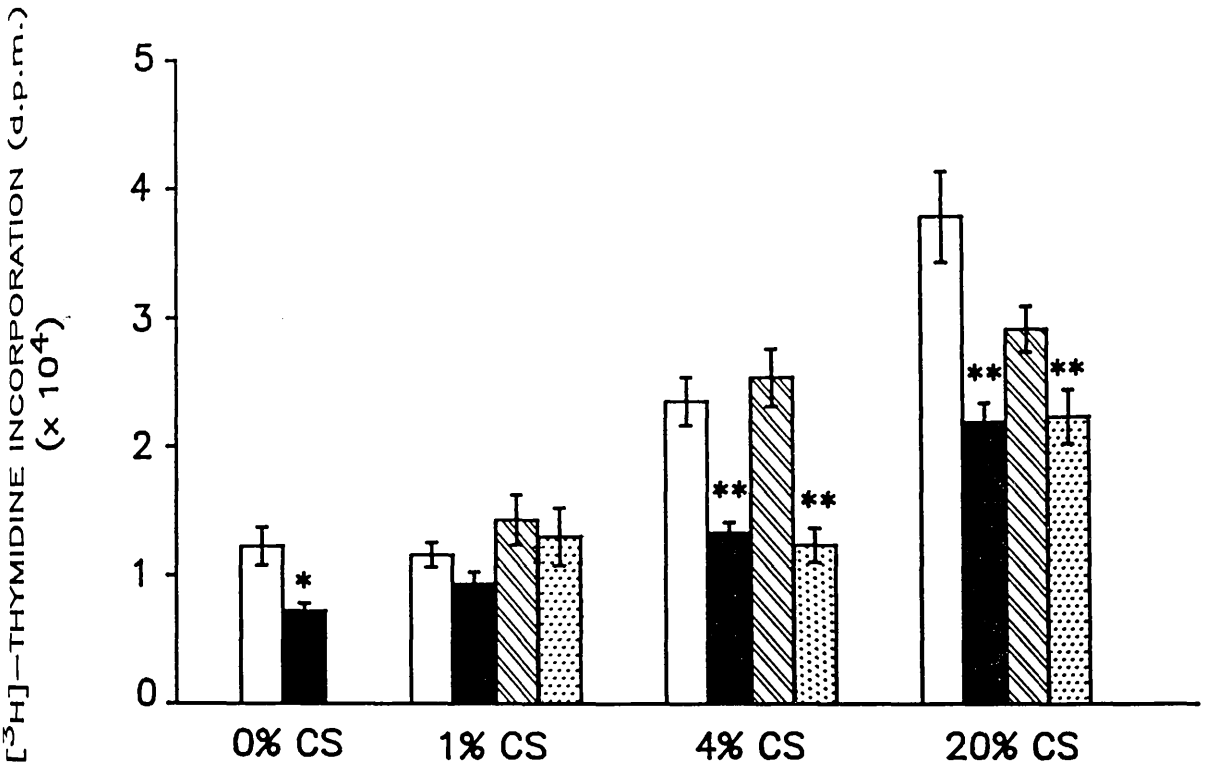


Figure 34: Effects of PMA and dibutyryl cyclic AMP on [^{3}H]-thymidine incorporation by PAEC grown in a range of serum concentrations. PAEC were seeded at a density of 1.5 x 10^4 cells/ cm^2 and allowed to grow for 24 hours in normal serum- supplemented DMEM. After a further 24 hour incubation in serum- free DMEM the cells were challenged with either no drugs (□), PMA (0.3µM, ■), dibutyryl cyclic AMP (30µM, ▨), or a combination of PMA and dibutyryl cyclic AMP (⊞) in serum- free, 1%, 4% and 20% serum- supplemented DMEM and pulsed with a mixture of [^{3}H]-thymidine (2µCi/ well) and thymidine (1µM) for 18 hours. Bars show the mean ± s.e. mean [^{3}H]-thymidine incorporation (n=6-12). * $P< 0.05$; ** $P< 0.005$; denotes a significant difference from the untreated cells at that particular serum concentration.

time course employed in the two methods: 18 hours in thymidine incorporation but 48 hours in the haemocytometeric experiments.

5.2.2. Effects of 8 bromo cyclic GMP

The membrane permeant analogue of cyclic GMP, 8 bromo cyclic GMP (30μM), when added twice daily to cells grown in normal serum-supplemented DMEM had no effect upon cell numbers throughout an 8 day period and had no effect on the ability of PMA (0.3μM) to reduce cell numbers at any time during the 8 day period (Figure 35).

5.2.3. Effects of glyceryl trinitrate and bradykinin

The effects of cyclic GMP on proliferation were investigated further by examining the effects of drugs which alter cyclic GMP content of PAEC.

Glyceryl trinitrate (1μM), which activates soluble guanylate cyclase through the formation of nitric oxide , and the stimulator of EDRF production, bradykinin (0.1μM), when added twice daily to cells grown in normal serum- supplemented DMEM had no effect on cell numbers when examined throughout a 6 day period (Figure 36). These results suggested that cyclic GMP had no effect on proliferation of PAEC in culture.

5.2.4. Effects of haemoglobin and methylene blue

The effects of two agents which lower intracellular cyclic GMP content by inhibiting the ability of spontanoeously- released EDRF to stimulate soluble gaunylate cyclase (Martin et al., 1988b), methylene blue and haemoglobin were examined on the

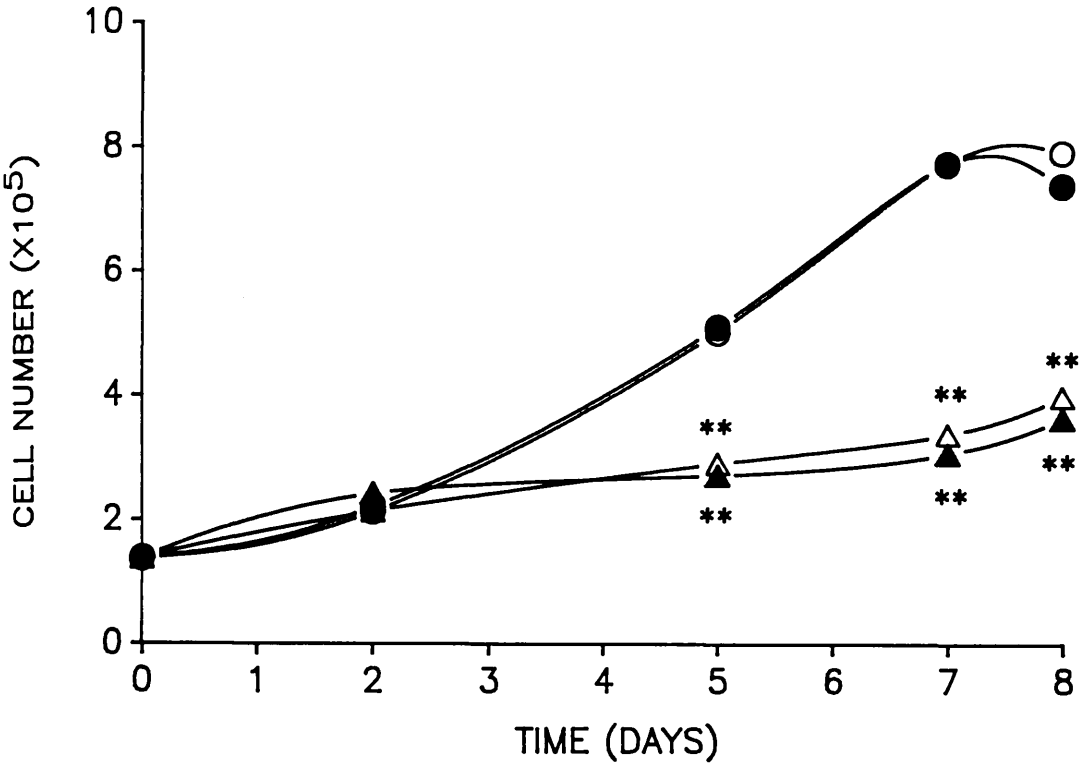


Figure 35: Effects of 8 bromo cyclic GMP on the antiproliferative action of PMA on PAEC. PAEC were seeded at a density of 1.3 x 10^4 cells/ cm^2 in normal serum- supplemented DMEM and received either no drugs (o), 8 bromo cyclic GMP (30µM,●), PMA (0.3µM,Δ), or a combination of PMA and 8 bromo cyclic GMP (▲), added twice daily. At the time points indicated the cells were counted by haemocytometry. Points show mean ± s.e. mean cell numbers (n=6); all s.e. means are contained within the symbols. * P< 0.005; ** P< 0.0005; denotes a significant difference from untreated cells on that day.

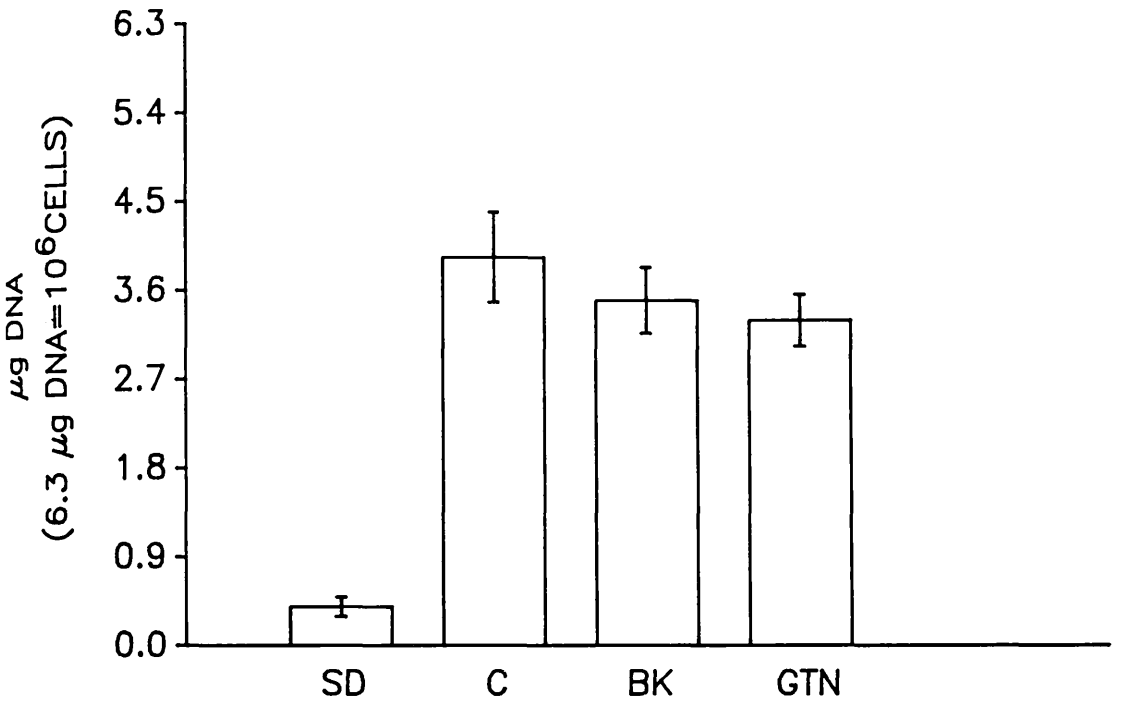


Figure 36: Effects of glyceryl trinitrate and bradykinin on the proliferation of PAEC. PAEC were seeded at a density (SD) equivalent to 0.04 µg DNA/ cm^2 in normal serum- supplemented DMEM and received either no drugs (C), bradykinin (0.1µM, BK), or glyceryl trinitrate (1µM, GTN). Drugs were added twice daily. The cells were allowed to grow for 6 days and the DNA content assessed fluorimetrically. Bars show the mean ± s.e. mean content of DNA (µg) (n=6).

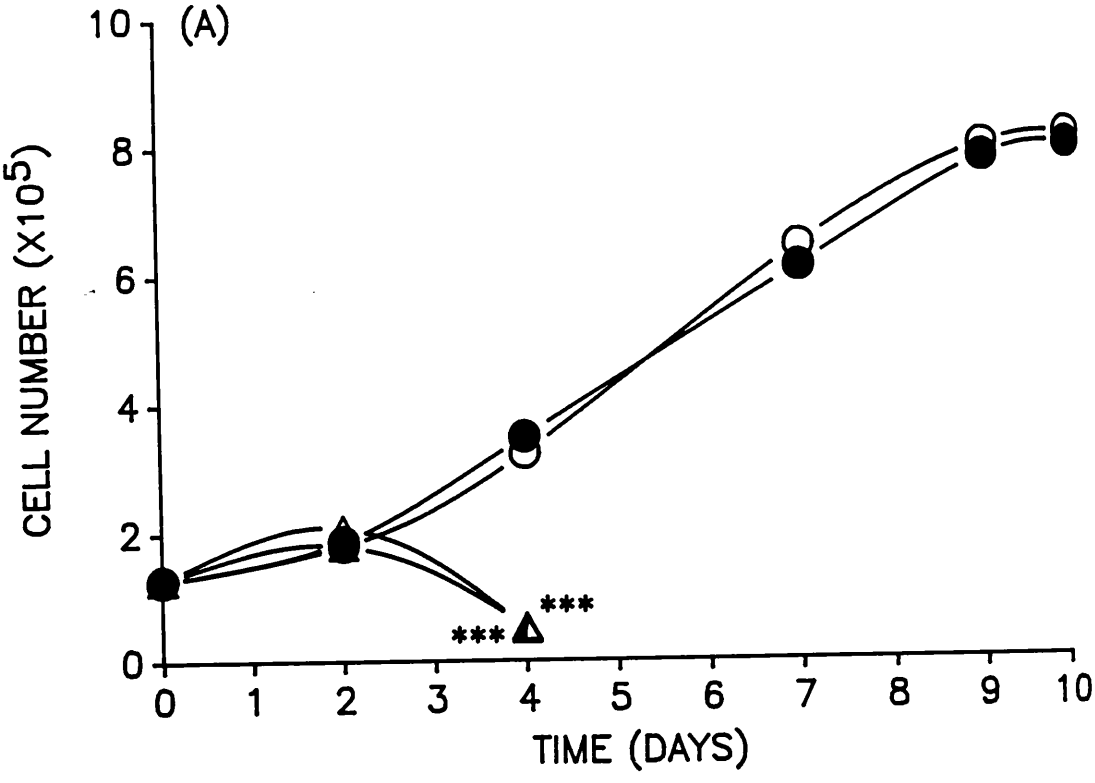
proliferation of PAEC. Methylene blue (10μM), when added once daily, and haemoglobin (10μM), when added twice daily, to cells grown in normal serum- supplemented DMEM each induced a reduction in cell numbers throughout a 10 day period: methylene blue caused a complete inhibition of proliferation whereas haemoglobin reduced cell numbers by 44 ± 4% (n=6) at day 10 (Figure 37).

The possibility that these reductions in cell numbers were mediated via a decrease in cellular cyclic GMP content was investigated by examining the effects of agents which elevate endothelial cyclic GMP content.

8 bromo cyclic GMP (30μM), when added twice daily to cells grown in normal serum- supplemented DMEM had no effect on cell numbers, or on the ability of methylene blue (10μM) to reduce cell numbers, but blocked the ability of haemoglobin (10μM) to reduce cell numbers throughout a 10 day period (Figure 37).

The atrial natriuretic factor, atriopeptin II (10nM), when added twice daily to cells grown in normal serum- supplemented DMEM had no effect on cell numbers, had no effect on the ability of methylene blue (10μM) to reduce cell numbers, but blocked the ability of haemoglobin (10μM) to reduce cell numbers after a 4 day period (Figure 38).

The nitrovasodilator, sodium nitroprusside (1μM), when added twice daily to cells grown in normal serum- supplemented DMEM had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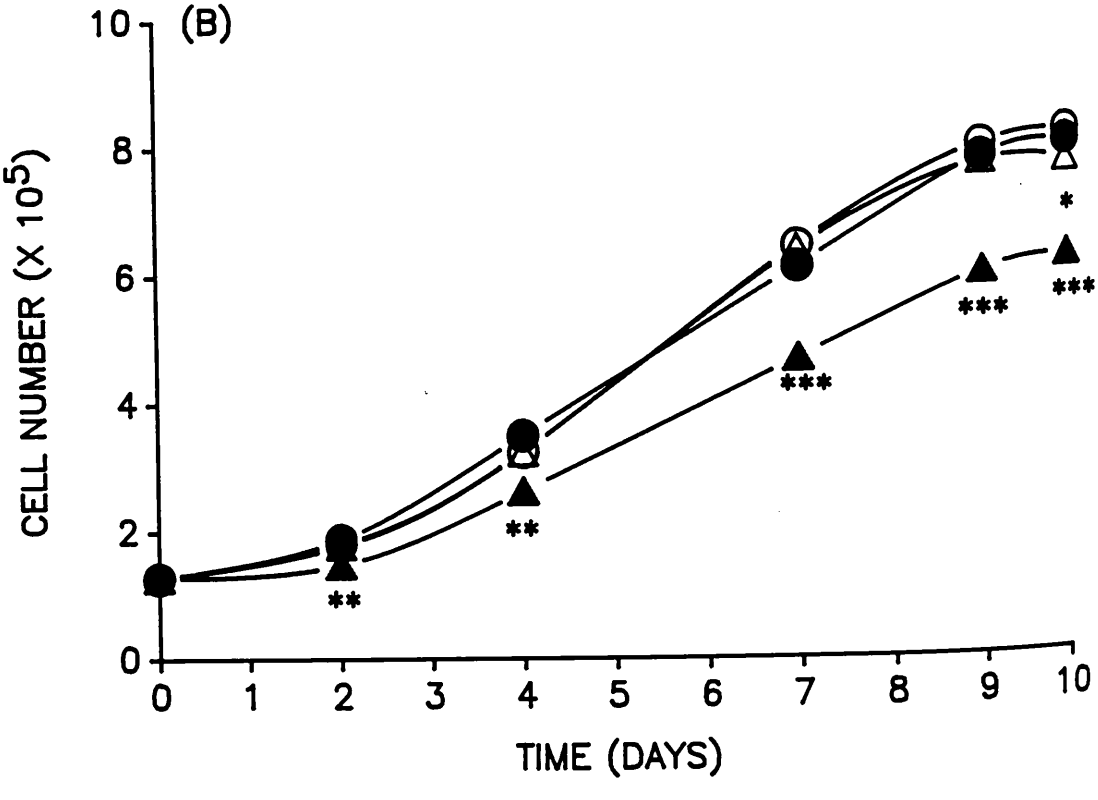
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Figure 37: 8 bromo cyclic GMP reverses the inhibitory effect of haemoglobin but not of methylene blue on proliferation of PAEC. PAEC were seeded at a density of 10^4 cells/ cm^2 in normal serum-supplemented DMEM.

(A) Cells received either no drug (o), methylene blue (10μM, ▲), 8 bromo cyclic GMP (30μM, ●), or a combination of methylene blue and 8 bromo cyclic GMP (Δ).

(B) Cells received either no drug (o), haemoglobin (10μM, ▲), 8 bromo cyclic GMP (30μM, ●), or a combination of haemoglobin and 8 bromo cyclic GMP (Δ). Drugs were added twice daily with the exception of methylene blue which was added once daily. At the time points indicated, the cells were counted by haemocytometry. Points show the mean ± s.e. mean cell numbers (n=6); all s.e. means are contained within the symbols. * $P< 0.05$; ** $P< 0.005$; *** $P< 0.0005$; denotes a significant difference from untreated cells on that day.

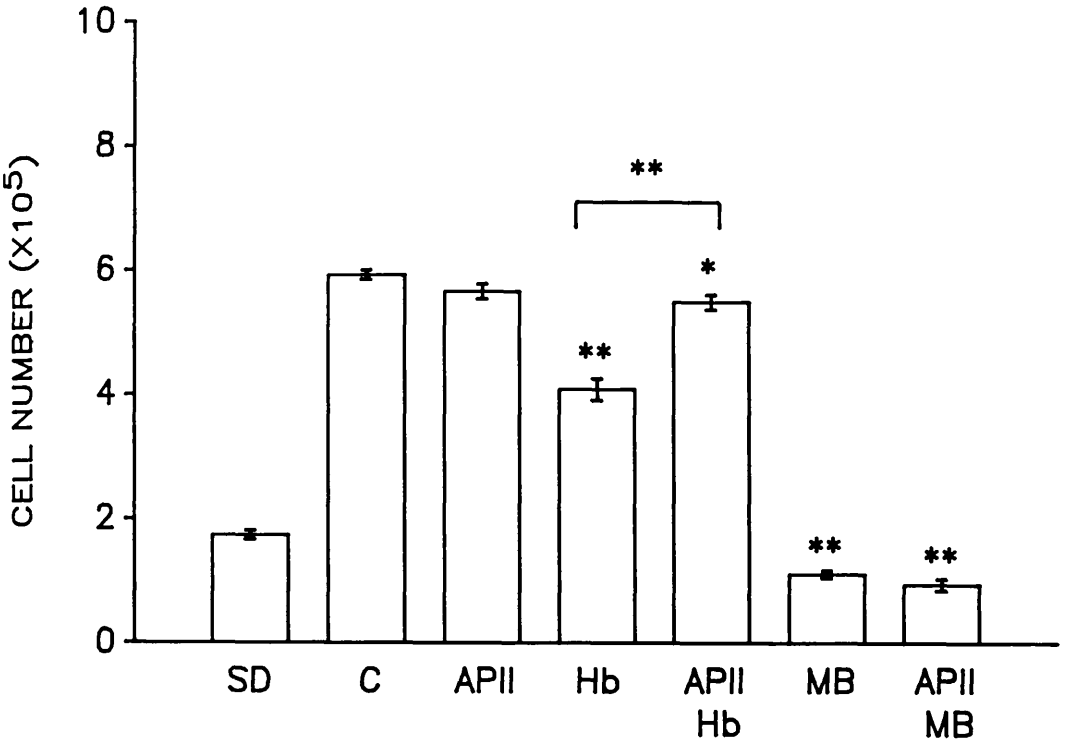


Figure 38: The ability of atriopeptin II to reverse the inhibitory action of haemoglobin but not of methylene blue on proliferation of PAEC. PAEC were seeded at a density of 1.7 x 10^4 cells/cm^2 and grown in normal serum- supplemented DMEM and received either no drug (C), atriopeptin II (10nM, AP II), haemoglobin (10μM, Hb), methylene blue (10μM, MB), a combination of AP II and Hb, or a combination of AP II and MB. Drugs were added twice daily with the exception of methylene blue which was added once daily. The cells were allowed to grow for 4 days and then counted by haemocytometry. Bars show mean cell number ± s.e. mean (n=6). * $P< 0.05$; ** $P< 0.005$; denotes a significant difference from untreated cells, or, between groups joined with a bracket.

no effect on cell numbers, had no effect on the ability of methylene blue (10μM) to reduce cell numbers but blocked the ability of haemoglobin (10μM) to reduce cell numbers after a 4 day period (Figure 39).

5.2.5. Effects of L-NMMA

EDRF has been identified as nitric oxide and vascular endothelial cells are known to synthesize nitric oxide from the terminal guanidino nitrogen atom(s) of L-arginine(Palmer et al., 1988b; Schmidt et al., 1988). The L-arginine analogue, N^G-monomethyl L-arginine (L-NMMA), but not its D- enantiomer, inhibits the synthesis of nitric oxide by inhibiting the converting enzyme, nitric oxide synthase (Rees et al., 1989; 1990). The effects of L-NMMA and D-NMMA were examined on the proliferation of PAEC.

L-NMMA (300μM) and D-NMMA (300μM) when added twice daily to cells grown in normal serum- supplemented DMEM each induced slight reductions in cell numbers in a 4 day period: 6 ± 1% (n=6) and 9 ± 1% (n=6), respectively (Figure 40). Since both enantiomers had equal activity, it is unlikely that the reduction in cell numbers resulted from loss of EDRF activity.

5.2.6. Effects of dipyridamole

To evaluate the effects of phosphodiesterase inhibition on proliferation of PAEC in culture the effects of dipyridamole were examined.

