University of Bath

PHD

# The cardiovascular effects of long chain acyl carnitines and novel ester derivatives

Criddle, David N.

*Award date:*
1990

*Awarding institution:*
University of Bath

Link to publication

**General rights**
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal ?

**Take down policy**
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Download date: 13. May. 2019

THE CARDIOVASCULAR EFFECTS OF LONG CHAIN ACYL CARNITINES AND NOVEL ESTER DERIVATIVES.

Submitted by David N. Criddle

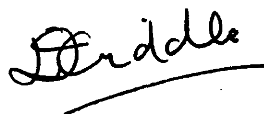
for the degree of Ph.D.

of the University of Bath, 1990.

Copyright.

Attention is drawn to the fact that copyright of this thesis rests with its author. This copy of the thesis has been supplied on the condition that anyone who consults it is understood to recognize that its copyright rests with its author and that no quotation from the thesis and no information derived from it may be published without the prior written consent of the author.

This thesis has been made available for consultation within the University Library and may be photocopied or lent to other libraries for the purpose of consultation.



David N. Criddle

UMI Number: U601450

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript
and there are missing pages, these will be noted. Also, if material had to be removed,
a note will indicate the deletion.

UMI U601450
Published by ProQuest LLC 2013. Copyright in the Dissertation held by the Author.
Microform Edition © ProQuest LLC.
All rights reserved. This work is protected against
unauthorized copying under Title 17, United States Code.

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

UNIVERSITY OF BATH
LIBRARY
23 | - 8 AUG 1991
PhD.
50539172

To Mum and Dad.

Thank you for your constant
love and support over
the years.

## ACKNOWLEDGEMENTS.

I am very grateful to Dr. Brian Woodward who has taught me so much over the past years, not only as a supervisor but as a friend.

It has also been my privilege to work with Dr. Mike Gwilt, who made my industrial experience both educational and enjoyable. Many thanks to Pfizer Ltd. for use of their facilities and the Staff who helped make my stay a pleasant one.

I would like to thank Drs. George Dewar and Bernard Wathey for their invaluable input into matters chemical, devoting much spare time and effort in the process. I am also grateful to Jane Mitchell and the William Harvey Research Institute for their help with the EDRF bioassay cascade experiments.

Finally, I would like to express my gratitude to all the Staff of the Animal House, Departmental technicians and many others, too numerous to mention individually, for providing assi stance wherever needed.

# SUMMARY.

This study has investigated the cardiovascular effects of long chain acyl carnitines and novel derivatives primarily using the coronary and mesenteric vascular beds, and thoracic aorta of the rat.

In the heart, long chain acyl carnitines caused an irreversible depression of myocardial contractility whilst exerting predominantly vasoconstrictor effects on the coronary vessels. Palmitoyl carnitine also caused vasoconstriction in quiescent mesenteric vessels, an effect not mediated via voltage-dependent calcium channels or $\alpha$-adrenoceptors, whilst having no effect on aorta. However, if vascular tone was artificially raised in mesenteric vascular beds using $\alpha$-adrenoceptor agonists, the vasoconstrictor response to palmitoyl carnitine was greatly potentiated, whereas if endothelin-1 was used to precontract the preparations palmitoyl carnitine elicited vasodilation. In contrast, the response to palmitoyl carnitine was very variable if high potassium was used to raise vascular tone. In precontracted aortae, however, palmitoyl carnitine produced less marked effects, eliciting small, gradual declines in vascular tone.

Esterification of palmitoyl carnitine changed the profile of activity of the drug from a vasoconstrictor to that of a potent dilator. Basic structural alterations of the molecule revealed that increasing the size of the ester group did not appreciably affect the activity of the drug, whereas increasing the fatty acyl chain length by two carbon atoms markedly reduced the vasodilator action. The vasodilator effects of the isopropyl ester of palmitoyl carnitine ($P1P^{i}$) were most marked in resistance vessels, such as the coronary and mesenteric vascular beds, the drug having little effect on the thoracic aorta. In the perfused heart the vasodilator action of $P1P^{i}$ did not appear to be mediated via the release of prostaglandins, EDRF or adenosine, activation of ATP-dependent potassium channels, or by stimulation of muscarinic or bradykinin $B_2$-receptors.

In mesenteric vascular beds precontracted with phenylephrine, endothelin or high potassium, $P1P^{i}$ elicited dose-related vasodilations, effects that were biphasic in the latter case. Additionally, in potassium-depolarised preparations this acyl carnitine ester also antagonized the contractile responses to calcium. In common with palmitoyl carnitine, $P1P^{i}$ had less marked effects on precontracted aortae, and also had no action on guinea-pig taenia-coli preparation. Electrophysiological studies carried out using guinea-pig papillary muscle, taenia coli and rabbit ear artery indicated that $P1P^{i}$ was without effect on resting membrane potential. Finally, the *in vivo* action of $P1P^{i}$ was transient compared with verapamil which produced a more sustained fall in blood pressure at lower doses in the anaesthetised rat.