As shown in Section 4.1.1. (Figure 10), PAEC contain two phosphodiesterase isozymes (Souness et al., 1990), and dipyridamole is a

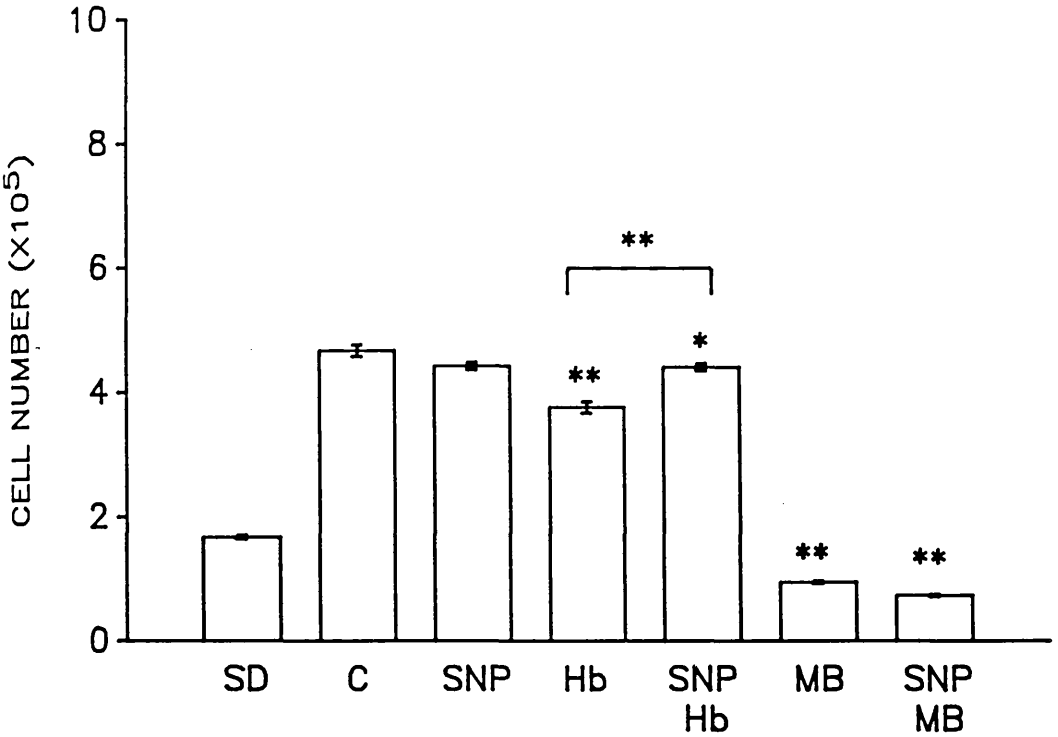


Figure 39: The ability of sodium nitroprusside to reverse the inhibitory action of haemoglobin but not of methylene blue on proliferation of PAEC. PAEC were seeded at a density of 1.7 x 10^4 cells/ cm^2 and grown in normal serum- supplemented DMEM. Cells received either no drug (C), sodium nitroprusside (1µM, SNP), haemoglobin (10µM, Hb), methylene blue (10µM, MB), a combination of SNP and Hb, or a combination of SNP and MB. Drugs were added twice daily with the exception of methylene blue which was added once daily. The cells were allowed to grow for 4 days and then counted by haemocytometry . Bars show the mean cell number ± s.e. mean (n=6). * P< 0.05; ** P< 0.005; denotes a significant difference from untreated cells, or, between groups joined with a bracket.

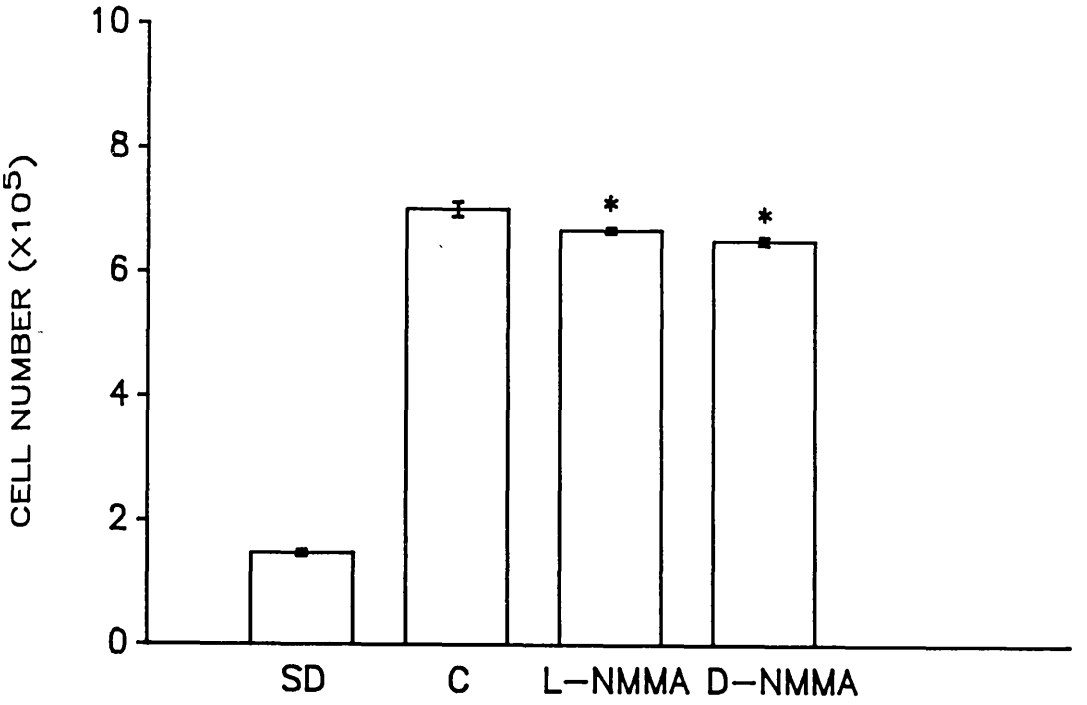


Figure 40: Effects of N^G- monomethyl L- arginine (L-NMMA) and N^G-monomethyl D- arginine (D-NMMA) on the proliferation of PAEC. PAEC were seeded at a density (SD) of 1.5 x 10^4 cells/ cm^2 in normal serum- supplemented DMEM and received either no drug (C), L-NMMA (300μM), or D-NMMA (300μM). Drugs were added twice daily. Cells were allowed to grow for 6 days and then counted by haemocytometry . Bars show mean cell number ± s.e. mean (n=6).
* $P< 0.05$ denotes a significant difference from untreated cells.

potent inhibitor of both. Dipyridamole (25μM) when added twice daily to cells grown in serum- supplemented DMEM produced a 53 ± 1% (n=6) reduction in cell numbers in a 6 day period. Haemoglobin (10μM), which inhibits the stimulation of soluble guanylate cyclase, when added twice daily, produced a slight inhibiton of proliferation by itself of 10 ± 2% (n=6) but had no effect upon the ability of dipyridamole (25μM) to inhibit proliferation (Figure 41). It is likely therefore that the reduction in cell numbers induced by dipyridamole resulted from the reduced hydrolysis of cyclic AMP.

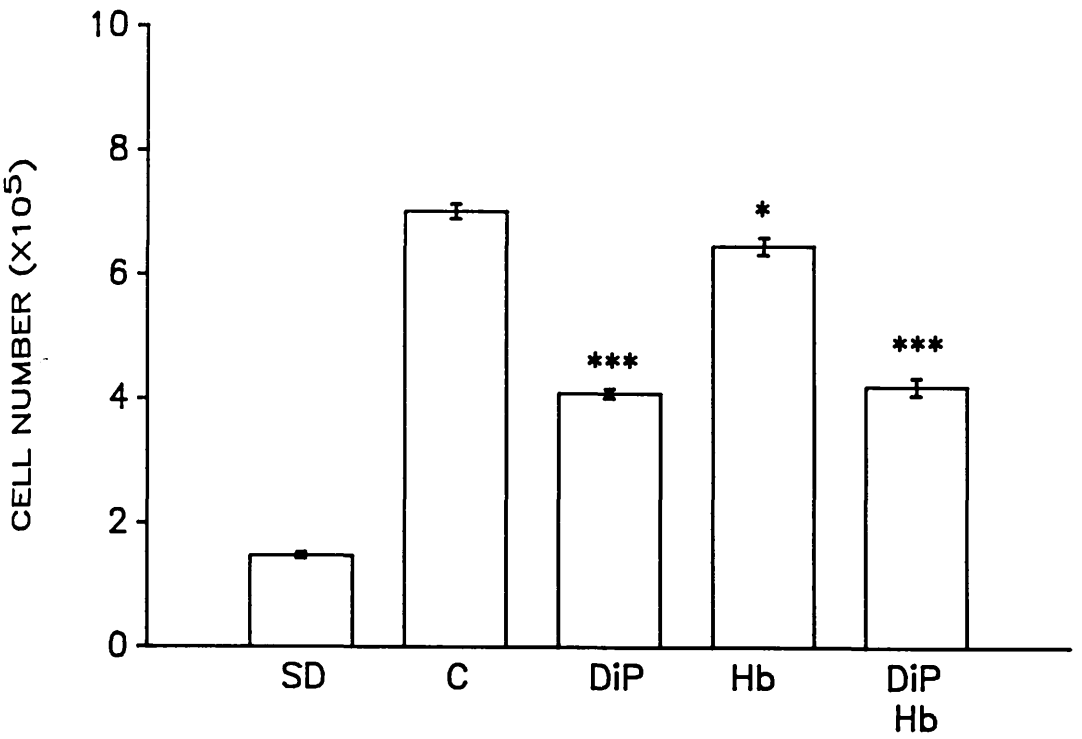


Figure 41: Effects of haemoglobin on the antiproliferative action of dipyridamole on PAEC. PAEC were seeded at a density (SD) of 1.5 x 10^4 cells/ cm^2 in normal serum- supplemented DMEM and received either no drug (C), dipyridamole (25µM, DiP), haemoglobin (10µM, Hb), or, a combination of DiP and Hb. Drugs were added twice daily. The cells were allowed to grow for 6 days and then counted by haemocytometry . Bars show mean cell number ± s.e. mean (n=6). * P< 0.005; *** P< 0.0005; denotes a significant difference from untreated cells.

6.1. PROLIFERATION OF RAT AORTIC SMOOTH MUSCLE CELLS

6.1.1. Effects of serum on the proliferation of rat ASMC

Since vascular smooth muscle cell proliferation plays a key role in the pathogenesis of atherosclerosis, identification of intracellular messenger pathways utilised in controlling proliferation is important. Smooth muscle cells are known to exhibit growth dependence upon platelet- derived mitogens (Fager et al., 1988). The effects of growing rat ASMC in a range of serum concentrations was therefore examined.

Rat ASMC were seeded at a density of 1.4×10^4 cells/ cm^2 and the concentration dependence of serum (2 to 20%) in stimulating growth was observed throughout an 11 day period: growth in serum-supplemented DMEM was clearly concentration- dependent (Figure 42). Cell numbers remained constant in 2% serum- supplemented DMEM but increased in higher concentrations. Unlike pig aortic endothelial cells, which when confluent become density-inhibited, rat ASMC even in the highest concentrations of serum continued to proliferate when confluent. Serum therefore contains mitogens that stimulate growth of rat ASMC.

6.1.2. Effects of phorbol 12-myristate 13- acetate on proliferation of rat ASMC

There are conflicting reports on the actions of protein kinase C (PKC) on the proliferation of smooth muscle cell obtained from identical or different sources. A stimulation of proliferation has been reported for smooth muscle cells obtained from bovine pulmonary artery and aorta (Dempsey et al., 1990; Doctrow & Folkman, 1987); rat aorta (Owen, 1985) and rabbit aorta (Kariya

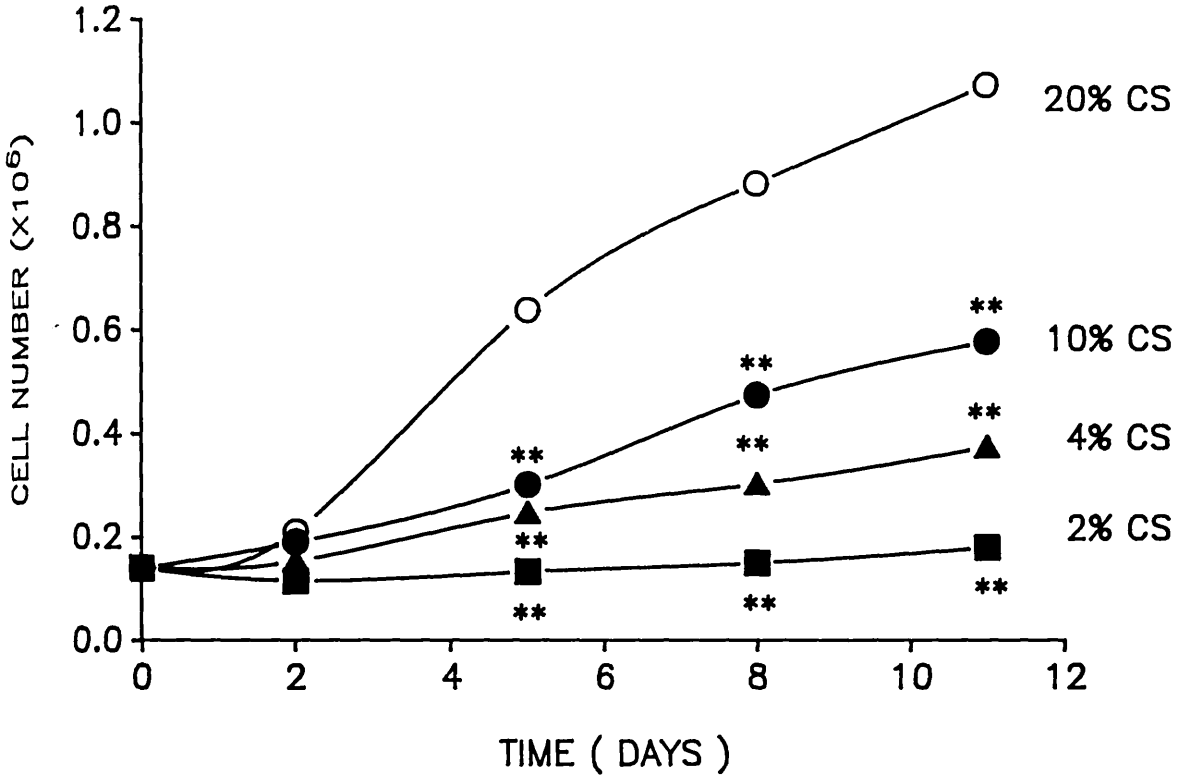


Figure 42: The serum- dependent growth of rat ASMC in vitro. Rat ASMC were seeded at a density of 1.4 x 10^4 cells/ cm^2 and grown in either normal serum- supplemented (20% CS, o), 10% serum- supplemented (10% CS, ●), 4% serum- supplemented (4% CS, ▲), or 2% serum- supplemented (2% CS, ■) DMEM. At the time points indicated, the cells were counted by haemocytometry. Points show mean cell number (n=6); all s.e. mean are contained within the symbols. ** P< 0.005 denotes a significant difference from 20% CS treated cells on that day.

et al., 1987a). In contrast, inhibition of proliferation has been observed for smooth muscle cells obtained from rat aorta (Kihara et al., 1989) and rabbit aorta (Kariya et al., 1987b; Fukumoto et al., 1988). One study demonstrated the ability of PMA to stimulate or inhibit the proliferation of rabbit aortic smooth muscle cells when the cells were either grown in plasma- derived serum or whole blood serum, respectively. In view of these conflicting reports, an attempt was made to determine if stimulation of PKC modulates the ability of rat ASMC to proliferate in serum- supplemented DMEM by examining the effects of phorbol 12-myristate 13- acetate (PMA).

Rat ASMC were seeded at a density of 1.5 $x10^4$ cells/ cm^2 and grown in DMEM in a range of serum concentrations (4, 10 and 20%). PMA (0.3µM), added twice daily after an initial 24 hour plating down period had no effect upon cells grown at any serum concentration throughout a 12 day period (Figure 43).

6.1.3. Effects of adrenoceptor stimulation on proliferation of rat ASMC

Recent reports indicate that an increase in plasma level of catecholamines is a major risk factor in the development of atherosclerosis (Helin et al., 1970; Kones, 1979; Kukreja et al., 1981) and that this a consequence of adrenoceptor -mediated stimulation of vascular smooth muscle cell growth (Nakaki et al., 1989). The effects of adrenoceptor agonists upon rat ASMC proliferation was therefore examined. To study the effects of the various drug treatments on growth of rat ASMC, the serum concentration was reduced to a sub- maximal level of 10%. This

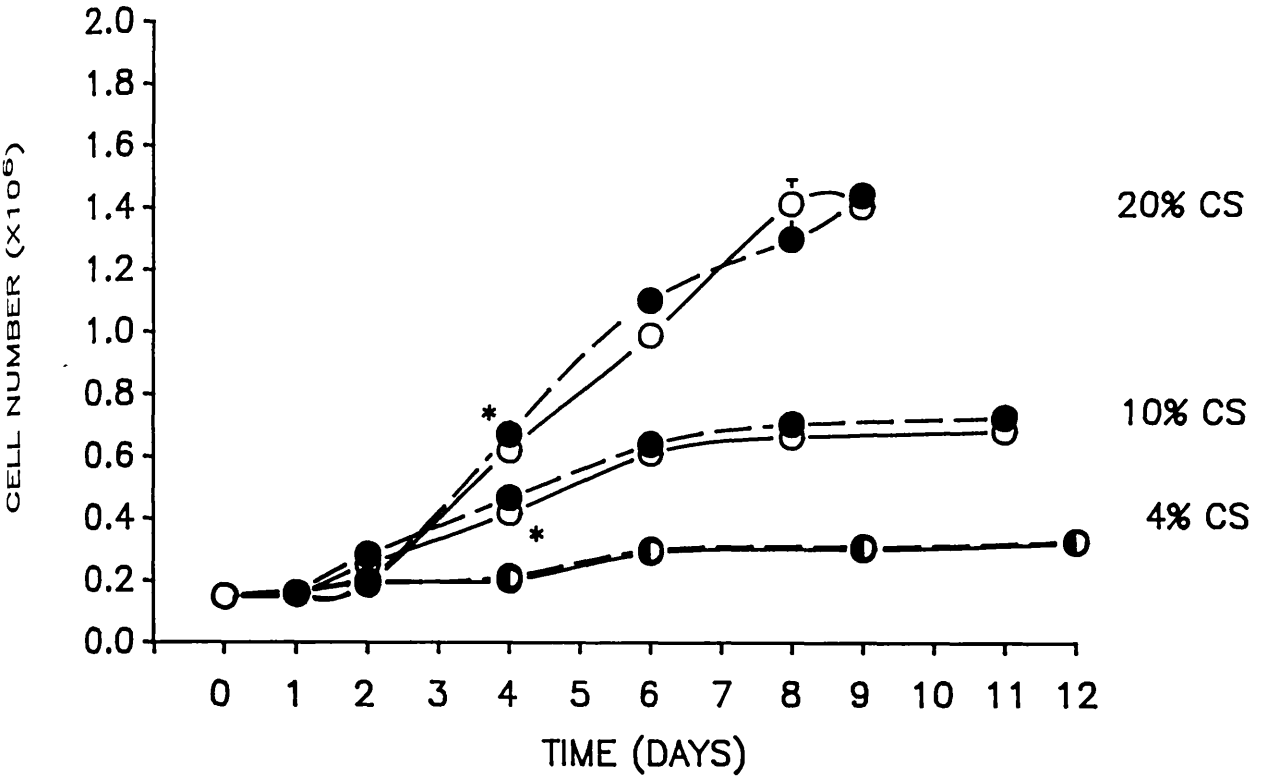


Figure 43: Effects of phorbol myristate acetate (PMA) on the proliferation of rat ASMC grown in serum- containing DMEM. Rat ASMC were seeded at a density of 1.5 x 10^4 cells/ cm^2 and grown in either normal serum- supplemented (20% CS), 10% serum- supplemented (10% CS) or 4% serum- supplemented (4% CS) DMEM. Cells received either no drug (o) or PMA (0.3μM, ●) which was added twice daily after an initial 24 hour plating down period. At the time points indicated the cells were counted by haemocytometry . Points show mean cell number ± s.e. mean (n=6); when error bars are not seen thay are contained within symbols. * P< 0.05 denotes a significant difference from untreated cells on that day.

theoretically should permit examination of the effects of drugs which inhibit or stimulate growth and prevent the possibility of a mitogenic effect being masked through maximal stimulation of growth growth in 20% serum- supplemented DMEM.

The α_1- adrenoceptor agonist, phenylephrine (0.1µM-1mM), was added twice daily after an initial 24 hour plating down period to cells grown in 10% serum- supplemented DMEM. No effect on cell numbers was observed after 4 days growth at the lower concentrations of phenylephrine (0.1-10µM) but cell numbers were increased at the higher concentrations (0.1 and 1mM): the increases in cell number were 17 ± 4% (n=6) and 30 ± 6% (n=6), respectively (Figure 44).

The α_2- adrenoceptor agonist , clonidine (0.1µM-1mM), was added twice daily after an initial 24 hour plating down period to cells grown in 10% serum- supplemented DMEM. No effect on cell numbers was observed after 4 days growth in clonidine concentrations of 0.1µM to 0.1mM, but at a concentration of 1mM, cell numbers were reduced by 73 ± 2% (n=6, Figure 45).

The unstable and non-selective β- adrenoceptor agonist, isoprenaline (30µM), was added twice daily after an initial 24 hour plating down period to cells grown in 10% serum- supplemented DMEM but had no effect upon cell numbers after 4 days growth (Figure 46).

The effects of stable β- adrenoceptor agonists were then examined. The selective β_1- adrenoceptor agonist, dobutamine (10µM)

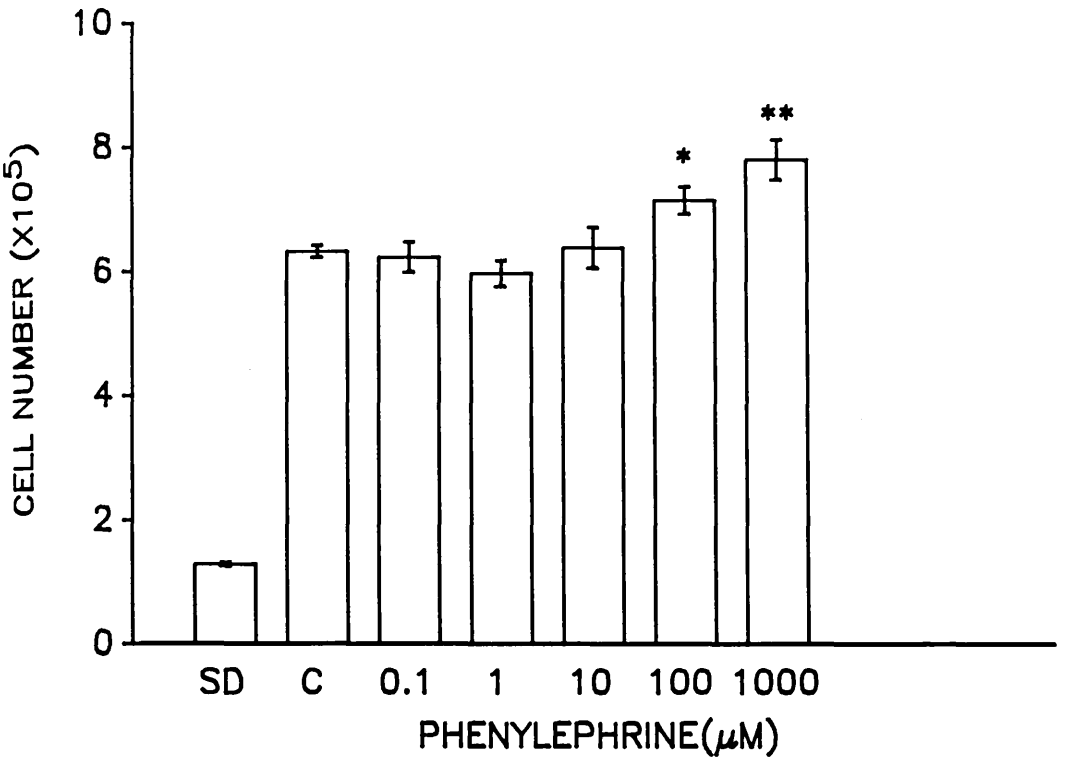


Figure 44: Concentration- effect relationship showing the effects of phenylephrine on proliferation of rat ASMC. Rat ASMC were seeded at a density (SD) of 1.3 x 10^4 cells/ cm^2 in 10% serum-supplemented DMEM. Cells received either no drug (C) or phenylephrine (0.1μM- 1mM) added twice daily after an initial 24 hour plating down period. The cells were allowed to grow for 4 days and then counted by haemocytometry. Bars show mean cell number ± s.e. mean (n=6). * $P< 0.05$; ** $P< 0.005$; denotes a significant difference from untreated cells.

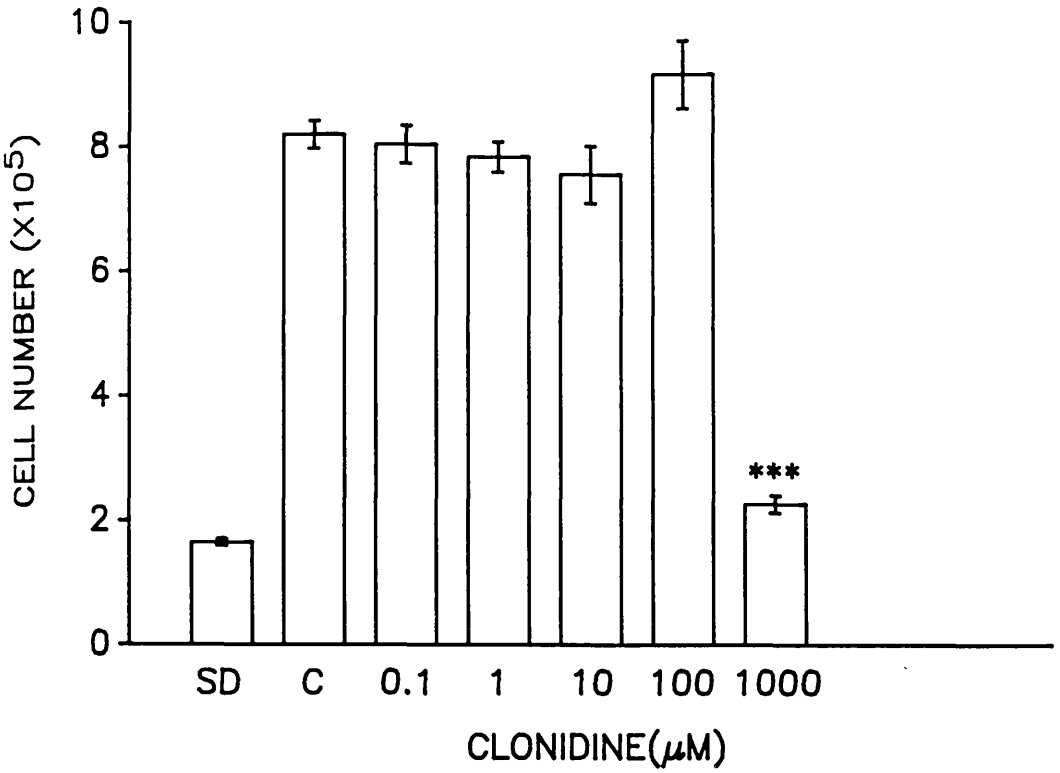


Figure 45: Concentration- effect relationship showing the effects of clonidine on proliferation of rat ASMC. Rat ASMC were seeded at a density (SD) of 1.4 x 10^4 cells/ cm^2 in 10% serum- supplemented DMEM. Clonidine (0.1μM- 1mM) was added twice daily after an initial 24 hour plating down period. The cells were allowed to grow for 4 days and then counted by haemocytometry . Bars show mean cell numbers ± s.e. mean (n=6). *** P< 0.005 denotes a significant difference from untreated cells.

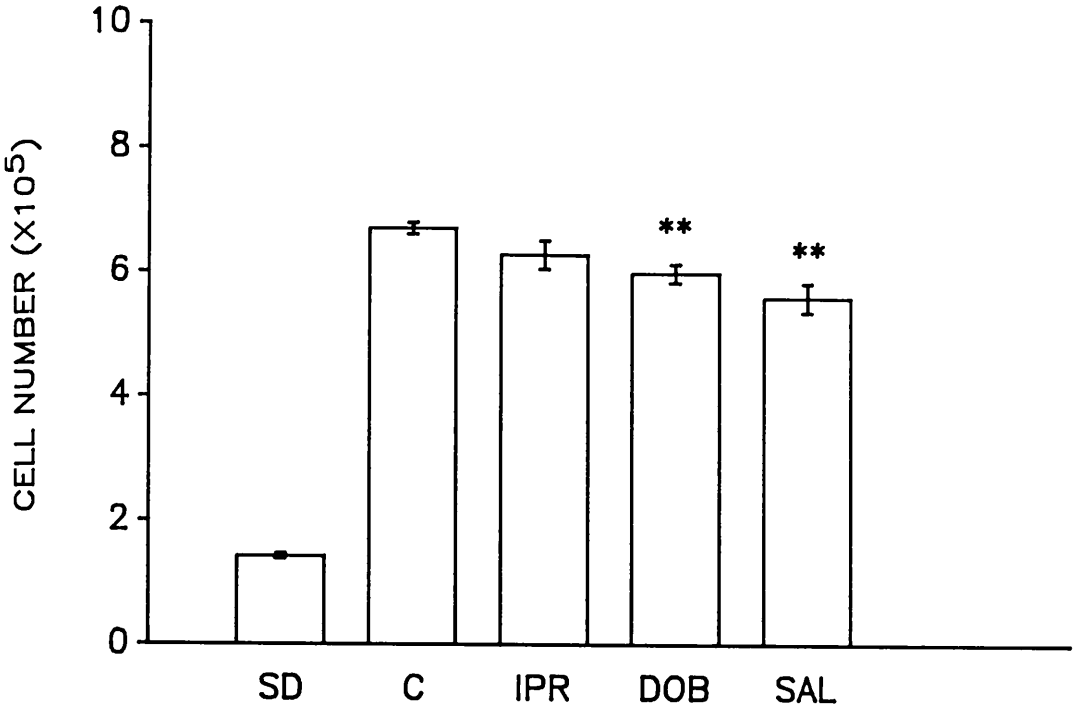


Figure 46: Effects of isoprenaline, dobutamine, and salbutamol on the proliferation of rat ASMC. Rat ASMC were seeded at a density (SD) of 1.4 x 10^4 cells/ cm^2 in 10% serum- supplemented DMEM and received either isoprenaline (30µM, IPR), dobutamine (10µM, DOB), or salbutamol (10µM, SAL) added twice daily after an initial 24 hour plating down period. The cells were allowed to grow for 4 days and then counted by haemocytometry . Bars show the mean cell number ± s.e. mean (n=6). ** P< 0.005 denotes a significant difference from untreated cells.

and the selective β_2- adrenoceptor agonist, salbutamol (10μM), were added twice daily after an initial 24 hour plating down period to cells grown in 10% serum-supplemented DMEM. These analogues reduced cell numbers after 4 days growth by 13 ± 3% (n=6) and 21 ± 4% (n=6), respectively (Figure 46). It is likely therefore that activation of α_1- adrenoceptors stimulates and β-adrenoceptors inhibits proliferation of rat ASMC.

6.2. EFFECTS OF CYCLIC AMP ON PROLIFERATION OF RAT ASMC

It is known that the effector pathway for β- adrenoceptor activation is via activation of adenylate cyclase (Watson & Abbott, 1989). It was possible that the reduction in cell numbers induced by dobutamine and salbutamol was mediated by an increase in cellular cyclic AMP content. In keeping with this, increasing cellular cyclic AMP content has been reported to reduce DNA synthesis by human ASMC cultured from atherosclerotic lesions (Tertov et al., 1984). This suggests that cyclic AMP may have a role in regulating the growth of vascular smooth muscle cells. The effects of various agents which increase cyclic AMP content by activation of adenylate cyclase or by inhibiton of the cyclic AMP specific phosphodiesterase were therefore examined on the proliferation of rat ASMC.

6.2.1. Effects of dibutyryl cyclic AMP

The membrane permeant analogue of cyclic AMP, dibutyryl cyclic AMP (30μM-1mM), was added twice daily after an initial 24 hour plating down period to cells grown in 10% serum- supplemented DMEM. This analogue had no effect on cell numbers after 4 days growth at a lower concentration of 30μM, but reduced cell numbers

at the higher concentrations of 100μM and 1mM by 29 ± 3% (n=6) and 66 ± 2% (n=6), respectively (Figure 47). Even at the highest concentration (1mM), dibutyryl cyclic AMP failed to stimulate trypan blue uptake (Figure 48).

6.2.2. Effects of forskolin

The activator of adenylate cyclase, forskolin (30μM), when added twice daily after an initial 24 hour plating down period to cells grown in 20% serum- supplemented DMEM, produced a marked reduction in cell numbers compared with untreated cells throughout an 8 day period: an inhibition of 57 ± 2% (n=6) was observed at day 8 (Figure 49). The ability of forskolin to reduce cell numbers was observed over the concentration range 1μM to 100μM: the maximum reduction obtained after 4 days growth at 100μM was 90 ± 1% (n=6, Figure 50). The inactive analogue, dideoxy forskolin (1μM-30μM) lacked the ability of forskolin to reduce cell numbers when assessed after 4 days growth (Figure 50). The solvent for forskolin and dideoxy forskolin, DMSO (0.1%), reduced cell numbers by itself but only by less than 10%. It is likely therefore that the ability of forskolin to reduce cell numbers results from activation of adenylate cyclase and the subsequent increase in cellular cyclic AMP content.

6.2.3. Effects of M & B 22948 and rolipram

Vascular smooth muscle is known to contain three phosphodiesterase isozymes: two cyclic GMP phosphodiesterases, one continuously active (Type V) and the other stimulated by the calcium-calmodulin complex (Type I), and a cyclic AMP phosphodiesterases (Type IV) (Lugnier et al., 1986; Schoeffter et al., 1987). The

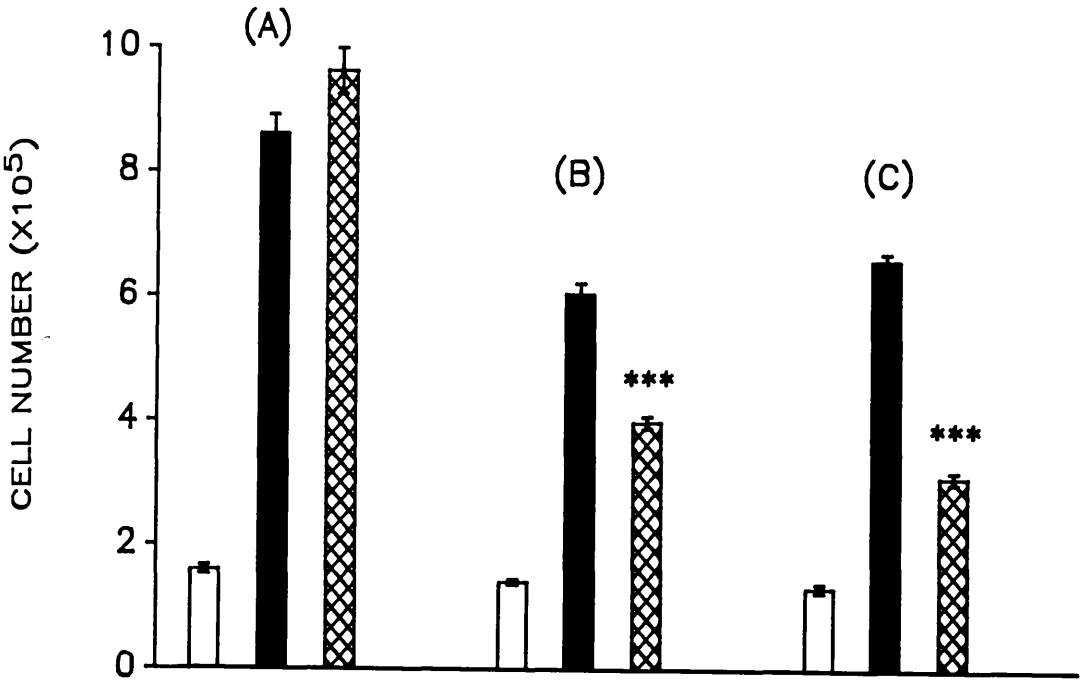


Figure 47: Concentration- effect relationship showing the ability of dibutyryl cyclic AMP (DBcAMP) to inhibit proliferation of rat ASMC. Rat ASMC were seeded at a density (▭) of 1.3- 1.6 x 10^4 cells/ cm^2 in 10% serum- supplemented DMEM.

(A) Cells received either no drugs (■) or DBcAMP (30µM, ▩).

(B) Cells received either no drugs (■) or DBcAMP (100µM, ▩).

(C) Cells received either no drugs (■) or DBcAMP (1mM, ▩).

Drugs were added twice daily after an initial 24 hour plating down period. Cells were allowed to grow for 4 days and then counted by haemocytometry. Bars show mean cell number ± s.e. mean (n=6). *** $P< 0.0005$ denotes a significant difference from untreated cells.

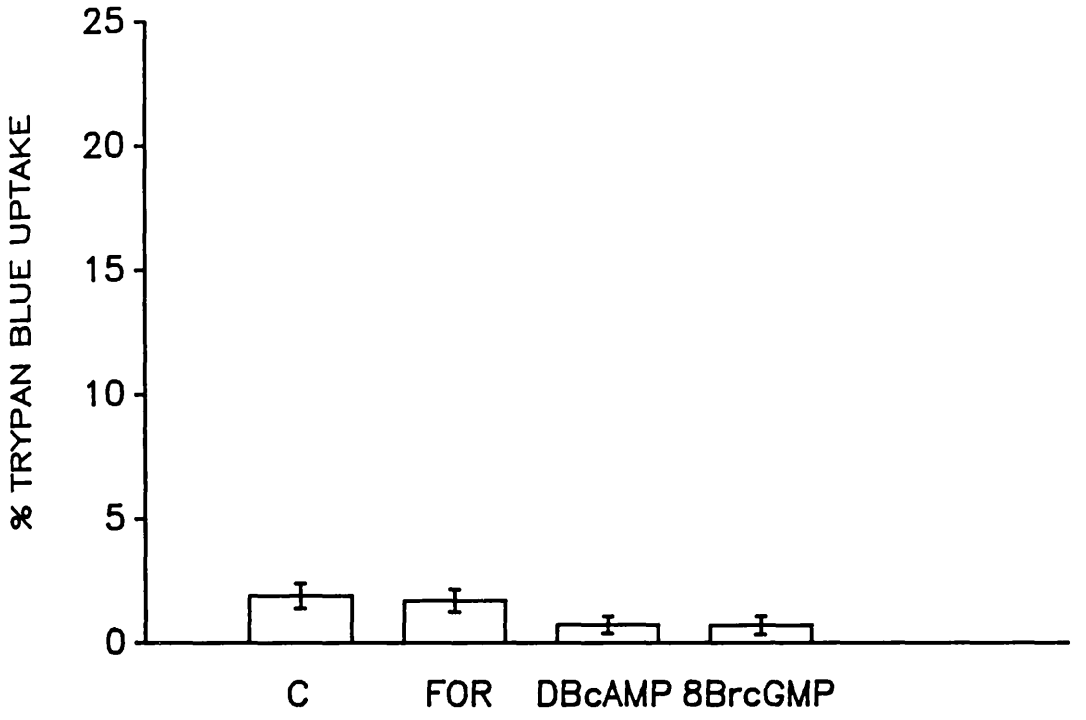


Figure 48: Effects of forskolin (FOR), dibutyryl cyclic AMP (DBcAMP) and 8 bromo cyclic GMP (8Br cGMP) on trypan blue uptake by rat ASMC. Rat ASMC were seeded at a density of 1.2 x 10^4 cells/ cm^2 in 10% serum- supplemented DMEM with the exception of forskolin- treated cells which were grown in 20% normal serum-supplemented DMEM. Cells received either no drugs (C), FOR (10µM), DBcAMP (1mM) and 8Br cGMP (1mM), added twice daily after an initial 24 hour plating down period. The cells were allowed to grow for 4 days and then treated with trypan blue solution (0.1% v/v in 0.9% NaCl) for 30 minutes. Bars show mean ± s.e. mean percentage of cells taking up trypan blue (n=6).

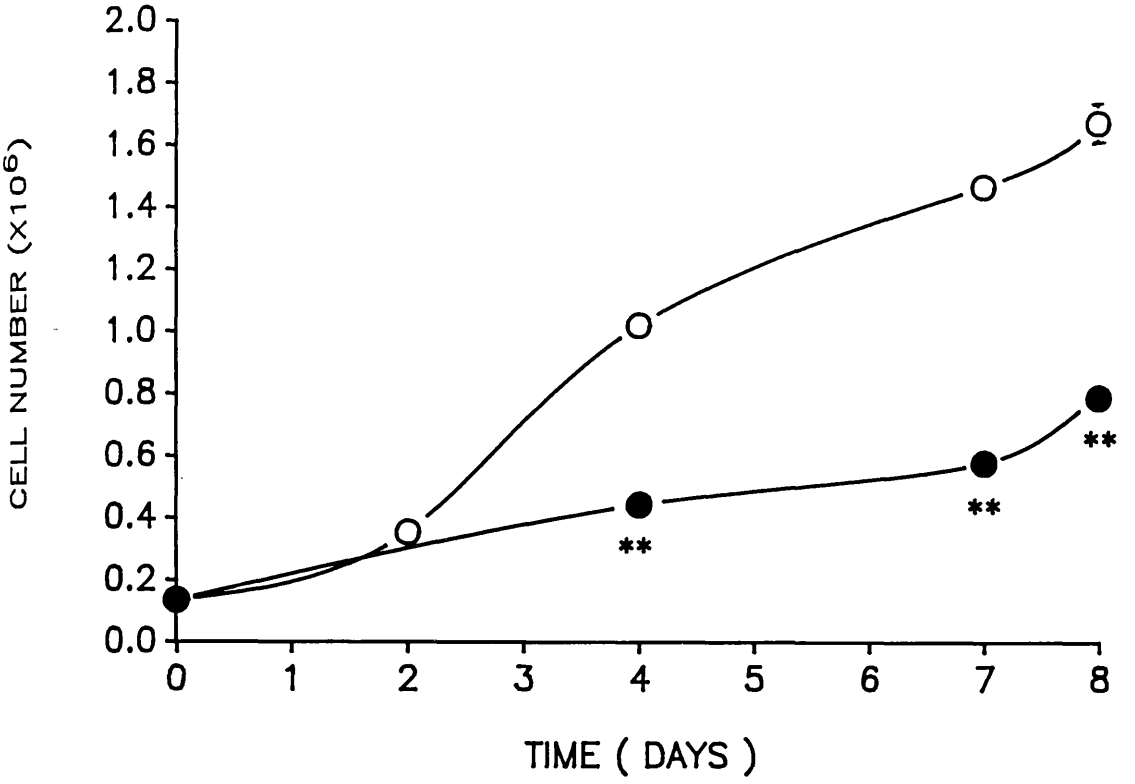


Figure 49: Effects of forskolin (FOR) on the proliferation of rat ASMC. Rat ASMC were seeded at a density of 1.4 x 10^4 cells/ cm^2 in 20% serum- supplemented DMEM and received either no drug (o) or forskolin (30µM, ●), added twice daily after an initial 24 hour plating down period. At the time points indicated, the cells were counted by haemocytometry . Points show mean cell numbers (n=6); all s.e. means are contained within the symbols.
** P< 0.005 denotes a significant difference from untreated cells on that day.

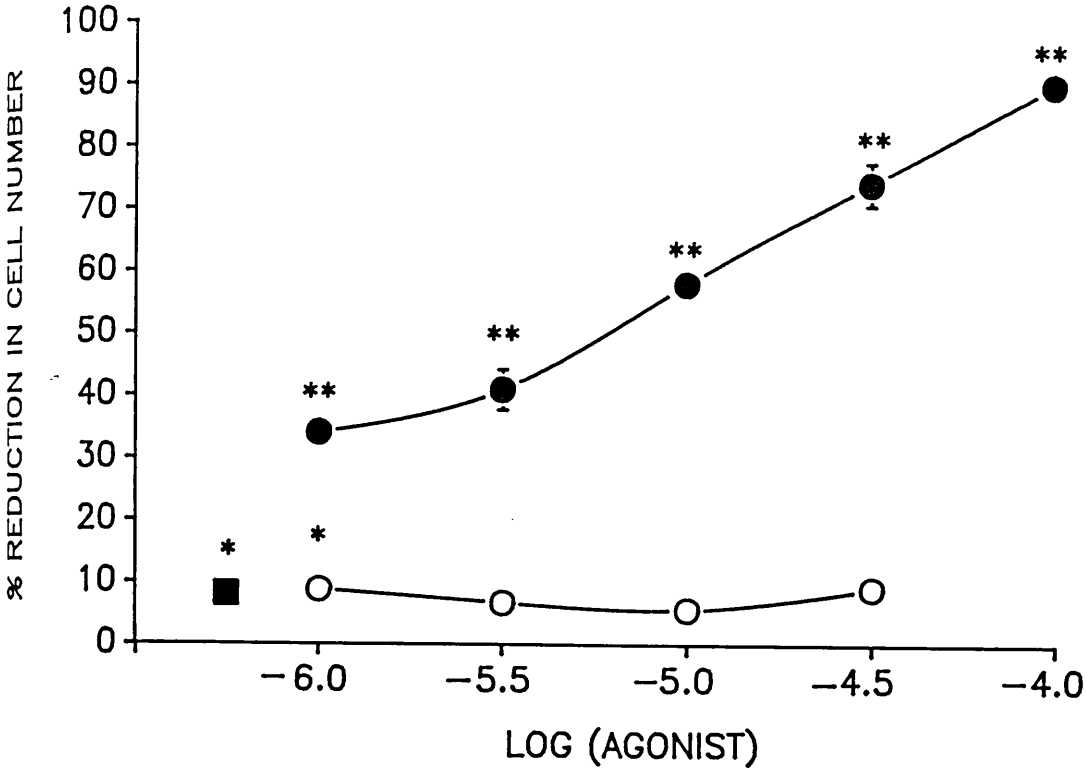


Figure 50: Concentration- effect curves showing the ability of forskolin but not of the inactive dideoxy forskolin to inhibit proliferation of rat ASMC. Rat ASMC were seeded at a density of 1.2×10^4 cells/ cm^2 in 20% serum- supplemented DMEM. Forskolin (1μM- 100μM, ●), dideoxy forskolin (1μM- 30μM, o) and DMSO (0.1% v/v, ■) were added twice daily after an initial 24 hour plating down period. The cells were allowed to grow for 4 days and then counted by haemocytometry . Results are expressed as the mean ± s.e. mean reduction (%) in cell numbers when compared with untreated cells (n=6); when error bars are not seen they are contained within symbols. * $P < 0.05$; ** $P < 0.005$; denotes a significant difference from untreated cells.

cyclic GMP phosphodiesterases are inhibited by M & B 22948 and the cyclic AMP phosphodisterase is inhibited selectively by rolipram.

To examine further the possibility that forskolin reduced cell numbers via an increase in cellular cyclic AMP, the effects of rolipram and M & B 22948 were examined alone and in the combination with forskolin.

Rolipram (30μM), when added twice daily after an initial 24 hour plating down period to cells grown in 20% serum- supplemented DMEM, produced a reduction 37 ± 2% (n=6) in cell numbers after 4 days growth and potentiated the inhibitory effect of forskolin (10μM) from 32 ± 4% to 57 ± 4% (n=6, Figure 51).

M & B 22948 (30μM), when added twice daily after an initial 24 hours plating down period to cells grown in 20% serum- supplemented DMEM produced a reduction in cell numbers after 4 days growth of 26 ± 5% and 48 ± 5% (n=6) in two seperate experiments (Figure 52), but did not potentiate the ability of forskolin (10μM) to reduce cell numbers after 4 days growth. In fact , M & B 22948 appeared to reverse the ability of forskolin to reduce cell numbers (Figure 52).

The potentiating action of rolipram further supports the concept of cyclic AMP being an inhibitor of proliferation of rat ASMC.