# CONTENTS.


Page.

Chapter 1. **INTRODUCTION.**

1.1. Long chain acyl carnitines. 1
a) Structure & formation. 1
b) Reported actions. 5
1) Calcium channel activation 5
2) Cardiac electrophysiology 5
3) Enzyme effects. 6
4) $\alpha$-adrenoceptors. 6
5) Acyl carnitines and disease. 7
1.2. Vascular smooth muscle. 10
a) Contractile/relaxant mechanisms. 10
b) Role of the endothelium. 11
c) Resistance & conduit vessels. 13
d) Acyl carnitines & blood vessels. 14

Chapter 2. **MATERIALS & METHODS.**

2.1. Isolated Langendorff perfused heart preparation. 16
2.2. $^{86}$Rubidium efflux measurement. 16
2.3. Isolated perfused mesenteric vascular bed preparation. 18
2.4. Thoracic aorta preparation. 19
2.5. Taenia-coli preparation. 19
2.6. Electrophysiology. 19
a) Guinea-pig papillary muscle. 19
b) Guinea-pig taenia-coli and rabbit ear artery. 20
2.7. *In vivo* anaesthetised preparation. 20
2.8. Statistical analysis. 22
2.9. Materials. 22

Chapter 3. **PHARMACOLOGY OF LONG CHAIN ACYL CARNITINES.**

3.1. Heart. 23
a) Control responses. 23
3.2. Mesenteric vascular bed. 28
a) Control responses. 28
b) Interactions with various agents. 28
1) Adrenoceptor agonists. 31
2) Potassium. 35
3) Endothelin. 35
4) Angiotensin. 40
c) Effect of phentolamine on potentiation of vascular response to palmitoyl carnitine. 40

d) Effect of antagonists on the vascular response to palmitoyl carnitine. 44
e) Effect of perfused palmitoyl carnitine on responses to phenylephrine. 44
f) Effect of lysophosphatidylcholine in the mesenteric vascular bed. 48
3.3. Thoracic aorta. 52
a) Control responses. 52
b) Interactions with various vasoactive agents. 52

Chapter 4. **DISCUSSION.** 56

Chapter 5. **PHARMACOLOGY OF LONG CHAIN ACYL CARNITINE ESTERS.**
Discovery of palmitoyl carnitine ethyl ester. 67
5.1. Heart. 71
a) Control responses. 71
b) Effect of agents on action of novel compounds. 81
1) Cyclo-oxygenase/lipoxygenase products.
2) Endothelium-derived relaxant factor(s). 85
3) Potassium channels. 88
4) Cholinergic action. 88
5) Bradykinin. 90
6) Palmitoyl carnitine. 90
7) Adenosine. 90
8) Phospholipase $A_2$. 93
c) Effect of PCE on $^{86}$rubidium efflux. 93
d) Effect of extracellular calcium concentration. 96
5.2. Mesenteric vascular bed. 98
a) Precontracted preparations. 98
1) Endothelin. 98
2) Phenylephrine. 98
3) Potassium. 98
5.3. Guinea-pig taenia-coli. 106
a) Effect of P1P$^i$ on calcium-induced contraction. 106
5.4. Aorta. 110
a) Precontracted tissues. 110
1) Phenylephrine. 110
2) Potassium. 113
3) Endothelin. 113
b) Effect on agonist response. 113
1) Phenylephrine. 113
2) Acetylcholine. 116

5.5. Electrophysiology. 120
a) Papillary muscle. 120
b) Rabbit ear artery. 121
c) Guinea-pig taenia-coli. 127
5.6. Influence of the endothelium. 129
a) Cultured endothelial cells. 129
b) Effect of endothelial damage on the vasodilator action of P1P$^{i}$. 129
5.7. *In vivo* responses to P1P$^{i}$. 131

Chapter 6. **DISCUSSION.** 135

Chapter 7. **CONCLUSIONS.** 143

**REFERENCES.** 147

**PUBLICATIONS BY THE AUTHOR.** 164

# CHAPTER 1.

# INTRODUCTION.

# 1) LONG CHAIN ACYL CARNITINES.

In recent years there has been considerable interest in the putative detrimental role of long chain acyl carnitines in the ischaemic myocardium. These fatty acid derivatives have been shown to accumulate in up to millimolar concentrations in the heart under conditions of ischaemia *(Liedtke et al.,1978)* whilst exogenous acyl carnitine has been shown to exert damaging biochemical, electrophysiological and mechanical effects *(Adams et al.,1979; Knabb et al.,1986; Nakaya & Tohse,1986).* Many of the studies so far, however, have involved the use of myocytes or subcellular fractions whilst little work has been carried out in intact hearts or isolated blood vessels. Therefore in the present study the effects of long chain acyl carnitines were examined in the Langendorff perfused heart, the mesenteric vascular bed and isolated aorta of the rat.