6.2.4. Effects of histamine

H_2- receptor activation normally results in the activation of

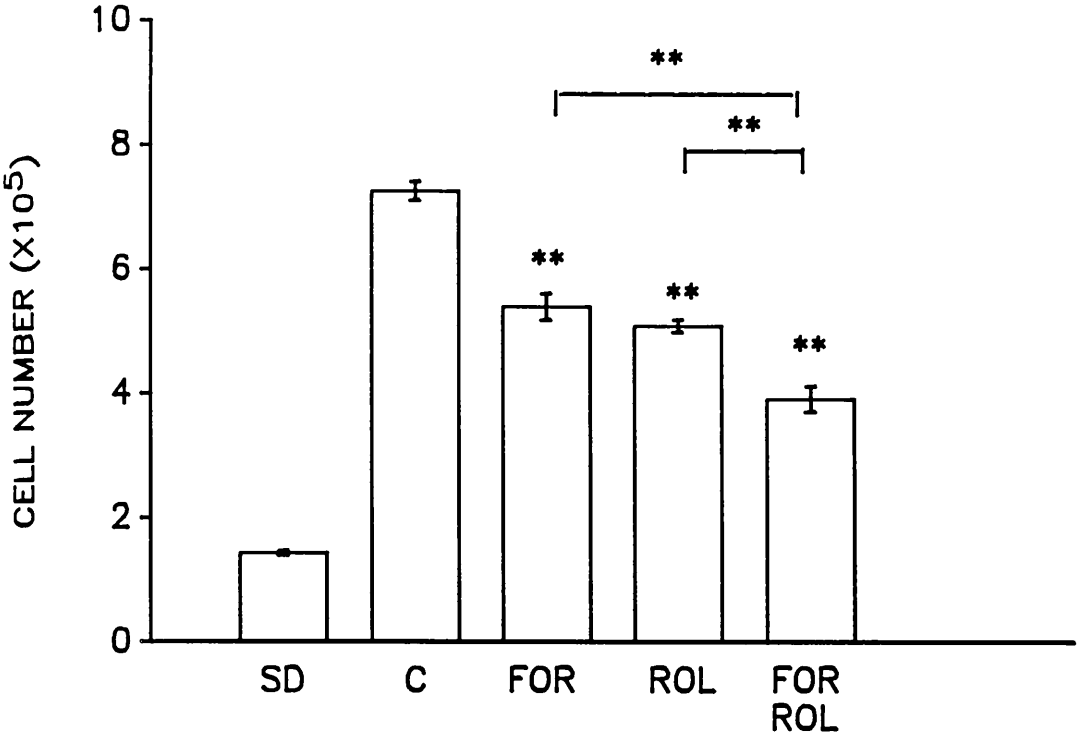
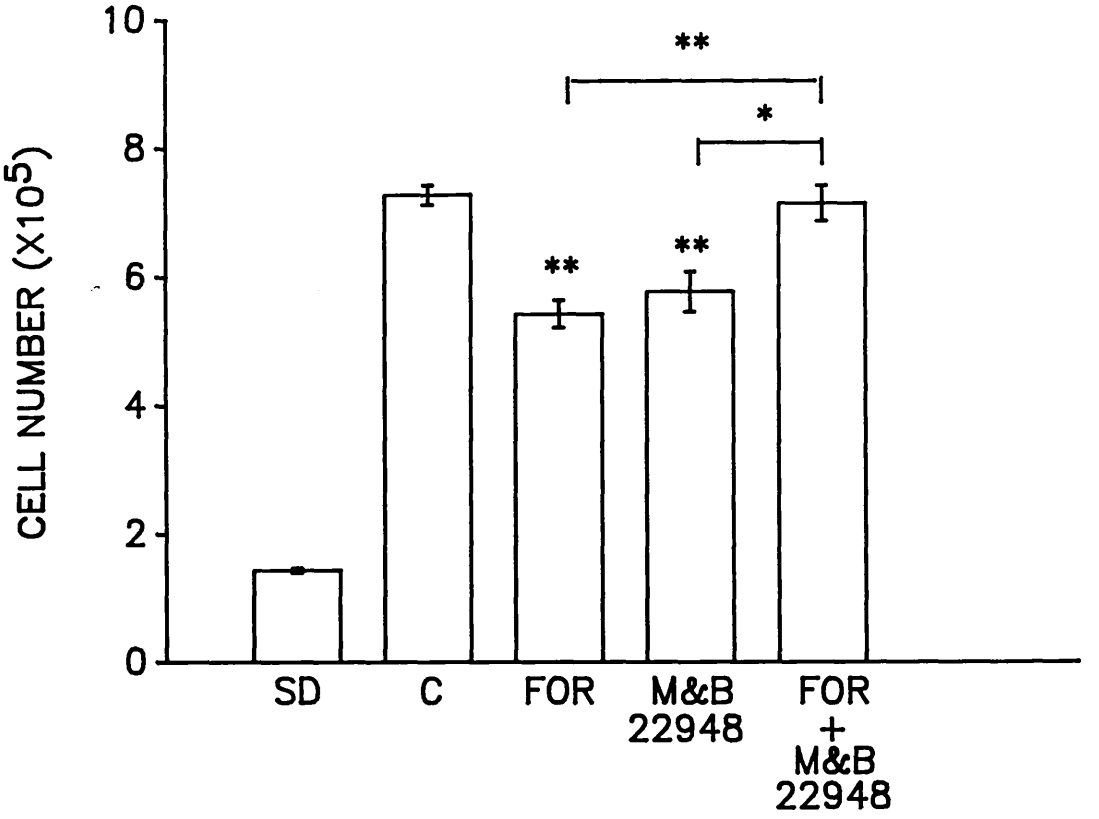


Figure 51: Effects of rolipram on the antiproliferative action of forskolin on rat ASMC. Rat ASMC were seeded at a density (SD) of 1.4×10^4 cells/ cm^2 in 20% serum- supplemented DMEM and received either no drug (C), forskolin (10μM, FOR), rolipram (30μM, ROL), or a combination of FOR and ROL. Drugs were added twice daily after an initial 24 hour plating down period. The cells were allowed to grow for 4 days and then counted by haemocytometry. Bars show the mean cell numbers ± s.e. mean (n=6).
** $P < 0.005$ denotes a significant difference from untreated cells, or, between groups joined with a bracket.


CELL NUMBER (X10^5)
10
8
6
4
2
0
**
*
**
**
SD
C
FOR
M&B 22948
FOR + M&B 22948

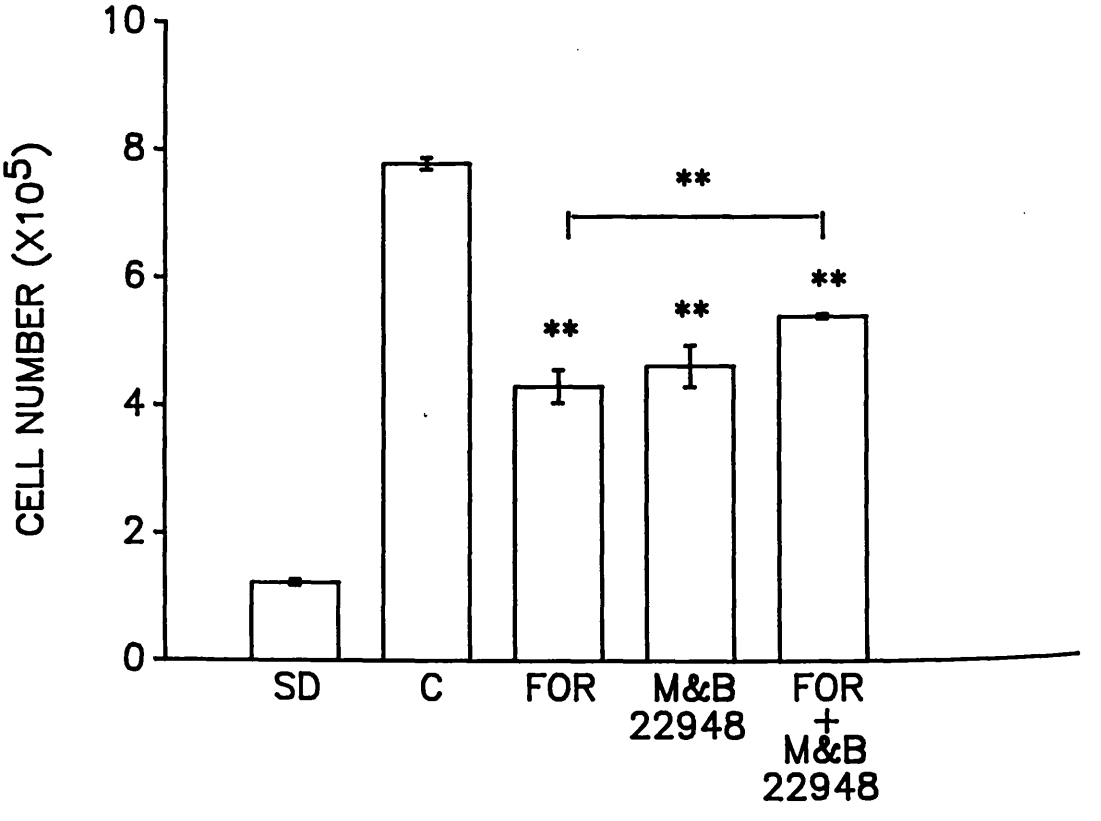

CELL NUMBER (X10^5)
10
8
6
4
2
0
**
**
**
**
SD
C
FOR
M&B 22948
FOR + M&B 22948

Figure 52: Effects of M & B 22948 on the ability of forskolin to inhibit proliferation of rat ASMC. Rat ASMC were seeded (SD) at a density of 1.4 x 10^4 cells/ cm^2 (top panel) or 1.2 x 10^4 cells/ cm^2 (bottom panel) in 20% serum- supplemented DMEM and received either no drugs (C), forskolin (10µM, FOR), M & B 22948 (30µM), or a combination of FOR and M & B 22948. Drugs were added twice daily after an initial 24 hour plating down period. The cells were allowed to grow for 4 days and then counted by haemocytometry. Bars show mean cell numbers ± s.e. mean (n=6).
* $P< 0.05$; ** $P< 0.005$; denotes a significant difference from untreated cells, or, between groups joined with a bracket.

adenylate cyclase (Hill, 1990). The possibility that histamine might inhibit proliferation of rat ASMC in culture through elevation of cyclic AMP content was therefore examined.

Histamine (1μM-1mM), when added twice daily after an initial 24 hour plating down period to cells grown in 10% serum- supplemented DMEM, reduced cell numbers in a concentration- dependent manner: the maximum reduction after 4 days growth in 1mM histamine was 44 ± 2% (n=6, Figure 53).

The possibility that histamine inhibited proliferation of rat ASMC through activation of H_2- receptors was examined by investigating the effect of cimetidine, an H_2- antagonist. Cimetidine (10μM), when added twice daily after an initial 24 hour plating down period to cells grown in 10% serum- supplemented DMEM had no effect by itself on cell numbers after 4 days growth, but blocked the ability of histamine (10μM) to reduce cell numbers (Figure 54).

6.3. EFFECTS OF CYCLIC GMP ON PROLIFERATION OF RAT ASMC

It has recently been reported that nitrovasodilators, which elevate levels of cyclic GMP, inhibit rat aortic smooth muscle cell growth in culture (Garg & Hassid, 1989). These workers further suggested that EDRF (identified as nitric oxide), the active principle generated by the nitrovasodilators, might be an endogenous regulator of smooth muscle cell proliferation in the arterial wall.

The effects of the various agents which increase cyclic GMP

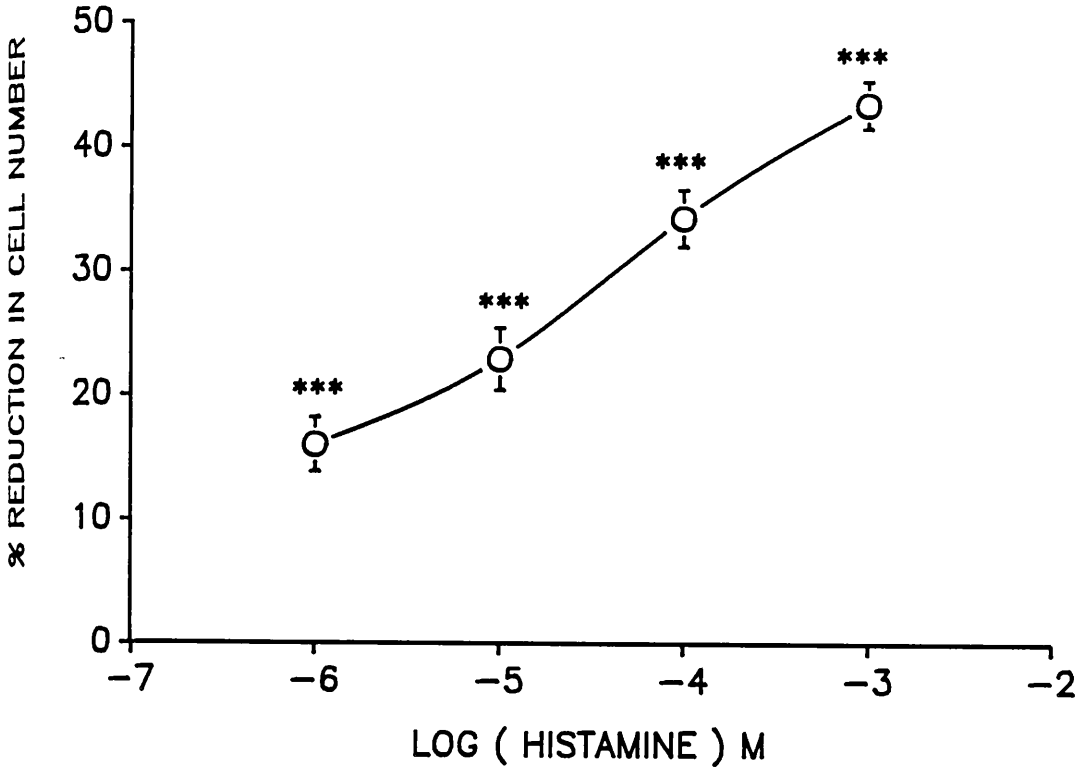


Figure 53: Concentration- effect curve showing the ability of histamine to inhibit proliferation of rat ASMC. Rat ASMC were seeded at a density of 1.2 x 10^4 cells/ cm^2 in 10% serum- supplemented DMEM. Histamine (1μM- 1mM) was added twice daily after an initial 24 hour plating down period. The cells were allowed to grow for 4 days and then counted by haemocytometry . The results are expressed as the mean ± s.e. mean reduction (%) of cell numbers compared with untreated cells (n=6). *** P< 0.0005 denotes a significant difference from untreated cells.

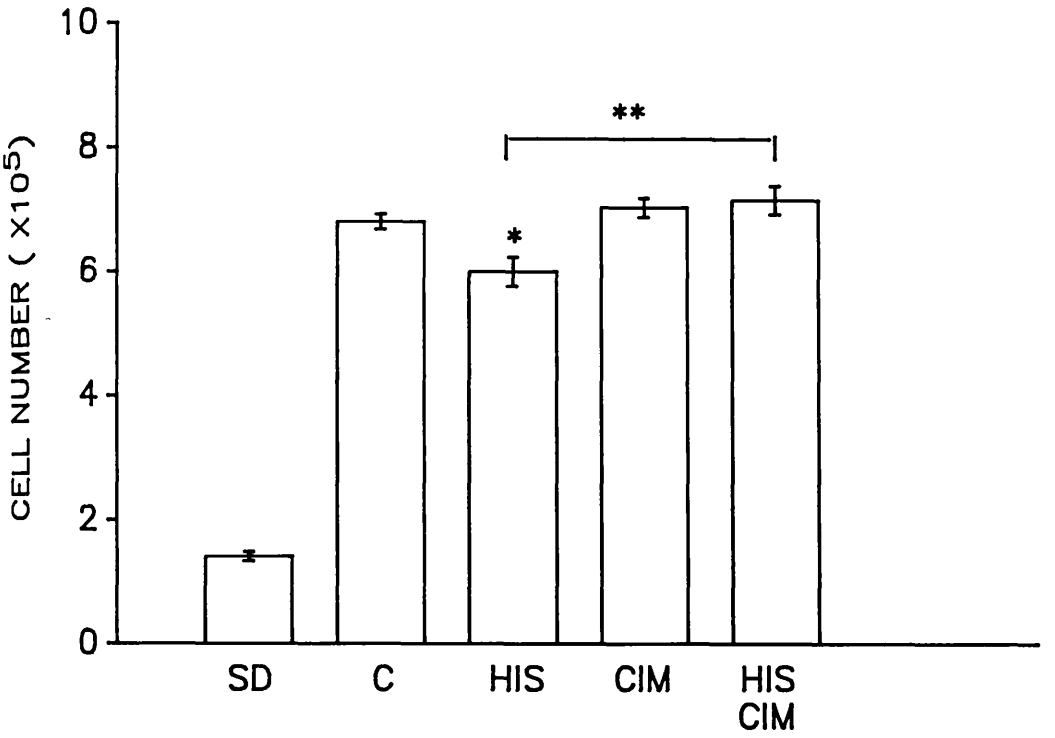


Figure 54: The ability of cimetidine to reverse the inhibitory action of histamine on rat ASMC proliferation. Rat ASMC were seeded at a density (SD) of 1.4 x 10^4 cells/ cm^2 in 10% serum-supplemented DMEM and received either no drug (C), histamine (10μM, HIS), cimetidine (10μM, CIM), or a combination of HIS and CIM. Drugs were added twice daily after an initial 24 hour plating down period. The cells were allowed to grow for 4 days and then counted by haemocytometry . Bars show mean cell numbers ± s.e. mean (n=6). * $P< 0.05$; ** $P< 0.005$; denotes a significant difference from untreated cells, or, between groups joined with a bracket.

content were therefore examined on the proliferation of rat ASMC. These agents included stimulants of soluble and particulate guanylate cyclase and an inhibitor of the cyclic GMP phosphodiesterases.

6.3.1. Effects of 8 bromo cyclic GMP and atriopeptin II

The membrane permeant analogue of cyclic GMP, 8 bromo cyclic GMP (0.1µM-1mM) was added twice daily after an initial 24 hour plating down period to cells grown in 10% serum- supplemented DMEM. It had no effect on cell numbers after 4 days growth at the lower concentrations of 0.1µM and 0.1mM, but reduced cell numbers by 17 ± 3% (n=6, Figure 55) at the higher concentration of 1mM. Even at the concentration of 1mM, however, 8 bromo cyclic GMP failed to increase trypan blue uptake (Figure 48). The atrial natriuretic factor, atriopeptin II (0.1µM), which activates particulate guanylate cyclase, was added twice daily after an initial 24 hour plating down period to cells grown in 20% serum-supplemented DMEM. It had no effect on cell numbers when assessed throughout an 8 day growth period (Figure 56).

6.3.2. Effects of glyceryl trinitrate and sodium nitroprusside

The nitrovasodilators, glyceryl trinitrate (0.1µM-1mM) and sodium nitroprusside (1µM-1mM), which activate soluble guanylate cyclase, were added twice daily after an initial 24 hours plating down period to cells grown in 10% serum- supplemented DMEM. Glyceryl trinitrate had no effect on cell numbers after 4 days growth in concentrations up to 0.1mM, but at 1mM, cell numbers were reduced by 17 ± 1% (n=6, Figure 57). Sodium nitroprusside had no effect on cell numbers at concentrations of 1µM to 0.1mM,

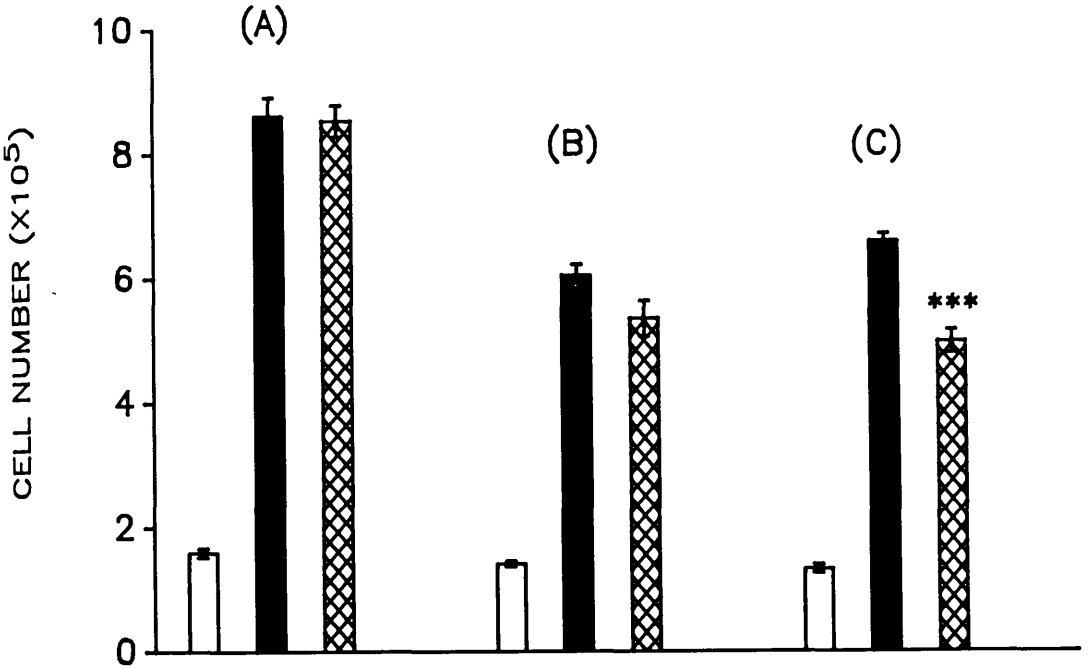


Figure 55: Concentration- effect relationship showing the ability of 8 bromo cyclic GMP to inhibit proliferation of rat ASMC. Rat ASMC were seeded at a density (▭) of 1.3- 1.6 x 10^4 cells/ cm^2 in 10% serum- supplemented DMEM.

(A) Cells received either no drug (■), or 8 bromo cyclic GMP (30µM, ⊠). (B) Cells received either no drug (■), or 8 bromo cyclic GMP (100µM, ⊠). (C) Cells received either no drug (■), or 8 bromo cyclic GMP (1mM, ⊠).

Drugs were added twice daily after an initial 24 hour plating down period. Cells were allowed to grow for 4 days and then counted by haemocytometry . Bars show mean cell numbers ± s.e. mean (n=6). *** P< 0.0005 denotes a significant difference from untreated cells.

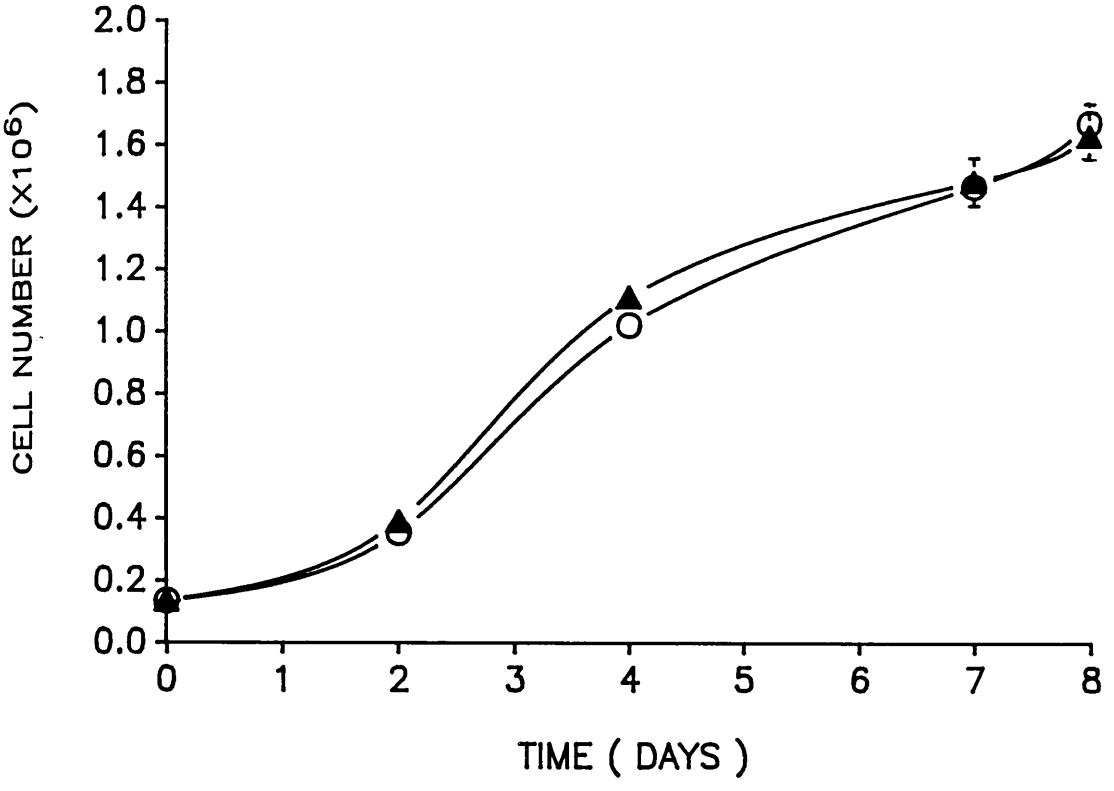
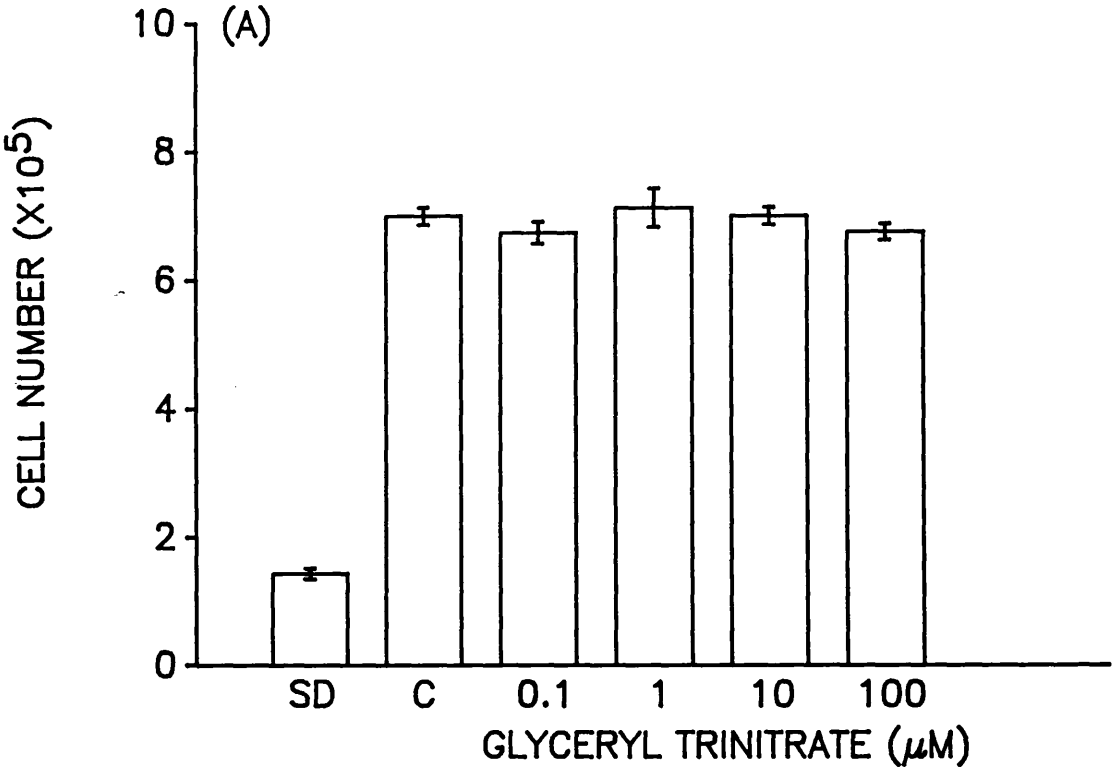


Figure 56: Effects of atriopeptin II on the proliferation of rat ASMC. Rat ASMC were seeded at a density of 1.4 x 10^4 cells/ cm^2 in 20% serum- supplemented DMEM and received either no drug (o), or, atriopeptin II (0.1μM, ▲). Drugs were added twice daily after an initial 24 hour plating down period. At the time points indicated, the cells were counted by haemocytometry. Points show mean cell numbers ± s.e. mean (n=6); when error bars are not seen they are contained within symbols.


(A)
CELL NUMBER (X10^5)
10
8
6
4
2
0
SD
C
0.1
1
10
100
GLYCERYL TRINITRATE (μM)

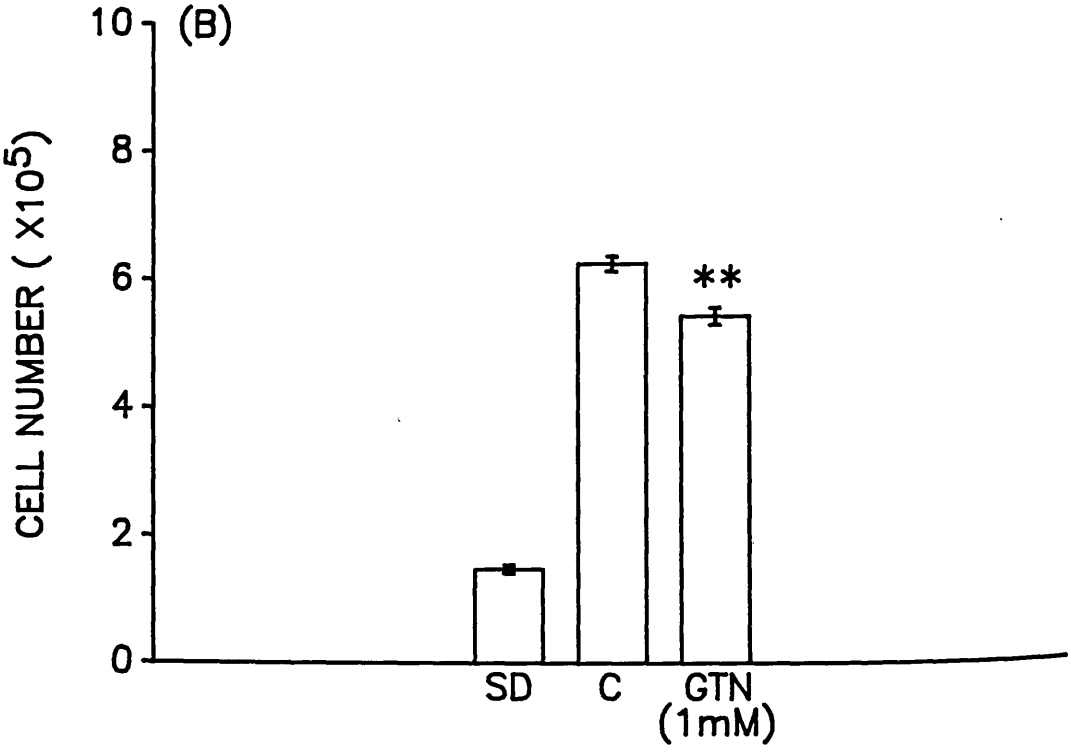

(B)
CELL NUMBER (X10^5)
10
8
6
4
2
0
**
SD
C
GTN
(1mM)

Figure 57: Concentration- effect relationship showing the effects of glyceryl trinitrate on proliferation of rat ASMC. Rat ASMC were seeded at a density (SD) of 1.4 x 10^4 cells/ cm^2 (A) and 1.4 X 10^4 cells/ cm^2 (B) in 10% serum- supplemented DMEM and received either no drug (C), glyceryl trinitrate (0.1μM- 100μM, GTN, A) or GTN (1mM, B). Drugs were added twice daily after an initial 24 hour plating down period. The cells were allowed to grow for 4 days and then counted by haemocytometry . Bars show mean cell numbers ± s.e. mean (n=6). ** P< 0.005 denotes a significant difference from untreated cells.

but reduced cell numbers at concentrations between 0.1mM and 1mM: the maximum reduction after 4 days growth was 107 ± 1% (n=6, Figure 58).

6.3.3. Effects of haemoglobin and M & B 22948 on the ability of sodium nitroprusside to inhibit proliferation of rat ASMC

In an attempt to determine if the ability of sodium nitroprusside to inhibit smooth muscle cell proliferation was mediated by nitric oxide- induced activation of soluble guanylate cyclase, the effects of haemoglobin and M & B 22948 were examined. Haemoglobin binds nitric oxide with high affinity thereby preventing it from activating soluble guanylate cyclase, and M & B 22948 inhibits cyclic GMP phosphodiesterase isozymes.

Haemoglobin (20µM) when added twice daily after an initial 24 hours plating down period to cells grown in 10% serum- supplemented DMEM had no effect on cell numbers after 4 days growth (data not shown). Furthermore, the sodium nitroprusside (0.1mM-1mM) induced reduction in cell numbers was not inhibited by the addition of haemoglobin (20µM, Figure 59).

M & B 22948 (30µM), when added twice daily after an initial 24 hours plating down period to cells grown in 10% serum- supplemented DMEM reduced cell numbers by 33 ± 2% (n=6, Figure 59) after 4 days growth. The sodium nitroprusside (0.1mM-1mM) induced reduction in cell numbers was not, however, potentiated following the addition of M & B 22948 (30µM, Figure 59).

These results suggested that the reduction in cell numbers in-

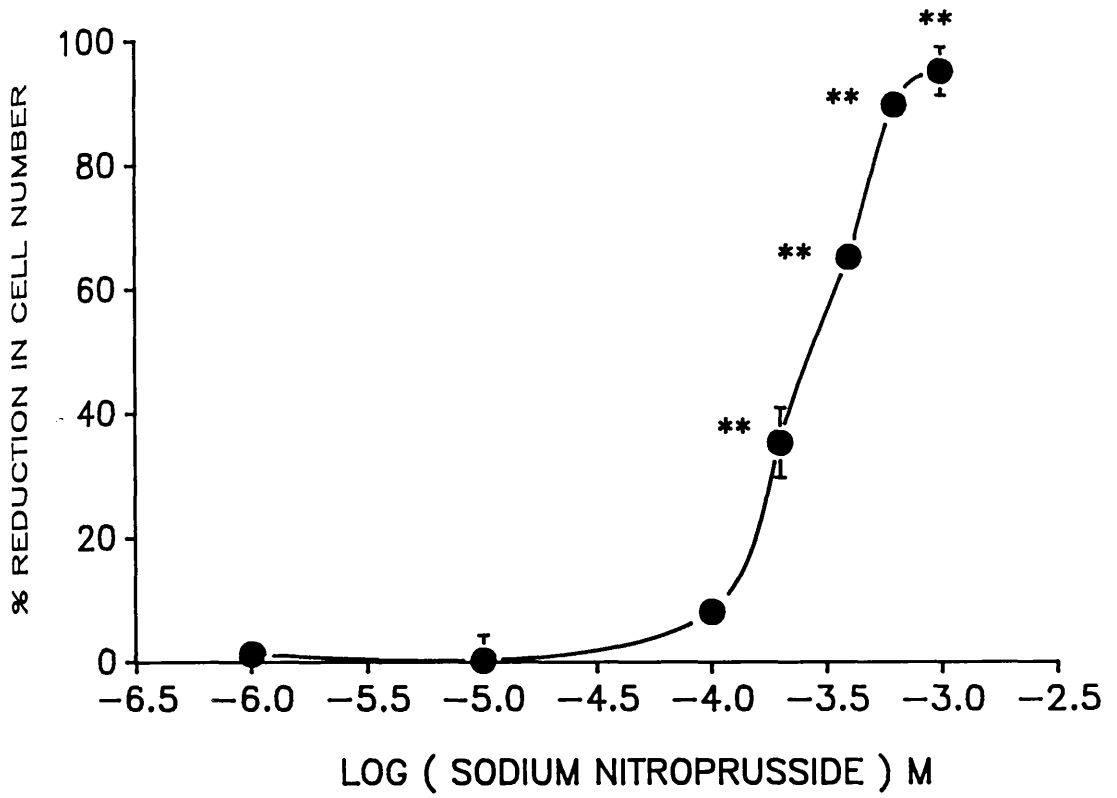


Figure 58: Concentration- effect curve showing the ability of sodium nitroprusside to inhibit proliferation of rat ASMC. Rat ASMC were seeded at a density of 1.5 x 10^4 cells/ cm^2 in 10% serum- supplemented DMEM. Sodium nitroprusside (1µM- 1mM) was added twice daily after an initial 24 hour plating down period. The cells were allowed to grow for 4 days and then counted by haemocytometry The results are expressed as the mean ± s.e. mean reduction (%) of cell number when compared with untreated cells (n=6-12); when error bars are not seen they are contained within symbols. **·P< 0.005 denotes a significant difference from untreated cells.

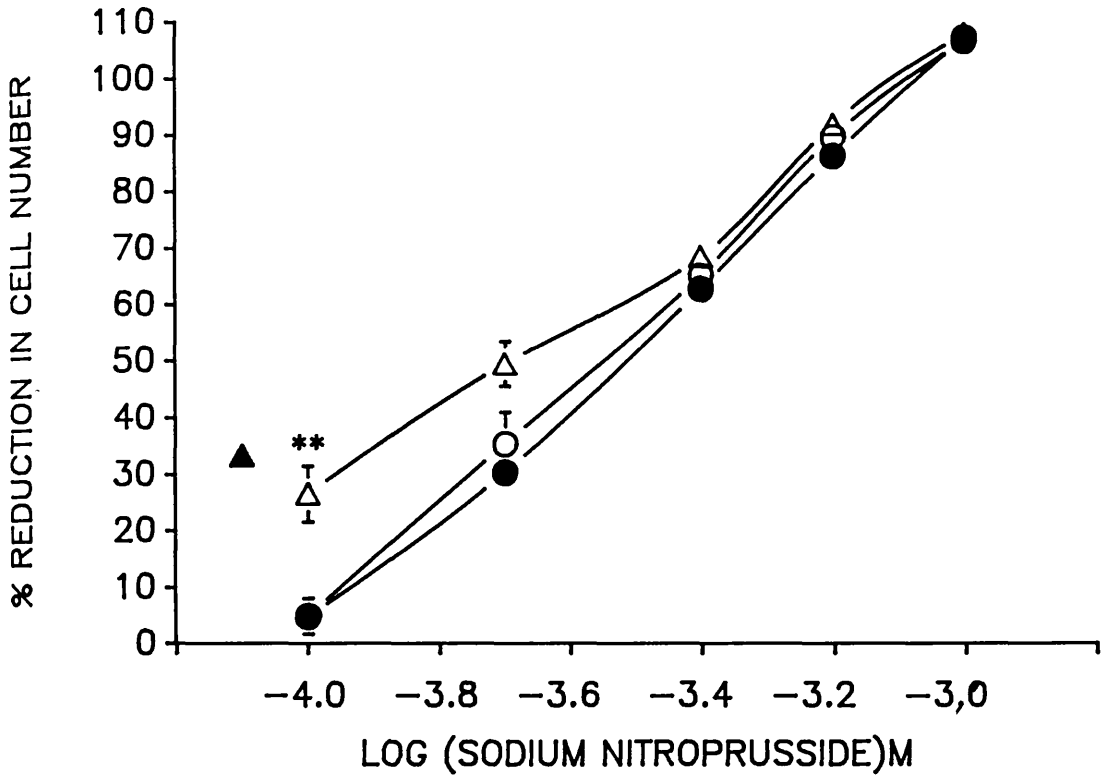


Figure 59: Effects of M & B 22948 and haemoglobin on the antiproliferative action of sodium nitroprusside on rat ASMC. Rat ASMC were seeded at a density of 1.4 x 10^4 cells/ cm^2 in 10% serum-supplemented DMEM and received either no drug, haemoglobin (20µM, data not shown), M & B 22948 (30µM, ▲), sodium nitroprusside (100µM- 1mM, o), a combination of sodium nitroprusside and haemoglobin (●), or a combination of sodium nitroprusside and M & B 22948 (Δ). Drugs were added twice daily after an initial 24 hour plating down period. Cells were allowed to grow for 4 days and then counted by haemocytometry . The results are expressed as mean ± s.e. mean reduction (%) of cell numbers compared with untreated cells (n=6); when error bars are not seen they are contained within the symbols. ** P< 0.005 denotes a significant difference from sodium nitroprusside treated cells.

duced by sodium nitroprusside was unrelated to stimulation of soluble guanylate cyclase, and resulted from a non- specific action. The possibility that the ability of sodium nitroprusside inhibited proliferation by a cytotoxic action was investigated by examining the accumulation of the vital stain, trypan blue. Sodium nitroprusside (0.1mM-1mM), over the same concentration range that inhibits proliferation, increased the accumulation of trypan blue in a concentration effect manner: at the highest concentration dose (1mM) almost all the cells accumulated trypan blue (Figure 60).

6.3.4. Effect of methaemoglobin on the ability of sodium nitroprusside to inhibit proliferation of rat ASMC

An alternative mechanism by which sodium nitroprusside could produce its antiproliferative actions on rat ASMC is through the production of cyanide. The effect of the oxidised form of haemoglobin, methaemoglobin, which binds to cyanide was therefore examined.

Methaemoglobin (5µM) was added twice daily after an initial 24 hour plating down period to cells grown in 10% serum- supplemented DMEM, had no effect by itself on cell numbers after 4 days growth. Furthermore, the sodium nitroprusside (0.1mM-1mM)-induced reduction in cell numbers was not inhibited by the addition of methaemoglobin (5µM, Figure 61). Generation of cyanide from the hydrolysis of sodium nitroprusside is unlikely therefore to contribute to the antiproliferative action.

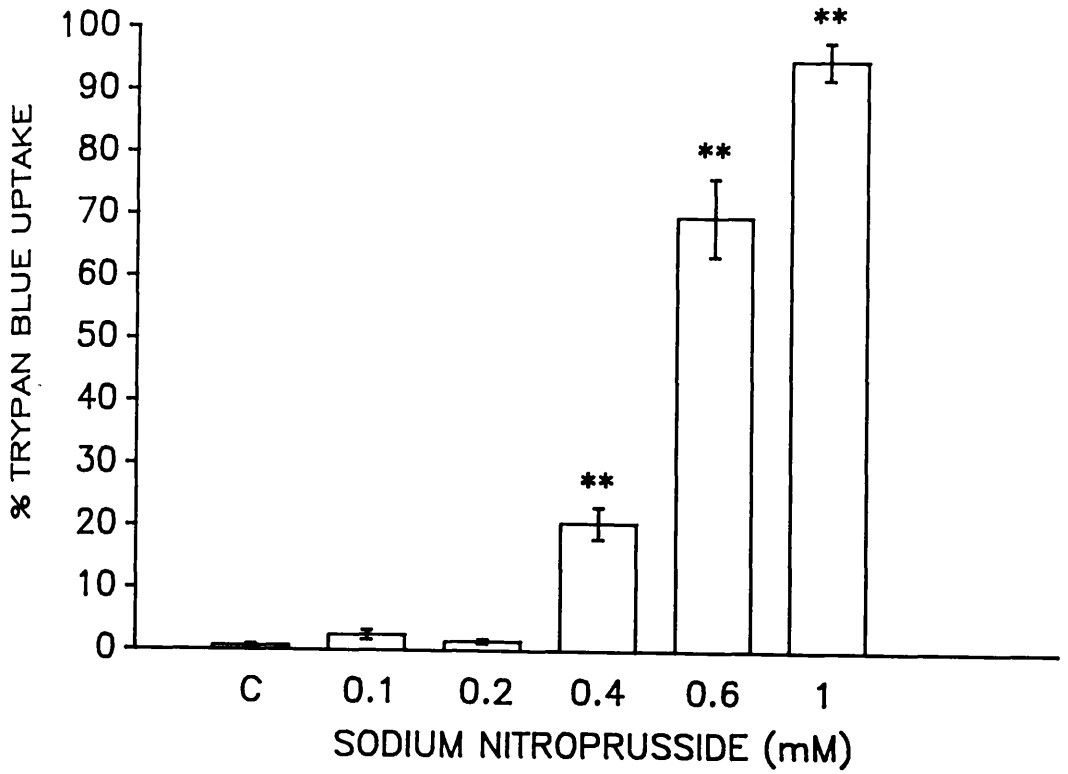


Figure 60: Effects of sodium nitroprusside on trypan blue uptake by rat ASMC. Rat ASMC were seeded at a density of 1.2 X 10^4 cells/ cm^2 in 10% serum- supplemented DMEM. Cells received either no drug (C), or sodium nitroprusside (0.1mM- 1mM), added twice daily after an initial 24 hour plating down period. The cells were allowed to grow for 4 days and then treated with trypan blue solution (0.1% v/v in 0.9% NaCl) for 30 minutes. Bars show mean ± s.e. mean percentage of cells taking up trypan blue (n=6). ** $P < 0.005$ denotes a significant difference from untreated cells.

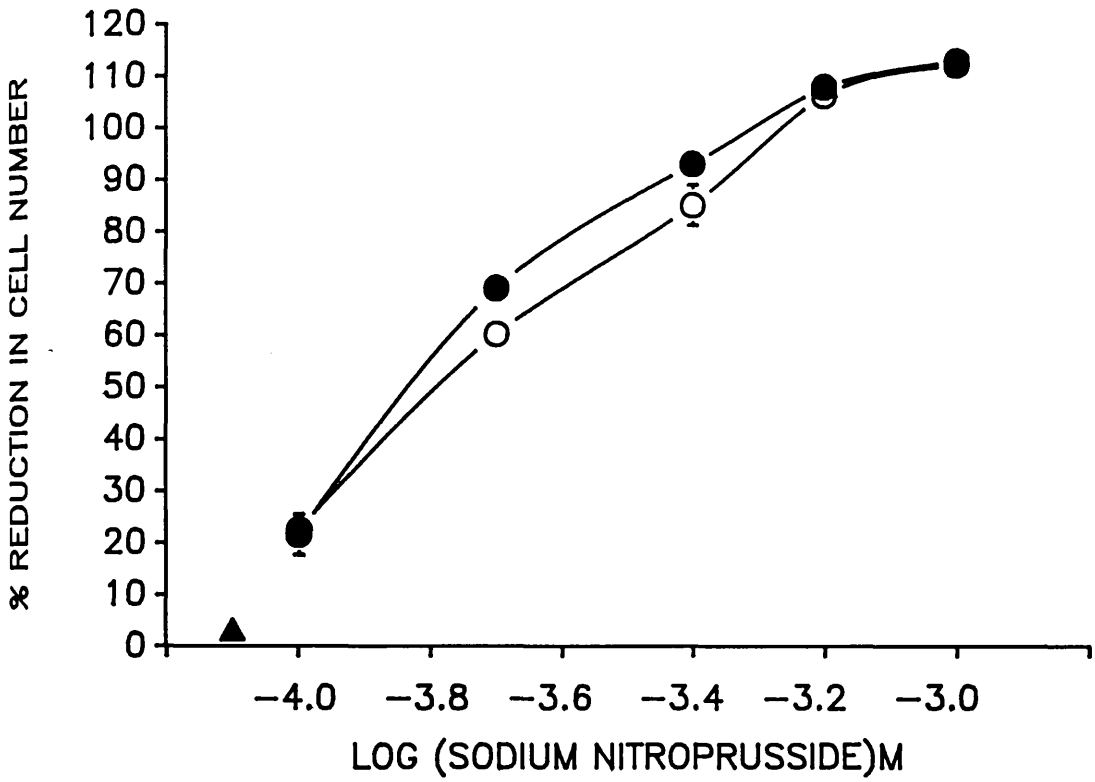


Figure 61: Effects of methaemoglobin on the antiproliferative action of sodium nitroprusside on rat ASMC. Rat ASMC were seeded at a density of 1.3 x 10^4 cells/ cm^2 in 10% serum- supplemented DMEM and received either no drug, methaemoglobin (5µM, ▲), sodium nitroprusside (100µM- 1mM, o), or a combination of sodium nitroprusside and methaemoglobin (●). Drugs were added twice daily after an initial 24 hour plating down period. Cells were allowed to grow for 4 days and then counted by haemocytometry . The results are expressed as mean ± s.e. mean reduction (%) of cell numbers compared with untreated cells (n=6); when error bars are not seen they are contained within the symbols.