## a) STRUCTURE & FORMATION.

Figure 1 illustrates the structure of palmitoyl carnitine, one of the major fatty acid metabolites that accumulates in the ischaemic heart. Long chain acyl carnitines are amphiphilic molecules in that they contain both hydrophobic and hydrophilic moieties and it has been suggested that many of their actions are simply due to this property *(Idell-Wenger et al.,1978; Katz & Messineo,1981).* The hydrophobic part of the molecule in this case is the long palmitoyl fatty acyl chain comprising 16 carbon atoms whilst the positively charged quaternary nitrogen atom and carboxylic acid group confer the hydrophilicity of the compound. At low concentrations, amphiphiles exist in monomeric form and can influence membrane fluidity by inserting into the hydrophobic lipid environment *(Watanabe et al.,1989)*, whilst at greater concentrations e.g. >13µM in the case of palmitoyl carnitine *(Piper et al.,1984)*, this class of compound can aggregate into micelles. In these various states amphiphiles can physically disrupt the lipid membrane via a non-specific 'detergent' action.

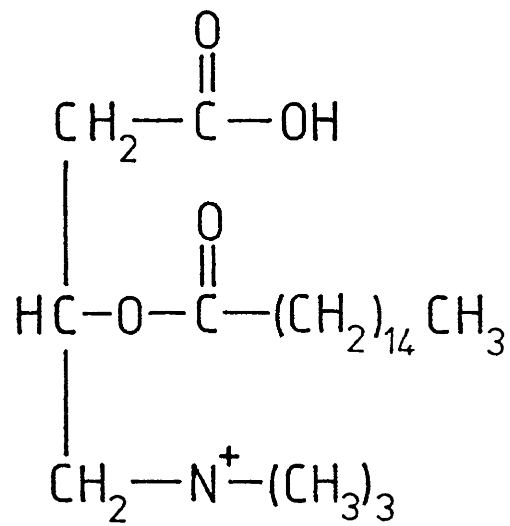



Figure 1. The structure of palmitoyl carnitine.

It has long been established that fatty acids are the preferential substrate of the myocardium and their metabolism in the heart is illustrated in figure 2. Free fatty acids taken up by the heart are rapidly esterified to fatty acyl CoA via a synthetase enzyme located on the outer mitochondrial membrane. Long chain acyl CoA does not readily cross the inner mitochondrial membrane and therefore the acyl group has to be transferred to a carrier molecule, carnitine, prior to $\beta$-oxidation. There are two carnitine acyl transferase enzymes (CATs) situated on either side of the inner mitochondrial membrane, as well as a translocase enzyme which is specific for the *l*-isomer *(Wolkowicz et al.,1982)*. Initially the acyl CoA is converted to acyl carnitine on the outside of the inner mitochondrial membrane, which is then translocated across the membrane and then acyl CoA is reformed on the inner side of the membrane. This latter step also releases carnitine which then is recycled back across the membrane via the translocase enzyme. Under physiological conditions the acyl CoA in the mitochondrial matrix can then undergo $\beta$-oxidation *(Pande,1975)* with the concomitant production of reduced nicotinamide adenine dinucleotide (NADH) and flavine adenine dinucleotide ($FADH_2$) which can then generate adenosine triphosphate (ATP).

However, under ischaemic conditions oxygen availability is insufficient for oxidation of NADH and $FADH_2$ and a build up of these reduced nucleotides occurs resulting in an inhibition of $\beta$-oxidation (for review see *Corr et al.,1984*). Consequently this causes a build up of intermediates such as long chain acyl CoA and acyl carnitine, the former occuring almost exclusively in the mitochondrial fraction whilst the latter predominantly in the cytosol. Therefore, accumulating cytosolic long chain acyl carnitine is likely to have access to the sarcolemma in ischaemia *(Idell-Wenger et al.,1978; Liedtke et al.,1978)*.