6.3.5. Effects of N^G-Nitro L- arginine

It has recently been reported that vascular smooth muscle cells generate low but measurable quantities of a labile relaxing factor which possesses pharmacological and chemical properties similiar to those of EDRF (Wood et al., 1990). The possibility that this endogenous factor has a role in controlling the proliferation of rat ASMC in culture was investigated by examining the effects of N^G-nitro L- arginine (L-NOARG). L-NOARG is a potent competitive inhibitor of the synthesis of nitric oxide from L-arginine and acts by inhibiting the converting enzyme, nitric oxide synthase (Rees et al., 1989; 1990).

L-NOARG (50μM) when added twice daily after an initial 24 hour plating down period to cells grown in 10% serum- supplemented DMEM, increased cell numbers by 17 ± 4% (n=6, Figure 62) after 4 days growth. It is possible therefore that L-NOARG increases cell numbers by inactivation of nitric oxide synthase.

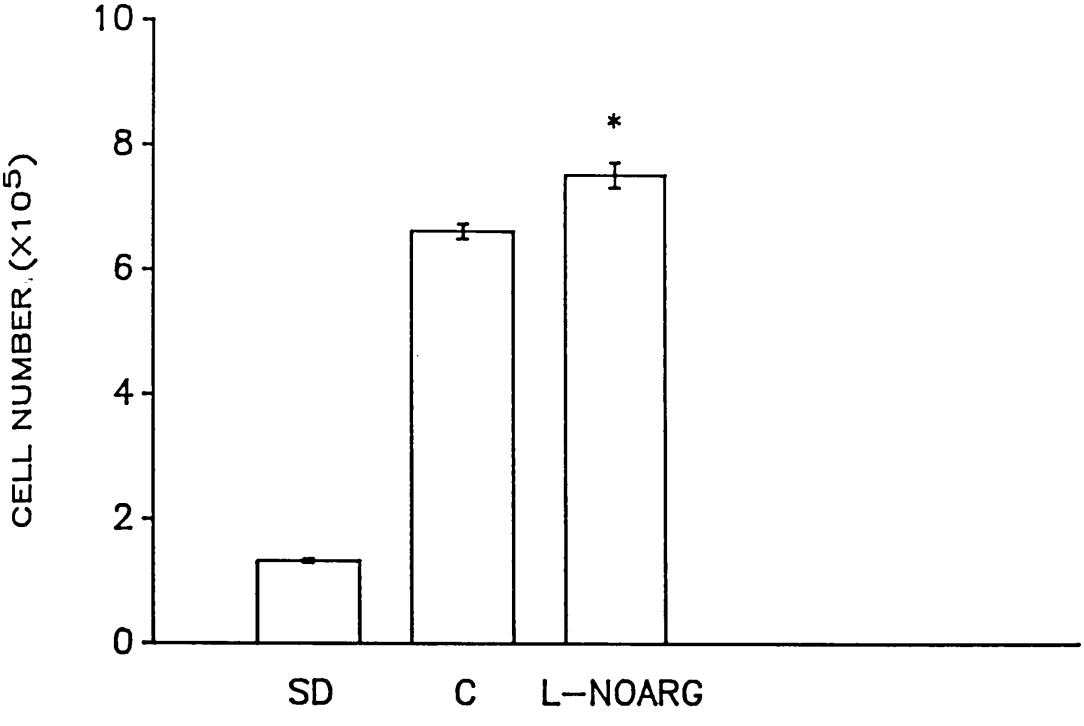


Figure 62: Effects of N^G- nitro L- arginine on the proliferation of rat ASMC. Rat ASMC were seeded at a density (SD) of 1.3 x 10^4 cells/ cm^2 in 10% serum- supplemented DMEM. Cells received either no drug (C),or L- NOARG (50µM) added twice daily after an initial 24 hour plating down period. Cells were allowed to grow for 4 days and then counted by haemocytometry . Bars show the mean cell numbers ± s.e. mean (n=6). * P< 0.05 denotes a significant difference from untreated cells.

DISCUSSION

7.1. PHOSPHODIESTERASE SUBTYPES IN PIG AORTIC ENDOTHELIAL CELLS

It is likely that as in other cells, cyclic nucleotides play a role as second messengers in endothelial cells. Several early studies demonstrated that the endothelium responded to certain vasoactive substances including catecholamines, histamine, acetylcholine, prostaglandins and angiotensin II by the elevation of either cyclic AMP or cyclic GMP content. This occured in endothelial cells cultured from a variety of sources including rabbit and bovine aortae and human umbilical vein (Buonassisi & Venter, 1976; Schafer et al., 1980; Makarski, 1981). More recently, atrial natriuretic peptides, nitrovasodilators and adenosine have been shown to elevate cyclic AMP or cyclic GMP content in endothelial cells cultured from bovine and pig aortae and human umbilical vein (Goldman et al., 1983; Brotherton, 1986; Leitman & Murad, 1986; Martin et al., 1988b; Schini et al., 1988; Legrand et al., 1989; 1990). These studies demonstrate that soluble and particulate guanylate cyclase and adenylate cyclase are present in vascular endothelial cells.

Several vascular responses have been shown to be under the influence of cyclic nucleotides, for example, elevation of cyclic AMP and cyclic GMP content may stimulate angiotensin converting enzyme activity (Krulewitz & Fanburg, 1986), and thromboxane A_2 synthesis (Fuller & Worthington, 1984), respectively. An early report described inhibition of prostacyclin production resulting from elevation of cyclic AMP content following treatment with the phosphodiesterase inhibitor, IBMX (Brotherton & Hoak, 1982). This conclusion has been revised, however, since subsequent work indicated that the IBMX- induced inhibition of prostacyclin

production was the result of a cyclic AMP- independent action of the inhibitor (Brotherton et al., 1982). The elevation of cyclic AMP content induced by prostacyclin in endothelial cells has been demonstrated to inhibit the release of EDRF (Shimokawa et al., 1988).

Furthermore, the elevation of cyclic GMP content by atrial natriuretic factor or by the membrane permeant analogue of cyclic GMP, 8 bromo cyclic GMP, has been demonstrated to inhibit agonist- induced release of EDRF from rabbit aorta (Evans et al., 1988; Hogan et al., 1989) and from cultured bovine aortic endothelial cells (Busse et al., 1988). Recent studies indicate that the inhibitory effect of cyclic GMP on EDRF release may be mediated through inhibition of calcium fluxes and IP_3 formation in response to EDRF releasing agents (Rapoport, 1986; Lang & Lewis, 1991).

The control of vascular permeability has also been demonstrated to be under the influence of cyclic nucleotides. Elevation of cyclic AMP content has an inhibitory action on the transfer of albumin across monolayers of endothelial cells cultured from pig aortic (Gudgeon & Martin, 1989), human umbilical vein (Yamada et al., 1990) and bovine pulmonary artery (Stelzner et al., 1989). There are, however, conflicting reports on the ability of cyclic GMP to inhibit permeability. Elevation of cyclic GMP has no effect on albumin transfer across monolayers of pig aortic endothelial cells (Gudgeon & Martin, 1989), but inhibits transfer across human umbilical vein endothelial cells (Yamada et al., 1990), respectively. It has been suggested that cyclic AMP and

cyclic GMP mediate their effects by the phosphorylation of cytoskeletal proteins via cyclic AMP- or cyclic GMP- dependent protein kinases (Stelzner et al., 1989; Yamada et al., 1990).

In view of these important intracellular actions of cyclic nucleotides, it is essential to understand how their activities are regulated. Cyclic nucleotides are known to be inactivated by phosphodiesterase (PDE) enzymes (Thompson & Appleman, 1971), but until recently little was known of the properties of PDE subtypes in vascular endothelial cells.

7.1.1. Purification and characterization of PDE activities in pig aortic endothelial cell homogenates

The inactivation of cyclic AMP and cyclic GMP is catalysed by cyclic nucleotide PDE. At least five distinct isozyme families exist with more than 20 different enzymes now recognised (Beavo & Reifsnyder, 1990). The five families are as follows: calcium/ calmodulin- dependent PDE (Type I), cyclic GMP- stimulated PDE (Type II), cyclic GMP- inhibited PDE (Type III), cyclic AMP- specific PDE (Type IV) and a cyclic GMP- specific PDE (Type V). These isozymes are found to be differentially expressed and regulated in different tissues and cell types. For example, the calcium/ calmodulin- dependent PDE (Type I) is located at high concentrations in the dendrites of Purkinje cells and cortical pyramidal cells of the rat brain (Kincaid et al., 1987), whereas the cyclic GMP-. specific PDE (Type V) is most abundant form in the retina (Hurwitz et al., 1985).

The following study of the PDE subtypes in pig aortic endothelial

cell homogenates was carried out in collaboration with Dr. J. E. Souness, Rhone- Poulenc Ltd., in his Dagenham laboratory.

The hydrolytic activity of the particulate and soluble fraction from PAEC homogenates was examined. It was found that both contained equal hydrolytic activity for cyclic AMP and cyclic GMP. Furthermore, their activities were not influenced by the addition of calcium (2mM)/ calmodulin (10 units/ ml). This indicates that the calcium/ calmodulin- dependent PDE (Type I) is absent from endothelial cells. The purification and characterization of the PDE subtypes present in PAEC was carried out by applying the supernantant (soluble) fraction of PAEC homogenates to a DEAE- Trisacyl chromatography.

The first peak of PDE activity eluted hydrolysed both cyclic AMP and cyclic GMP. Upon addition of cyclic GMP (1μM), the hydrolysis of cyclic AMP was stimulated two- fold, thus indicating that this first isozyme is allosterically regulated by cyclic GMP. Furthermore, as observed with crude homogenates, addition of calcium (2mM) and calmodulin (0.5 unit/ ml) did not augment the hydrolysis of cyclic GMP. The first peak was therefore characterized as Type II according to the criteria of Beavo & Reifsnyder (1990).

The second peak eluted was a PDE which selectively hydrolysed cyclic AMP and was neither stimulated nor inhibited by cyclic GMP. Again addition of calcium (2mM) and calmodulin (0.5 units/ ml) did not augment the hydrolysis of cyclic AMP. This enzyme was therefore characterized as Type IV PDE according to Beavo &

Reifsnyder (1990).

PAEC therefore contain two PDE isozymes, a cyclic AMP- specific PDE (Type IV) and a cyclic GMP- stimulated PDE (Type II). These findings are in agreement with those of Lugnier & Schini (1990) who demonstrated the presence of the same two PDE activites in cultured bovine aortic endothelial cells. One small difference between these two studies was that for bovine aortic endothelial cell homogenates, the majority of the hydrolytic activity for both cyclic nucleotides was found in the cytosolic fraction (more than 80%), whereas in our study, the percentages for soluble and particulate were 58% and 42%, respectively.

7.1.2. Selectivity of phosphodiesterase inhibitors on the cyclic GMP- stimulated PDE and on the cyclic AMP- specific PDE

An examination of the selectivity of various inhibitors on the isozymes present in PAEC homogenates was also carried out in collaboration with Dr. J. E. Souness, Rhone- Poulenc Ltd., in his Dagenham laboratory.

It was found that M & B 22948, a selective inhibitor of Type 1 PDE in smooth muscle, was a weak inhibitor of both PDE isozymes. Similiarly, SK & F 94120, a selective inhibitor of the cylic GMP-inhibited PDE (Type III), also exhibited only weak inhibitory activity against the two PDE isozymes. It was found, however, that dipyridamole and trequinsin, two non- selective inhibitors, potently inhibited both isozymes whereas, rolipram was found to selectively inhibit the cyclic AMP- specific PDE. These observations were similiar to those reported in cultured bovine aortic

endothelial cells homogenates (Lugnier & Schini, 1990). In there, M & B 22948 was a weak inhibitor of both isozymes, trequinsin and dipyridamole potently inhibited both isozymes, and rolipram was found a selective inhibitor of the cyclic AMP- specific PDE.

The first objective of this project was to investigate the functional roles of each of the two PDE isozymes in regulating the cellular cyclic AMP and cyclic GMP content. This was attempted by examining the effects of the inhibitors, dipyridamole, trequinsin and rolipram, on basal and agonist stimulated content of cyclic AMP and cyclic GMP. All these and subsequent experiments were carried out in Glasgow.

7.1.3. Cyclic GMP content of PAEC

It was found that primary cultures of pig aortic endothelial cells had a basal cyclic GMP content of 59.1 ± 5.7 fmol µg DNA^{-1} (n=34). Furthermore, haemoglobin and L- NMMA, which selectively block the activation of soluble guanylate cyclase by binding to EDRF (Martin *et al.*, 1985) and by inhibiting EDRF synthesis (Palmer *et al.*, 1988a; Rees *et al.*, 1989; 1990), respectively, were found to reduce the basal intracellular level of cyclic GMP. L- canavanine, which inhibits nitric oxide synthase in the macrophage (Hibbs *et al.*, 1987a) has been proposed to inhibit the synthesis of EDRF in rabbit aorta (Schmidt *et al.*, 1988), but this has been disputed (Rees *et al.*, 1989). L- canavanine did not, however, reduce the basal intracellular level of cyclic GMP content of PAEC. These observations suggest that the basal intracellular level of cyclic GMP is determined by the spontaneous production of EDRF by the endothelial cells

themselves. This finding is consistent with previous studies demonstrating that in the isolated aorta of the rat and rabbit, the basal level of cyclic GMP is related to spontaneous production of EDRF by the endothelial cells (Rapoport & Murad, 1983; Martin et al., 1986a; 1986b).

In PAEC bradykinin increased the intracellular cyclic GMP content through stimulation of EDRF production as previously described (Martin et al., 1988b). This increase was abolished by pretreating the cells with either haemoglobin or L- NMMA, but L-canavanine had no effect. From these findings it is clear that synthesis of EDRF determines the cyclic GMP content of PAEC, and this is inhibited by L-NMMA but not L- canavanine.

7.1.4. Role of the cyclic GMP- stimulated PDE in PAEC

It was likely that the cyclic GMP- stimulated PDE present in PAEC played an important regulatory role in controlling cyclic GMP content, since it was the one capable of hydrolysing this cyclic nucleotide.

In keeping with this, the non- selective inhibitor, dipyridamole, which had previously been found to inhibit the cyclic GMP-stimulated PDE in cell homogenates, induced a large increase in intracellular cyclic GMP content. This peaked within 5 to 10 minutes and then declined rapidly but remained elevated above control levels for a prolonged period. Several recent studies have indicated that efflux of cyclic nucleotides from cells might act together with phosphodiesterase enzymes to regulate cellular cyclic nucleotide content (Goldman et al., 1983; Schini et al.,

1989). Dipyridamole is known to block the actions of the nucleoside transporter protein in numerous cell types, including erythrocytes (Plagemann & Waffendin, 1988), endothelial cells (Pearson et al., 1978) and sarcoma 180 cells (Cabral et al., 1984). It was possible therefore that dipyridamole caused an elevation of cyclic GMP content in PAEC by blocking the efflux of this molecule from the cell and not by inhibiting the cyclic GMP-stimulated PDE. This was not the case, however. In untreated cells there was a small steady leakage of cyclic GMP into the bathing medium with time. Rather than blocking this leak, as would have been expected if the nucleoside transporter was blocked, dipyridamole elevated the cyclic GMP content of the bathing medium. Furthermore, this increase occured only after the intracellular increase had been established.

Thus, it is almost certain that dipyridamole increases intracellular cyclic GMP content by inhibiting the cyclic GMP-stimulated PDE and not the efflux of the cyclic nucleotide from the cells. The magnitude of the rise in cyclic GMP content stimulated by dipyridamole suggests a rapid intracellular hydrolysis of this cyclic nucleotide in PAEC. The elevation of cyclic GMP content was likely to have resulted from the activation of soluble guanylate cyclase by EDRF since both the rises in intracellular and extracellular content were abolished following pretreatment with haemoglobin.

Trequinsin, which had previously been found to inhibit the cyclic GMP- stimulated PDE in cell homogenates was also observed to elevate intracellular cyclic GMP content. This peaked after 15

minutes, fell thereafter, but remained elevated above basal levels for a prolonged period. This increase, like that induced by dipyridamole, was abolished by pretreatment with haemoglobin.

It is clear therefore that in PAEC the rises in cyclic GMP content following treatment with the PDE inhibitors, dipyridamole and trequinsin, were dependent upon the activation of soluble guanylate cyclase by EDRF synthesised by the endothelial cells themselves. Rolipram, which in cell homogenates had previously been found to inhibit only the cyclic AMP- specific PDE, had no effect on the intracellular cyclic GMP content. This, as expected, confirms that the cyclic AMP- specific PDE has no role in regulating the intracellular cyclic GMP content of PAEC.

The time course of the rise in cyclic GMP content stimulated by dipyridamole or trequinsin was complex: it reached a peak then fell to a plateau after roughly 15 minutes. It was possible that the inhibitors, dipyridamole and trequinsin, had a short duration of action, but as will be seen later, this was not the case. An alternative explanation, however, is that high intracellular concentrations of cyclic GMP might potentially inhibit the production of EDRF. Recent studies have demonstrated that atrial natriuretic factor and 8 bromo cyclic GMP,each reduce agonist-induced release of EDRF in the rabbit aorta and ear artery (Busse et al., 1988; Evans et al., 1988; Hogan et al, 1989). These observations suggest the presence of a negative feedback mechanism controlling the production of EDRF.

7.1.5. Role of the cyclic GMP- stimulated PDE in regulating

agonist- induced increases in cyclic GMP content in PAEC

Nitrovasodilators stimulate soluble guanylate cyclase through production of nitric oxide (Arnold et al., 1977; Katsuki et al., 1977; Craven & De Rubertis, 1978). These agents induce increases in the intracellular cyclic GMP content of endothelial cells cultured from a variety of sources including pig aorta and human umbilical vein (Brotherton, 1986; Martin et al., 1988b). The increases in intracellular cyclic GMP content stimulated by glyceryl trinitrate and sodium azide are blocked following pretreatment with either methylene blue or haemoglobin, two agents known to inhibit the stimulation of soluble but not particulate guanylate cyclase by EDRF (Martin et al., 1988b). In contrast, in bovine aortic endothelial cells, sodium nitroprusside induced only a small increase in cyclic GMP content (Schini et al., 1988). These workers suggested that the observed differences may be related to species variation, vascular origin of cells or time spent in culture. It has been shown that endothelial cells responsiveness declines with time spent in culture (Ager & Martin, 1983; Pearson et al., 1983; Needham et al., 1987).

In the present study, sodium nitroprusside was found to increase both the intracellular and extracellular (i.e. the Kreb's bathing medium) content of cyclic GMP. Examining the effects of inhibiting the cyclic GMP- stimulated PDE with dipyridamole on the sodium nitroprusside- induced rise in cyclic GMP content was complicated by the finding that the PDE inhibitor itself stimulated an increase. This, as described previously, peaked within a few minutes then fell to a lower sustained level after

around 35 minutes, possibly through a negative feedback inhibition of EDRF production. After this time, however, the PDE still appeared to be inhibited since subsequent stimulation with sodium nitroprusside resulted in augmented increases in cyclic GMP content.

The effects of inhibiting the cyclic GMP stimulated PDE were also examined on the increase in cyclic GMP content stimulated by activation of particulate guanylate cyclase. In these experiments the PAEC were pretreated with haemoglobin to prevent activation of soluble guanylate cyclase by the spontaneous produced EDRF. It was found that the atrial natriuretic factor, atriopeptin II, which activates particulate guanylate cyclase, elevated the intracellular cyclic GMP content in PAEC. The effect of atriopeptin II was concentration dependent. These findings are in agreement with those of several earlier studies describing the ability of atrial natriuretic peptides to increase the cyclic GMP content of bovine aortic, human umbilical vein and pig aortic endothelial cells (Leitman & Murad, 1986; Schini et al., 1988; Brotherton, 1986; Martin et al., 1988b). In one of these studies, it was observed that the atriopeptin II- induced increase in intracellular cyclic GMP content was associated with a time dependent efflux of this cyclic nucleotide into the bathing medium (Schini et al., 1988). This reinforces the suggestion that regulation of cyclic nucleotide content of endothelial cells is controlled by a combination of hydrolysis and efflux (Schini et al., 1989).

It was found that the non- selective PDE inhibitors,

dipyridamole and trequinsin, each enhanced the ability of atriopeptin II to increase the intracellular cyclic GMP content of PAEC. In contrast, the selective inhibitor of the cyclic AMP-specific PDE, rolipram, had no effect on the atriopeptin II-induced increase in intracellular cyclic GMP content.

These findings show that in PAEC the cyclic GMP- stimulated PDE controls basal cyclic GMP content and following stimulation of either soluble or particulate guanylate cyclase. They further show that the other enzyme present in PAEC, the cyclic AMP-specific PDE has no role in regulating either the basal or agonist- stimulated increases in cyclic GMP content.

7.1.6. Role of the cyclic AMP- specific PDE in PAEC

Our experiments with cell homogenates showed that both PDE isozymes present in PAEC, the cyclic AMP- specific PDE and the cyclic GMP- stimulated PDE, had the ability to hydrolyse cyclic AMP (Souness et al., 1990). The importance of the cyclic AMP-specific PDE (Type IV) in regulating the cyclic AMP content of intact PAEC was investigated by examining the effects of the selective inhibitor of this enzyme, rolipram.

The activator of adenylate cyclase, forskolin (Seaman & Daly, 1981), was found to have no effect on the intracellular cyclic AMP content at a concentration of 10μM but at 30μM, an elevation was observed. Rolipram elevated the intracellular cyclic AMP content by itself and enhanced the forskolin- induced increase. This result is in agreement with previous studies reporting the ability of forskolin and prostacyclin to elevate endothelial

cyclic AMP content and of cyclic AMP PDE inhibitors to enhance these rises (Leitman et al., 1986; Martin et al., 1988b). These findings show that the cyclic AMP- specific PDE regulates basal and agonist- stimulated cyclic AMP content in PAEC.

7.1.7. Role of the cyclic GMP- stimulated PDE in regulating cyclic AMP content in PAEC

Our studies with cell homogenates showed that cyclic AMP could be hydrolysed by the cyclic GMP- stimulated PDE (Souness et al., 1990). It was possible, therefore, that this PDE acted together with the cyclic AMP- specific PDE to control cyclic AMP hydrolysis in PAEC. This was difficult to determine, however, since no selective inhibitor of this PDE isozyme was available.

The non- selective inhibitor, dipyridamole, which inhibits both the cyclic GMP- stimulated PDE and cyclic AMP- specific PDE in cell homogenates, was found to have no effect on the intracellular cyclic AMP content at a concentration of 25μM, but at 100μM, an elevation was observed. Furthermore, dipyridamole (25μM and 100μM) was found to enhance isoprenaline- (a β- adrenoceptor agonist) and forskolin- induced increases in intracellular cyclic AMP content. Whether these rises occurred by inhibition of the cyclic GMP- stimulated PDE, the cyclic AMP- specific PDE, or both could not be determined.

7.1.8. Conclusion

In conclusion, pig aortic endothelial cells contain two PDE activities, a cyclic GMP- stimulated PDE (Type II) and a cyclic AMP- specific PDE (Type IV). The cyclic GMP- stimulated PDE

isozyme actively participates in the regulation of the intracellular content of cyclic GMP under basal conditions and following stimulation of both soluble and particulate guanylate cyclase. The cyclic AMP- specific PDE participates only in the regulation of the intracellular cyclic AMP content, since it lacks the ability to hydrolyse cyclic GMP. The cyclic GMP- stimulated PDE might have an additional role in regulating the cyclic AMP content, but definite proof of this must await the development of a selective inhibitor of this enzyme.

8.1. PROLIFERATION OF PAEC IN CULTURE

The objective of this part of the study were to investigate the effects of PKC activation by phorbol esters and of cyclic nucleotides on the proliferation of pig aortic endothelial cells in culture.

8.1.1. Effects of PKC activation on the proliferation of endothelial cells

The calcium- phospholipid dependent protein kinase, PKC, has been shown to play a role in signal transduction during several cellular responses including proliferation (Nishizuka, 1986). Basic FGF is a potent mitogen for endothelial cells and its ability to activate PKC has been previously demonstrated in many cell types. In Swiss 3T3 fibroblasts, induction of diacylgycerol formation and PKC activation was observed during the mitogenic response to basic FGF (Tsuda et al., 1985). In contrast, basic FGF was found to be mitogenic via a PKC- independent pathway in hamster fibroblasts (Magnaldo et al., 1986). This was shown to be the case since basic FGF lacked the ability to induce accumulation of inositol trisphosphate and activate PKC.

Recently, however, it has been reported that the mitogenic activity of basic FGF and of a structurally related mitogen, human hepatoma- derived growth factor (Klagsbrun et al., 1986; Presta et al., 1986), in endothelial cells is, at least in part, related to their ability to activate PKC. The response to these growth factors was blocked following pretreatment either with phorbol esters, which initially stimulate then down- regulate PKC, or a PKC inhibitor. This was seen in several endothelial cell types

including bovine cerebral cortex capillary (Daviet et al., 1990), bovine adrenal capillary (Doctrow & Folkman, 1987) and in a transformed foetal bovine aortic endothelial cell line (Presta et al., 1989a).

The importance of PKC activation in the proliferation of endothelial cells has been further elucidated by the use of phorbol esters. They have been shown to have differing actions on the proliferation of endothelial cells from different species and vascular origin. An inhibition of proliferation has been reported for endothelial cells obtained from human aorta (Hoshi et al., 1988b) and bovine adrenal capillary (Doctrow & Folkman, 1987). In contrast, a stimulation of proliferation has been observed in endothelial cells obtained from bovine cerebral cortex capillary (Daviet et al., 1989; 1990) and in a transformed foetal bovine aortic cell line (Presta et al., 1989a). No effect was observed on proliferation of endothelial cells obtained from foetal bovine aorta (Presta et al., 1989a), human omental microvasculature (Dupuy et al., 1989), bovine aorta (Doctrow & Folkman, 1987) and bovine adrenal capillary (Morris et al., 1988). Activation of PKC, therefore, plays an important role in the mitogenic response of endothelial cells.

8.1.2. Effect of PKC activation by PMA on the proliferation of PAEC as assessed by haemocytometry

In view of the above conflicting reports on the effects of phorbol esters on proliferation of endothelial cells, the effects of phorbol 12-myristate 13-acetate (PMA) was examined on PAEC. It was found that PMA significantly inhibited the proliferation of

PAEC in serum- containing medium over a period of 1 to 8 days. This finding is therefore in agreement with those of Hoshi et al. (1988b) and Doctrow & Folkman (1987). The inhibition of growth is unlikely to have resulted from a non- selective cytotoxic action since plating efficiency and cell viability, as assessed by uptake of the vital stain trypan blue was unaffected by PMA. This is in agreement with previous reports demonstrating that phorbol esters have no effect on either plating efficiency or viability of endothelial cells (Doctrow & Folkman, 1987; Hoshi et al., 1988b). The inhibition of proliferation by PMA was observed to be concentration- dependent and appeared to be due to the activation of PKC since the inactive phorbol ester, 4α- phorbol- 12,13- didecanoate, lacked antiproliferative activity.

The activation of PKC by PMA was further investiagted by examining the effects of a PKC inhibitor, staurosporine (Tamaoki et al., 1986). It was found that staurosporine inhibited serum- induced proliferation of PAEC throughout a 8 day period, but, failed to block the antiproliferative effects of PMA.

Recent studies have indicated the presence of multiple PKC isozymes in many cell types (Coussens et al., 1986; Nishizuka, 1988; Farago & Nishizuka, 1990). Different PKC isozymes have also been identified in PAEC (Uratsuji & DiCorleto, 1988). Why staurosporine blocks the serum- induced proliferation of PAEC is not clear. It is possible that it inhibits proliferation of PAEC by blocking the activation of a PKC isozyme(s) involved in the serum- induced mitogenic response. Alternatively, as staurosporine has been shown to be a non- selective inhibitor it may be

acting by a PKC- independent mechanism. For example, staurosporine inhibits not only PKC but also several other protein kinases, including tyrosine protein kinase, cyclic AMP- dependent protein kinase and calcium/ calmodulin- dependent protein kinase (Rüegg & Burgess, 1989).

The failure of staurosporine to block the antiproliferative activity of PMA in PAEC is surprising. If PMA does act by stimulating PKC, as seems likely from the lack of effect of the inactive phorbol ester, 4α- phorbol- 12,13- didecanoate, it is necessary to propose that PMA activates PKC isozyme(s) that is insensitive to blockade by staurosporine. There are, however, no other reports of the effects of PKC inhibitors on the antiproliferative effects of PMA on endothelial cells.

It has been shown, however, that in endothelial cell types where phorbol esters stimulate proliferation that staurosporine and another inhibitor, H-7, each block this stimulation. This has been seen in transformed foetal bovine aortic (Presta et al., 1989a) and bovine cerebral cortex capillary (Daviet et al., 1989; 1990) endothelial cells. It is possible, therefore, that where phorbol esters inhibit proliferation, this occurs by stimulation of a subtype(s) of PKC that is insensitive to staurosporine whereas when stimulation of proliferation is seen, this occurs by stimulation of an isozyme(s) that is sensitive to inhibition.

8.1.3. Effect of PMA on proliferation of PAEC as assessed by [^{3}H]-thymidine incorporation

In a study of the effects of PMA on the proliferation of PAEC, as

assessed by [^{3}H]-thymidine incorporation into DNA, an early stimulation of proliferation was observed followed by an inhibition (Uratsuji & DiCorleto, 1988). This dualistic action was considered by these authors to reflect the ability of phorbol esters to stimulate then down- regulate PKC. In the experiments discussed thus far where cell numbers was the index of proliferation, no early stimulation of proliferation was observed following treatment of PAEC with PMA. This lack of an early stimulation might have resulted because within the first time point examined, (48 hours), there had been a complete down- regulation and therefore disappearance of PKC activity. Uratsuji & DiCorleto (1988) had previously demonstrated a complete disappearance of PKC activity following treatment of PAEC for 24 hours with PMA (200nM). Measurement of [^{3}H]-thymidine incorporation represents a more sensitive index of proliferation, so experiments were repeated using this technique to see if an early stimulation was observed with PMA in PAEC.

It was found that serum- induced stimulation of [^{3}H]-thymidine incorporation by PAEC was concentration- dependent. Moreover, it was demonstrated that PMA significantly reduced [^{3}H]-thymidine incorporation in 0%, 4% and 20% serum- containing medium after an 18 hour incubation period. Even at the earlier time points examined (4, 8 and 12 hours), PMA was found to induce only inhibition of [^{3}H]-thymidine incorporation into PAEC with no stimulatory phase.

The PKC inhibitor, staurosporine (10nM), was found to have no effect on serum- induced incorporation of [^{3}H]-thymidine into

PAEC after an 18 hour incubation period, but at the higher concentration of 100nM, an inhibition of incorporation was observed. This contrasts slightly with the results from the haemocytometric experiments where staurosporine (10nM) inhibited serum- induced proliferation of PAEC over a period of 4 to 8 days. The possibility that this discrepancy resulted from the different incubation periods employed in the two experiments is supported by a recent report demonstrating that staurosporine inhibits the proliferation of rabbit aortic smooth muscle cells only after a 24 hour incubation period (Matsumoto et al., 1989).

In agreement with the earlier haemocytometric experiments, staurosporine at both concentrations (10 and 100nM) was without effect on the PMA- induced inhibition of [^{3}H]-thymidine incorporation by PAEC over the range of serum concentrations (1%, 4% and 20%). This as has already been discussed, may be explained by PMA inhibiting proliferation by activating an isozyme of PKC that is insenstive to blockade by staurosporine. It is not clear why the early stimulation of [^{3}H]-thymidine incorporation reported previously (Uratsuji & DiCorleto, 1988) could not be reproduced in this study despite experimental conditions being similiar. In this study, passage 1 PAEC only were used, whereas in the earlier study, PAEC between passage 4 and 13 were used. No other explanation for the differences reported in the two studies appears obvious.

These results, utilising two independent methods (haemocytometry and [^{3}H]-thymidine incorporation), suggest that activation of PKC powerfully inhibits the proliferation of PAEC.

8.1.4. Possible mechanisms by which phorbol esters mediate their antiproliferative action on PAEC

It is generally accepted that PKC exerts its actions on proliferation through changes in the phosphorylation state of proteins (reviewed in Takai et al., 1985; Nishizuka, 1986). This leads subsequently to altered expression of growth factors and oncogenes (Bikfalvi et al., 1990; Murphy et al., 1988; Blackshear, 1988; Colotta et al., 1988; Reuse et al., 1990) and the down- regulation of growth factor receptors (Hoshi et al., 1988b). The possibility that PMA inhibits proliferation of PAEC by alternative mechanisms was considered.

8.1.5. Oxygen- derived free radicals

PMA is known to stimulate the production of destructive, oxygen-derived free radicals by endothelial cells (Matsubara & Ziff, 1986), and these could potentially contribute to the antiproliferative action. In keeping with this possibility, paraquat, another agent known to generate free radicals (Minakami et al., 1990) was found to inhibit proliferation of PAEC in a concentration- dependent manner. At the highest concentration examined of 1mM, the antiproliferative effect was associated with detachment of cells and uptake of the vital stain, trypan blue. This is in contrast to the actions of PMA where inhibition of proliferation occurred with no evidence of cytotoxicity.

Furthermore, combined treatment with two agents which act extracellularly to remove superoxide anions and hydrogen peroxide, superoxide dismutase and catalase, respectively, had no effect on proliferation or the antiproliferative actions of PMA or

paraquat. The possibility that both PMA and paraquat mediate their actions by the intracellular generation of radicals which cannot be removed by exogenous superoxide dismutase and catalase was considered. An earlier study had already indicated that the antiproliferative action of paraquat on E. coli was mediated via intracellular generation and this was unaffected by superoxide dismutase and catalase (Minakami et al., 1990). It was found in PAEC that two lipid soluble agents known to act as intracellular radical scavangers, vitamin E and BHT, each had no effect by themselves on proliferation or on the antiproliferative action of PMA, but both reversed the antiproliferative action of paraquat. This demonstrates that the intracellular production of oxygen-derived free radicals is responsible for the antiproliferative actions of paraquat in PAEC. In contrast, it is unlikely that the generation of oxygen- derived free radicals contributes significantly to the ability of PMA to inhibit proliferation of PAEC.

8.1.6. Cyclic nucleotides

An interaction between phorbol esters and cyclic nucleotides has been previously reported. In an interleukin- dependent T cell line, agents which elevate the cellular cyclic AMP content inhibit phorbol 12,13- dibutyrate- induced growth (Goto et al., 1988). Furthermore, PMA- induced contraction of vascular smooth muscle in the rabbit ear artery is reversed by elevation of cyclic AMP content (Rasmussen et al., 1984). However, it was found that neither of the membrane permeant analogues, dibutyryl cyclic AMP and 8 bromo cyclic GMP, had any effect on the ability of PMA to inhibit the proliferation of PAEC. Using the more sensitive index of proliferation, dibutyryl cyclic AMP had no

effect by itself, and was found to be without effect on the PMA-induced inhibition of [^{3}H]-thymidine incorporation in these cells.

These findings show that neither the production of oxygen- derived free radicals nor an interaction with cyclic nucleotides is likely to contribute to the antiproliferative actions of PMA on PAEC. It is likely that the antiproliferative action of PMA on PAEC is mediated by the phosphorylation of unidentified proteins.

8.2. EFFECTS OF CYCLIC NUCLEOTIDES ON CELLULAR PROLIFERATION

Cyclic nucleotides have been shown to act as intracellular messengers in the regulation of proliferation of a variety of cell types (Friedman, 1981). Cyclic AMP has been found to have either a growth inhibitory or growth stimulatory action: inhibition of proliferation was observed with BHK 21/13 hamster cells, HeLa cells, human normal fibroblasts, rat thymic- and B- lymphocytes (Bürk, 1968; Ryan & Heidrick, 1968; MacManus & Whitfield, 1969; Hollenburg & Cuatrecasas, 1975; Muraguchi et al., 1984), whereas stimulation of proliferation was observed in mammary epithelial cells, hepatocytes and Swiss 3T3 fibroblasts (Yang et al., 1980; McGowan et al., 1981; Rozengurt et al., 1983b; O'Neill et al., 1985). The effects of cyclic AMP on cellular proliferation are therefore strictly dependent on the cell type being examined.

8.2.1. Effect of elevated cyclic AMP content on the proliferation of PAEC

This study showed that when cell numbers were counted, the membrane permeant analogue of cyclic AMP, dibutyryl cyclic AMP,

profoundly inhibited the proliferation of PAEC in serum- containing medium during an extended period in culture (8 days). In contrast, in shorter experiments (18 hours) where [^{3}H]-thymidine incorporation was measured, dibutyryl cyclic AMP apparently failed to inhibit proliferation. It is possible that this discrepancy results from the different incubation periods employed in the two experiments.

The finding that dibutyryl cyclic AMP inhibits proliferation is in agreement with Leitman et al. (1986) who found a similiar inhibition of proliferation on bovine aortic endothelial cells. Furthermore, Leitman et al. (1986) demonstrated that the inhibition of proliferation of bovine aortic endothelial cells did not result from a cytotoxic action since treatment with forskolin, cyclic AMP PDE inhibitors or dibutyryl cyclic AMP did not increase the release of lactate dehydrogenase.

Elevation of cyclic AMP content does not, however, inhibit proliferation of all endothelial cell types. In bovine coronary microvascular (Meininger & Granger, 1990), foetal bovine aortic (Presta et al., 1989a) and human dermal microvascular (Davidson & Karasek, 1981) endothelial cells, elevation of cyclic AMP content has been shown to stimulate proliferation.

8.2.2. Effect of elevated cyclic GMP content on the proliferation of PAEC

Unlike for cyclic AMP, the effects of cyclic GMP on cellular proliferation are less well documented. It has been reported, however, that elevated levels of cyclic GMP inhibit the prolif-

eration of rat glomerular mesangial cells in culture (Johnson et al., 1988). Furthermore, one previous study found that membrane permeant analogues of cyclic GMP have only a slight inhibitory effect on the proliferation of bovine aortic endothelial cells (Leitman et al., 1986).

Endothelial cells contain both soluble and particulate forms of guanylate cyclase (Brotherton, 1986; Leitman & Murad, 1986; Martin et al., 1988b; Schini et al., 1988). In endothelial cells, as in other cell types (Arnold et al., 1977; Katsuki et al., 1977), the soluble form of guanylate cyclase is activated by nitrovasodilators, such as sodium nitroprusside (Brotherton, 1986; Schini et al., 1988) or glyceryl trinitrate (Martin et al., 1988b) or even by the endothelial cell's own EDRF (Martin et al., 1988b) and the particulate form can be stimulated by atrial natriuretic factors (Brotherton, 1986; Leitman & Murad, 1986; Martin et al., 1988b; Schini et al., 1988).

In this study it was found that stimulation of soluble guanylate cyclase by glyceryl trinitrate, sodium nitroprusside or bradykinin, which stimulates EDRF production, had no effect on proliferation of PAEC in culture. Activation of particulate guanylate cyclase by the atrial natriuretic peptide, atriopeptin II also had no effect on the proliferation of PAEC. Furthermore, the membrane permeant analogue of cyclic GMP, 8 bromo cyclic GMP, was also without effect on proliferation of PAEC. These observations initially suggested that cyclic GMP had no effect on the proliferation of PAEC.

this turned out, however, not to be the case, since haemoglobin and methylene blue, two agents known to lower endothelial cell cyclic GMP content by inhibiting the ability of spontaneously released EDRF to stimulate soluble guanylate cyclase (Martin et al., 1985; 1988b), each inhibited the proliferation of PAEC in serum- containing medium in culture.

In the case of haemoglobin, the inhibition was of the order of 20- 40 % and was specific, since it was reversed by the addition of agents which elevate intracellular cyclic GMP content i.e. sodium nitroprusside, atriopeptin II or the membrane permeant analogue, 8 bromo cyclic GMP. The ability of these agents to reverse the antiproliferative effect of haemoglobin, but to have no stimulating effects in the absence of haemoglobin is difficult to explain. One possible explanation is that a certain level of cyclic GMP, generated by the endogenous production of EDRF, is required to exert an all-or-none permissive action on cell growth. According to this scheme, elevating cyclic GMP content further cannot increase the permissive action. Only by reducing the cyclic GMP content e.g. by using haemoglobin can the permissive action be inferred. Further work is required, however, to test this hypothesis.

In contrast to haemoglobin, the inhibitory effect of methylene blue on proliferation was not reversed by sodium nitroprusside, atriopeptin II or 8 bromo cyclic GMP. It is likely, therefore, to reflect an action unrelated to guanylate cyclase inhibition. It is possible that the antiproliferative action of methylene blue on PAEC is due to its ability to produce oxygen- derived free

radicals (Marshall et al., 1988). This explanation would be consistent with the earlier observations in this study that paraquat, an agent known to generate free radicals, potently inhibits the proliferation of PAEC.

EDRF is now recognised to be nitric oxide (Palmer et al., 1987), and its biosynthetic pathway has been identified. L- arginine is the physiological precursor from which EDRF is produced by the action of the enzyme, nitric oxide synthase (Palmer et al., 1988a; Schmidt et al., 1988; Rees et al., 1989). The L- arginine analogue, N^G- monomethyl L- arginine (L- NMMA) inhibits the synthesis of nitric oxide by inhibiting the enzyme, nitric oxide synthase, but its enantiomer, D- NMMA is completely inactive (Rees et al., 1989; 1990).

In this study the possibility that EDRF exerts a permissive role in controlling proliferation of PAEC was investigated by examining the actions of L- NMMA on proliferation. It was found, however, that although L- NMMA did inhibit proliferation of PAEC in culture, this property was one shared with the inactive D- isomer, D- NMMA. This would suggest that both L- NMMA and D- NMMA exerts their antiproliferative actions on PAEC via a mechanism independent of EDRF.

In conclusion, the inhibitory effect of haemoglobin and its reversal following elevation of cyclic GMP content suggests that EDRF exerts a permissive action in regulating the proliferation of PAEC in culture through stimulation of soluble guanylate cyclase. It remains to be determined whether or not EDRF exerts

such a permissive effect on proliferation of endothelial cells from all species and vascular sites.

It has been observed that there is reduced release of EDRF by aortas from humans and animals in atherosclerosis (Guerra et al., 1989). This impaired release of EDRF may augment the development of the atherosclerotic lesion by several mechanisms. Firstly, EDRF has been shown to possess anti- thrombotic properties (Radomski et al., 1987a; 1987b; 1987c; Hawkins et al., 1988; Sneddon & Vane, 1988). Its loss might, therefore, promote the aggregation and adhesion of platelets to the vessel wall. The localised release of platelet mitogens, including PDGF, could therefore stimulate the migration and proliferation of the underlying smooth muscle cells. Secondly, EDRF has been proposed to inhibit smooth muscle proliferation (Garg & Hassid, 1989), and so reduced production of this agent might lead to enhanced proliferation. Thirdly, as already discussed, EDRF may play a permissive role in controlling endothelial cell proliferation. Reduced production of EDRF in atherosclerosis might reduce the rate of re- endothelialization of denuded or damaged areas. This would permit greater adhesion of platelets to the exposed underlying collagen.

8.2.3. Role of PDE isozymes in regulating proliferation of PAEC

Two PDE isozymes, a cyclic GMP- stimulated PDE (Type II) and a cyclic AMP- specific PDE (Type IV), are found in pig aortic endothelial cells and have been demonstrated to play a role in regulating the intracellular cyclic AMP and cyclic GMP content (Souness et al., 1990; this study). The effects of inhibitors of

these PDE isozymes on cyclic nucleotide content has been discussed earlier (Chapter 7). The effects of these inhibitors were examined on the proliferation of PAEC in culture.

It was found that the non- selective PDE inhibitor, dipyridamole, which elevates the intracellular cyclic AMP and cyclic GMP content (this study), inhibited the proliferation of pig aortic endothelial cells in serum- containing medium. Haemoglobin blocked the ability of dipyridamole to raise the cyclic GMP content by blocking the actions of EDRF (Figure 13) but did not inhibit the reduction in proliferation. It is likely, therefore, that the antiproliferative action of dipyridamole is mediated by the increase in intracellular content of cyclic AMP and not cyclic GMP. This result is in agreement with an earlier study demonstrating that elevation of cyclic AMP content by cyclic AMP PDE inhibitors inhibits the proliferation of bovine aortic endothelial cells in culture (Leitman et al., 1986).

Since dipyridamole inhibits the two PDE isozymes located in PAEC it is not possible to conclude if one or both of these enzymes has the ability to regulate the cell's growth by controlling the cyclic AMP content. To answer this definitely requires the development of selective inhibitors of each of the PDE isozymes.

8.2.4. Conclusion

In conclusion, this part of the study shows that phorbol 12-myristate 13- acetate (PMA) powerfully inhibits the proliferation of pig aortic endothelial cells in serum- containing medium. This antiproliferative action of PMA was not mediated by the stimulat-

ed production of oxygen- derived free radicals or an interaction with cyclic nucleotides, and is likely to have resulted from PKC-stimulated phosphorylation of as yet unidentified proteins. Furthermore, cyclic AMP was found to inhibit proliferation, whereas cyclic GMP was found to have a permissive role on the proliferation of pig aortic endothelial cells.

9.1. PROLIFERATION OF RAT AORTIC SMOOTH MUSCLE CELLS IN CULTURE

The objectives of this part of the study were to investigate the effects of PKC activation by phorbol esters, and of cyclic nucleotides on the proliferation of rat aortic smooth muscle cells in culture.

Smooth muscle cells located within the blood vessel wall are responsible for maintaining the vascular tone via contraction and relaxation. These cells upon examination are found to contain large amounts of thick and thin myofilaments and are described to be in the contractile phenotype (Chamley- Campbell et al., 1979). As well as maintaining vascular tone, smooth muscle cells play a role in wound repair, but abnormal migration and proliferation can lead to the development of several vascular diseases, for example, atherosclerosis. In this condition, the cells are observed to undergo a phenotypic transformation: they lose their contractile machinery and the ability to contract, and now contain large amounts of rough endoplasmic reticulum, free ribosomes and Golgi appartus. Furthermore, the cells become capable of synthesizing and secreting an extracellular matrix. All of these changes prepare the cells for proliferation. This state is described as the synthetic phenotype (Chamley- Campbell et al., 1979).

Freshly isolated smooth muscle cells are observed to undergo this phenotypic transformation after 5 to 6 days in culture before they proliferate in response to serum. Cells that have undergone less than five cell doublings will return to the contractile phenotype upon reaching confluency, but if they have undergone

more than five, the cells will remain permanently in the synthetic phenotype (Chamley- Campbell et al., 1981).

9.1.1. Serum- dependent proliferation of rat ASMC in culture

Adult smooth muscle cells have been shown to secrete a PDGF- like growth factor and possess cell surface receptors to this mitogen thus making them responsive to it (Walker et al., 1986; Seifert et al., 1984; Sjöland et al., 1988). Proliferation of human aortic smooth muscle cells has been found to be dependent on the presence of PDGF (Fager et al., 1988). These cells did not grow in plasma- derived serum or serum- supplemented medium in the presence of an antiserum to PDGF (Fager et al., 1988). Furthermore, PDGF has been observed to be a potent mitogen for bovine aortic (Banskota et al., 1989) and rat aortic (Tomita et al., 1987; Takagi et al., 1988; Abell et al., 1989; Kihara et al., 1989; Majack et al., 1990) smooth muscle cells. PDGF is now regarded as the main mitogen present in serum (Seppä et al., 1982).

Upon examining the effects of serum on proliferation of rat ASMC in this study, it was found that proliferation was stimulated in a concentration- dependent manner over a period of 11 days. This result is in agreement with previous studies where an increase in serum concentration produced a corresponding increase in either cell numbers or [^{3}H]-thymidine incorporation in bovine aortic (Jonzon et al., 1985) and rat aortic (Kihara et al., 1989) smooth muscle cells. Furthermore, monkey and rat ASMC maintained in the absence of serum or in a low concentrations of serum (0.5 - 2%) will survive for a period of days but proliferate only at a

greatly reduced rate (Chamley- Campbell et al., 1979; Jonzon et al., 1985; this study). The growth dependence of smooth muscle cells on the presence of PDGF is further demonstrated by the fact that cultured smooth muscle cells lack the ability to grow in plasma- supplemented medium (Ross et al., 1974; Fager et al., 1988). This is in contrast to cultured endothelial cells which can proliferate equally well in either serum- or plasma- supplemented medium (Kazlauskas & DiCorleto, 1985). It is likely therefore that serum contains growth factors which are mitogenic for rat ASMC in culture. This not only includes PDGF (Tomita et al., 1987; Takagi et al., 1988; Abell et al., 1989; Majack et al., 1990), but other platelet- derived mitogens such as EGF (Owen, 1985; Tomita et al., 1987; Takagi et al., 1988) and TFGβ (Majack et al., 1990).

9.1.2. Effects of PKC activation on proliferation of rat ASMC

The mitogenic response to PDGF in rat and rabbit aortic smooth muscle cells was reduced significantly by pretreatment with either phorbol esters, or a PKC inhibitor, H-7 (Kariya et al., 1987a; Tagaki et al., 1988). This suggests that PKC may play a regulatory role in the response of smooth muscle cells to PDGF. Not all findings are consistent with this, however, since no reduction in PDGF- induced mitogenesis was observed in rat ASMC pretreated with another PKC inhibitor, polymixin B (Kihara et al., 1989).

The role of PKC activation in the proliferation of vascular smooth muscle cells has been further investigated by examining

the effects of phorbol esters. An inhibition of proliferation has been reported for smooth muscle cells obtained from rat aorta (Kihara et al., 1989) and rabbit aorta (Kariya et al., 1987b; Fukumoto et al., 1988). In contrast, a stimulation of proliferation has been observed for smooth muscle cells obtained from bovine pulmonary artery and aorta (Dempsey et al., 1990; Doctrow & Folkman, 1987), rat aorta (Owen, 1985; Takagi et al., 1988), and rabbit aortic (Kariya et al., 1987a). The ability of PMA to either stimulate or inhibit the proliferation of rat and rabbit ASMC was found to be dependent on the culture conditions employed (Owen, 1985; Takagi et al., 1988; Kihara et al., 1989; Kawahara et al., 1988). Inhibition of proliferation was observed when the cells were cultured in 10% whole blood serum, whereas, stimulation was observed in serum- free or plasma- derived serum- supplemented medium.

It was found in this study, however, PMA had no effect on proliferation of rat ASMC grown in either low or high concentrations of serum throughout a twelve day period. At this moment, there is no clear explanation why PMA lacked activity on rat ASMC grown in conditions that favoured inhibiton of proliferation (high serum concentrations) or stimulation of proliferation (low serum concentrations).

9.1.3. Effects of adrenoceptor activation on proliferation of rat ASMC

Circulating catecholamines have been reported to be a major risk factor in the development of atherosclerosis in animals and humans (Helin et al., 1970; Kones, 1979; Kukreja et al., 1981).

Abnormal migration and proliferation of vascular smooth muscle cells are early events occurring during the development of an atherosclerotic lesion and catecholamines are known to modulate the proliferation of vascular smooth muscle cells through interactions with specific adrenergic receptors.

Blaes & Boissel (1983), Bauch et al. (1987) and Bell & Madri (1989) reported that catecholamines had a stimulatory effect on smooth muscle cell proliferation, and that this required the presence of serum. It has been proposed that adrenoceptor activation acts synergistically with growth factors present in serum to stimulate proliferation of smooth muscle cells (Nakaki et al., 1989). Furthermore, it was reported that noradrenaline, an adrenoceptor agonist, produced a biphasic effect on proliferation of rat aortic smooth muscle cells in 10% serum- containing medium (Nakaki et al., 1989). These workers proposed that the stimulatory and inhibitory effects of noradrenaline on proliferation were mediated through different receptors, with stimulation through α_1- and inhibition through β_2- adrenoceptors. Furthermore, it was proposed that expression of the adrenergic signal- transduction systems depended on cell density and number of cell doublings: in smooth muscle cells seeded at low density, α_1- adrenoceptor stimulation predominated resulting in stimulation of proliferation, whereas in cells seeded at a higher density or in cells of high passage number, β_2- adrenoceptor stimulation predominated resulting in inhibition of proliferation. These findings may be evidence of the existence of a subtle negative feedback mechanism regulating the growth of smooth muscle cells within the arterial wall.

α_1- adrenoceptor activation has been demonstrated to stimulate the proliferation of several other cells types in culture, including 3T3 cells, bovine aortic endothelial cells and rat hepatocytes (Sherline & Mascardo, 1984; Cruise et al., 1985). It was found in the present study that the α_1- adrenoceptor agonist, phenylephrine, stimulated the proliferation of rat ASMC in 10% serum- supplemented medium. This stimulation was observed to be concentration dependent over the range of 0.1mM to 1mM and produced a maximum increase in cell numbers of 30 ± 6%. It is known that α_1- adrenergic receptor activation induces phosphatidylinositol hydrolysis (Watson & Abbott, 1989). This results in an accumulation of two second messengers, diacylglycerol, which is the endogenous activator of protein kinase C (Bell, 1986) and inositol trisphosphate, which stimulates the release of stored calcium (Putney, 1987; Rana & Hokin, 1990). Calcium influx is also stimulated through receptor- operated and voltage operated channels (Benham & Tsien, 1987). From the present study, it is impossible to state which one(s) of the second messengers are involved in the mitogenic response of phenylephrine.

Although the α_1- adrenoceptor agonist, phenylephrine, stimulated proliferation in serum- containing medium the α_2- adrenoceptor agonist, clonidine had no such effect. It is likely, therefore, that the action of phenyephrine found in this study did indeed result from activation of α_1- adrenceptors.

In this study, it was found that the non- selective β- adrenoceptor agonist, isoprenaline, was without effect on the proliferation of rat ASMC whereas the selective β_1- and β_2- adrenoceptor

agonists, dobutamine and salbutamol, respectively, both inhibited proliferation. Nakaki et al. (1989) found, however, that isoprenaline was more potent than dobutamine and salbutamol. A possible explaination for the difference in effects of isoprenaline between this study and that of Nakaki et al. (1989) may be related to the experimental protocol employed. Nakaki et al. (1989) examined the effect of isoprenaline on rat ASMC in serum- free medium by measuring [^{3}H]-thymidine incorporation over a 24 hour incubation period. In the present study, the effect of isoprenaline was examined over a period of 4 days by counting cell numbers grown in serum- supplemented medium. It is possible that within this time the inhibitory effect of isoprenaline had been masked by the growth stimulatory action of serum. Alternatively, the lack of effect of isoprenaline could have reflected its short half- life. Nevertheless, both studies agree that β_2- adrenoceptor stimulation inhibits the proliferation of rat ASMC in culture.

It is known that β- adrenoceptor activation leads to stimulation of adenylate cyclase and increases in cellular cyclic AMP content (Watson & Abbott, 1989). It is likely therefore that dobutamine and salbutamol inhibited proliferation of rat ASMC by elevating cyclic AMP content. Further evidence that cyclic AMP inhibits the proliferation of vascular smooth muscle cells is suggested by the effects of adenosine and prostaglandins.

Adenosine and certain of its analogues acting via A_1 or A_2 receptors have been shown to have a dualistic effect on the proliferation of rat aortic smooth muscle cells (Jonzon et al., 1985). It

was proposed that the stimulatory and inhibitory effects of adenosine and its analogues were mediated through different receptors, with stimulation through A_1- and inhibition through A_2- receptors, respectively (Jonzon et al., 1985). A_2- receptor activation is known to involve activation of adenylate cyclase and elevation of cyclic AMP content (Watson & Abbott, 1989), whereas A_1- receptor activation inhibits the stimulation of adenylate cyclase (Watson & Abbott, 1989). It is possible that adenosine, which is present in plasma following metabolism of ATP by many cell types (Gordon, 1986), could prevent proliferation of smooth muscle cells in the arterial wall by activation of A_2- receptors, and therefore prevent or inhibit the development of atherosclerosis.

Furthermore, several prostaglandins (PGE_1, PGD_2 and PGI_2) have been shown to inhibit serum- induced DNA synthesis in smooth muscle cells obtained from rat aorta (Nilsson & Olsson, 1984) and human aorta (Tertov et al., 1984). This inhibitory effect may be related to the ability of prostaglandins to elevate cyclic AMP content (Nilsson & Olsson, 1984). β- adrenoceptor agonists might also have potential anti- atherosclerotic actions through inhibition of smooth muscle proliferation.

9.2. EFFECTS OF CYCLIC NUCLEOTIDES ON PROLIFERATION OF RAT ASMC

9.2.1. Effects of cyclic AMP on proliferation of rat ASMC

In keeping with the concept of an inhibitory effect of cyclic AMP, it was found in the present study that the membrane permeant analogue of this cyclic nucleotide, dibuytryl cyclic AMP, and the stimulator of adenylate cyclase, forskolin (Seaman & Daly, 1981),

both profoundly inhibited the proliferation of rat ASMC in serum-containing medium. The inhibitions of proliferation induced by dibutyryl cyclic AMP or forskolin were observed to be concentration- dependent. These results are in agreement with several studies demonstrating that membrane permeant analogues of cyclic AMP, or forskolin or cyclic AMP PDE blockers inhibit the proliferation of rat brain microvascular (Kempski et al., 1987), rabbit aortic (Fukumoto et al., 1988) and human aortic (Tertov et al., 1984) smooth muscle cells in culture. The antiproliferative actions of dibutyryl cyclic AMP and forskolin in this study were found not to result from a cytotoxic effect as assessed by the vital stain, trypan blue. Furthermore, the antiproliferative action of forskolin was clearly due to activation of adenylate cyclase since the inactive forskolin analogue, dideoxy forskolin and the solvent, DMSO, both lacked the antiproliferative action of forskolin.

To further examine if the forskolin- induced inhibiton of proliferation was mediated via an elevation of cellular cyclic AMP content, the effects of two PDE inhibitors were examined. Smooth muscle cells from rat, bovine and human aortas are known to contain three PDE activities, two cyclic GMP PDEs (one continuously active [Type V], the other stimulated in the presence of calmodulin and calcium [Type I]), and a cyclic AMP- specific PDE (Type IV)(Lugnier et al., 1986; Schoeffter et al., 1987). Inhibition of the cyclic GMP PDE isozymes by M & B 22948 and of the cyclic AMP PDE by rolipram has been previously shown to result in increases in smooth muscle cell cyclic GMP and cyclic AMP content, respectively (Lugnier et al., 1986; Schoeffter et al.,

1987).

In this study, the selective cyclic AMP PDE inhibitor, rolipram, was found to inhibit proliferation of rat ASMC by itself in serum- containing medium and to potentiate the forskolin- induced inhibition of proliferation. This strengthens the concept that forskolin mediates its antiproliferative actions via an elevation of intracellular cyclic AMP content. Furthermore, M & B 22948, the selective cyclic GMP PDE inhibitor, was found to inhibit the proliferation of rat ASMC by itself in serum- containing medium. Whether this inhibition of proliferation resulted from an accumulation of cyclic GMP is not clear. However, in two seperate experiments M & B 22948 was observed to reverse the antiproliferative actions of forskolin on rat ASMC. There is no clear explanation for this action at the present time.

Histamine, an important mediator of inflammation (Killackey et al., 1986; Movat, 1987) is primarily secreted from mast cells, basophils and the platelets of certain species, and possibly from endothelial cells (Riley & West, 1966; Graham et al., 1955; Saxena et al., 1989). Histamine is known to produce relaxation of smooth muscle through H_2- receptor- linked activation of adenylate cyclase and resultant increases in intracellular cyclic AMP content (reviewed by Hill, 1990). The possibility that histamine as well as possessing vasodilator activity might have a role in tissue growth and repair has been reported. Support for this suggestion comes from several studies where the activity of histidine decarboxylase, the enzyme which synthesizes histamine from histadine, was elevated in a number of rapidly proliferating

tissues (Ishikawa et al., 1970; Watenabe et al., 1981; Bartholeyns & Fozard, 1985). Furthermore, histamine has been reported to stimulate the proliferation of human microvascular endothelium (Marks et al., 1986) and bovine aortic endothelium (Bell & Madri, 1989). The growth stimulatory effect was mediated via H_1- receptors as the H_1- antagonist, clemastine fumarate, selectively blocked this mitogenic response (Marks et al., 1986).

In the present study, it was found that histamine inhibited the proliferation of rat ASMC in serum- containing medium. This antiproliferative action of histamine was observed to be concentration- dependent and appeared due to activation of H_2- receptors since it was blocked by the H_2- antagonist, cimetidine. It is therefore possible that histamine (secreted by mast cells, basophils, platelets or endothelial cells) could play a role in inhibiting the proliferation of smooth muscle cells in the arterial wall thus preventing the development of atherosclerosis.

In summary, it is likely that the inhibiton of proliferation of rat ASMC produced by forskolin, cyclic AMP PDE inhibitors, histamine and β- adrenoceptor agonists resulted from elevation of cyclic AMP content.

9.2.2. Effects of cyclic GMP on proliferation of rat ASMC

Recent evidence has been accumulating that cyclic GMP acts as the intracellular mediator generated by several vasodilators. These include EDRF, recently identified as nitric oxide (Palmer et al., 1987), atrial natriuretic peptides and the nitrovasodilators, such as sodium nitroprusside and glyceryl trinitrate (Rapoport &

Murad, 1983; Lincoln & Fisher- Simpson, 1984; Grace et al., 1988). As discussed earlier, histamine (this study), adenosine (Jonzon et al., 1985), β- adrenoceptor agonists (this study; Nakaki et al., 1989) and prostaglandins (Nilsson & Olssen, 1984; Tertov et al., 1984) possess not only vasodilator properties, but have also been shown to inhibit the proliferation of smooth muscle cells in culture by elevating the intracellular cyclic AMP content. By analogy, therefore, it was possible that agents which relaxed vascular smooth muscle by elevating cyclic GMP content might also be able to inhibit the proliferation of vascular smooth muscle cells.

It was found in the present study that atriopeptin II, which activates particulate guanylate cyclase (Leitman & Murad, 1986), had no effect on the proliferation of rat ASMC in serum- containing medium. This is in contrast to earlier studies indicating that atrial natriuretic factor inhibits the proliferation of rat and rabbit ASMC induced by either PDGF or whole blood serum (Abell et al., 1989; Kariya et al., 1989). The difference in results may be related to the experimental procedure used. In the studies of Abell et al. (1989) and Kariya et al. (1989), [^{3}H]-thymidine incorporation after 24 hours was used as the index of proliferation. In this study, however, cell numbers throughout a 8 day period were counted with 48 hours as the first time point examined. It is possible that within the 48 hours the growth inhibitory effects of AP II had been masked by the mitogenic actions of serum.

Nitrovasosdilators stimulate soluble guanylate cyclase through

production of nitric oxide (Arnold et al., 1977; Katsuki et al., 1977; Craven & De Rubertis, 1978). In this study, the two nitrovasodilators, glyceryl trinitrate and sodium nitroprusside were each found to inhibit the proliferation of rat ASMC in serum-containing medium. The inhibitory effect of these agents was observed only at high concentrations i.e. 1mM for glyceryl trinitrate and 0.1 - 1mM for sodium nitroprusside. These concentrations are 100-1000 times greater than those required for relaxation of vascular smooth muscle (Lincoln & Fisher-Simpson, 1984; Martin et al., 1986b; Grace et al., 1988). The antiproliferative effect of sodium nitroprusside and glyceryl trinitrate, therefore, may be unrelated to the ability of these agents to elevate cyclic GMP content.

The effector molecule generated from the nitrovasodilators is nitric oxide (Arnold et al., 1977; Katsuki et al., 1977: Craven & De Rubertis, 1978). Nitrovasodilators can mimic therefore the actions of endogenously produced EDRF, which has been identified as nitric oxide (Palmer et al., 1987). It had been previously shown that several nitrovasodilators including sodium nitroprusside, S- nitroso- N- acetylpenicillamine and isosorbide dinitrate, inhibited the proliferation of rat and rabbit aortic smooth muscle cells in culture (Garg & Hassid, 1989; Kariya et al., 1989). On the basis of the actions of nitrovasodilators it was suggested by Garg & Hassid (1989) that EDRF released from endothelial cells may act as an endogenous regulator of smooth muscle cell growth within the arterial wall.

A recent study demonstrated that smooth muscle cells of

endothelium- denuded rings of bovine intrapulmonary arterial produce low but measurable quantities of a labile relaxing factor possessing pharmacological and chemical properties similiar to those of EDRF (Wood et al., 1990). On the scheme of Garg & Hassid (1989), it was possible that this smooth muscle- derived EDRF also had a regulatory role in controlling proliferation of smooth muscle cells within the arterial wall. In keeping with this hypothesis, it was found in the present study that N^G- nitro L-arginine, a competitive inhibitor of nitric oxide synthase, the enzyme that forms nitric oxide from L- arginine, stimulated the proliferation of rat ASMC by 17 ± 4% in serum- containing medium. The stimulation is more likely to have resulted from a non- selective action, however, since as will be seen later, haemoglobin, which inhibits the action of nitric oxide, did not share this property.

Recently, a third potential source of nitric oxide has been identified that could regulate smooth muscle proliferation in the arterial wall. It has been demonstrated that macrophages have the ability to generate nitric oxide from the terminal guanidino nitrogen atom(s) of L- arginine (Hibbs et al., 1987b; Stuehr et al., 1989; Kwon et al., 1990; Tayeh & Marletta, 1990). Macrophages secrete much higher quantities of nitric oxide than endothelial cells, and such quantities are clearly cytotoxic for many cell types (Hibbs et al., 1987a; Drapier & Hibbs, 1988; KrÖncke et al., 1991). It is possible, therefore, that nitric oxide inhibits the proliferation of smooth muscle cells not by stimulating soluble guanylate cyclase, but by a cytotoxic action. This is supported by the observation made in the present study

that sodium nitroprusside induced accumulation of the vital stain, trypan blue, in the same concentration range over which it inhibited proliferation: at the highest concentration examined i.e. 1mM almost all the smooth muscle cells accumulated trypan blue. In sharp contrast to the present study Garg & Hassid (1989) reported that the antiproliferative actions of the nitrovasodilators on rat ASMC were not due to cytotoxicity assessed by release of lactate dehydrogenase, staining with trypan blue or cell detachment. These workers assessed cytotoxicity only after a short incubation time (20 hours) whereas in the present study the cytotoxic effects were evident after 24 hours.

A possible explanation for the cytotoxic actions of sodium nitroprusside is that one of its degradation products is the metabolic poison, cyanide. This appeared not to be the case, however, since in this study, it was found that the addition of methaemoglobin, which binds cyanide (Stadie, 1920; Drabkin & Austin, 1935) did not reverse the antiproliferative action of sodium nitroprusside.

Haemoglobin has been shown to bind nitric oxide avidly (Martin et al., 1985). However, in this study, the antiproliferative action of sodium nitroprusside was not reversed by haemoglobin at concentrations up to 20μM. Garg & Hassid (1989), however, showed that at 50μM, inhibition was reversed. It is likely that a high concentration of haemoglobin was required to bind the large quantities of nitric oxide generated by the high concentration of sodium nitroprusside. Furthermore, the ability of superoxide dismutase to potentiate the actions of the nitrovasodilator in

inhibiting proliferation of smooth muscle (Garg & Hassid, 1989) is consistent with inhibition being mediated by nitric oxide. Superoxide anions have previously been shown to destroy nitric oxide (Gryglewski et al., 1986; Rubanyi & Vanhoutte, 1986a; 1986b).

Although it is likely that nitric oxide is responsible for the nitrovasodilator- induced inhibition of smooth muscle proliferation, it is not clear if stimulation of soluble guanylate cyclase is involved. In the present study, 8 bromo cyclic GMP at a high concentration of 1mM did inhibit proliferation of rat ASMC without increasing uptake of trypan blue , thus confirming a previous report on rabbit ASMC (Kariya et al., 1989) It is not certain, however, if the inhibiton was mediated by cyclic GMP protein kinase, or a non- specific action.

Other evidence suggesting that sodium nitroprusside inhibits proliferation by a cyclic GMP- independent mechanism is that the cyclic GMP PDE inhibitor, M & B 22948, did not potentiate the action of sodium nitroprusside. A recent study reported that the antiproliferative action of several nitrovasodilators, including sodium nitroprusside in Balb/ c3T3 fibroblasts is mediated by a cyclic GMP- independent mechanism (Garg & Hassid, 1990). These cells are known to lack soluble guanylate cyclase. Furthermore, nitric oxide has recently been shown to induce several cyclic GMP- independent responses, including decreased cytosolic free calcium in Balb c/3T3 cells (Garg & Hassid, 1991) and ADP- ribosylation of a 39 kDa protein in platelets (Brüne & Lapetina, 1989). These effects suggest the existence of an alternative

signalling transduction pathway for nitric oxide. In the study of Garg & Hassid (1990), the antiproliferative action of the nitrovasodilators appeared not to be due to cytotoxicity as assessed by release of lactate dehydrogenase, staining with trypan blue or cell detachment. These workers assessed cytotoxicity after 24 hours, but after 4 days treatment with the nitrovasodilator, S-nitroso- N- acetylpenicillamine, increased cell detachment was, clearly evident.

It is possible, therefore, that high concentrations of nitric oxide can inhibit proliferation by a cyclic GMP- independent transduction mechanism, but the margin for safety before cytotoxicity is seen is very small. It is unlikely, therefore, that EDRF acts as an endogenous regulator of smooth muscle cell growth in the arterial wall.

9.2.3. Conclusion

In conclusion, this part of the study shows that activation of protein kinase C by phorbol esters has no effect on the proliferation of rat aortic smooth muscle cells in culture. Furthermore, elevation of cyclic AMP content inhibits the proliferation of these cells without evidence of cytotoxicity. The effects of elevating cyclic GMP were less clear. 8 bromo cyclic GMP does inhibit smooth muscle proliferation at high concentrations but the inhibition of proliferation induced by sodium nitroprusside was probably mediated by a cyclic GMP- independent cytotoxic action.

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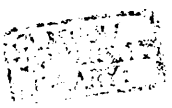
Appendix I. Purification of Endothelial Cell PDEs

PAEC (100- 150 million) grown in 250 ml culture flasks (Nunclon) were washed three times with ice- cold phosphate- buffered saline before being scrapped from the surface and removed to a centrifuge tube (50 ml). The cells were then centrifuged at 2000 g for 5 minutes and, after the phosphate- buffered saline had been removed, the pellet was stored at - 70°C until required.

The cell pellet was homogenized in 6 vol. of Tris/ HCl 20 mM, pH 7.5 containing magnesium acetate 2 mM, dithiothreitol 1 mM, EDTA 5 mM and aprotinin (2000 units/ ml) with a Dounce homogenizer. The homogenate was then centrifuged at 105000 g for 60 minutes and the supernatant (20 ml) applied to a DEAE- Trisacryl column (7 cm X 0.9 cm) pre- equilibrated with column buffer (Tris/ HCl 20 mM, magnesium acetate 2 mM, dithiothreitol 1 mM, N^{α}- tosyl- L-lysychloromethane hydrochloride 20μM, pH 7.5). The column was washed with two successive linear gradients of NaCl (0- 150 mM in 80 ml and 150- 400 mM in 70 ml) in column buffer; 2 ml fractions were collected, assayed, and for short term storage at -20°C, ethylene glycol was added to a final concentration of 30% (v/v). Assays on the pooled peak fractions were performed within 48 hr after homogenization of the cells.



Appendix II. Measurement of PDE Activity

PDE activity was determined by the two- step radioisotope method previously described by Thompson *et al*. (1979). The reaction mixture contained Tris/ HCl 20 mM, pH 8.0, $MgCl_2$ 10 mM, 2-mercaptoethanol 4 mM and bovine serum albumin 0.05 mg/ ml. The concentration of substrate (cyclic [^{3}H] AMP or cyclic [^{3}H] GMP) was 1μM.

IC_{50} values (concentration which produced 50% inhibition of substrate hydrolysis) for the compounds examined were determined from concentration- response curves, in which concentrations ranged from 0.1μM to 1 mM. Three concentration responses were generated for each inhibitor examined.