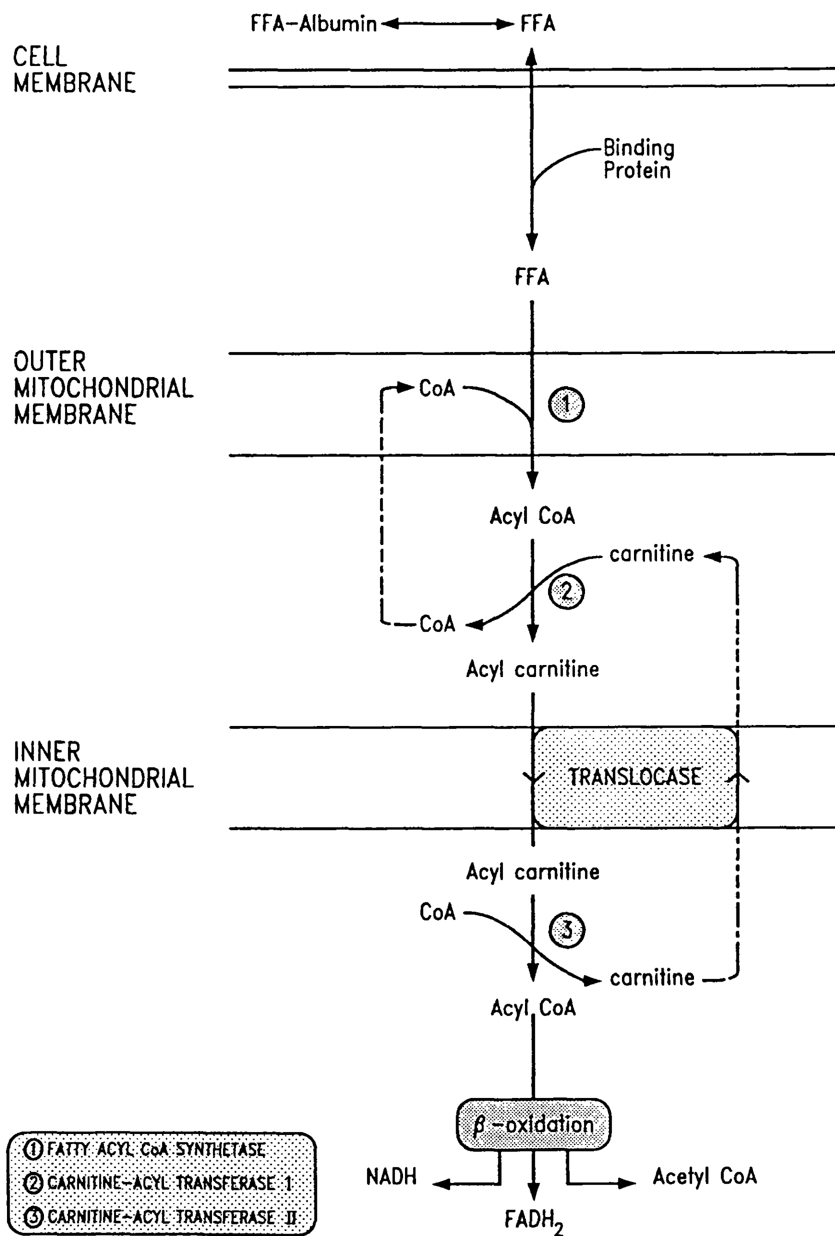



Figure 2. Schematic representation of the metabolism of free fatty acids (FFA) in the myocardium.

b) REPORTED ACTIONS.

1. Calcium Channel Activation.

In recent years it has been suggested that palmitoyl carnitine is an endogenous modulator of calcium channel function *(Spedding & Mir,1987)* on the basis that the effects of palmitoyl carnitine in $K^+$-depolarised smooth muscle resemble those of the dihydropyridine $Ca^{2+}$-channel activator, Bay K 8644. Previously it has been shown that palmitoyl carnitine augments myocardial calcium current in avian ventricular muscle, possibly via an effect on membrane surface charge *(Inoue & Pappano, 1983)*. Further evidence for a modulatory role of this fatty acid metabolite on calcium channels has come from studies showing a positive inotropic effect of the drug at low concentrations (0.5-5μM) in chick myocytes, with higher concentrations reversing the inhibitory effects of the calcium antagonists, verapamil and nisoldipine *(Duncan et al.,1986; Patmore et al.,1989)*. Recently it has been speculated that palmitoyl carnitine has a site of action at the sarcoplasmic reticulum in the heart, tightly linked to the calcium channel *(Spedding et al.,1989)*.

2. Cardiac Electrophysiology.

Dysrhythmias induced by myocardial ischaemia are responsible for most sudden deaths due to coronary artery disease *(Armstrong et al.,1972)* and although these appear to arise from specific electrophysiological changes leading to ventricular fibrillation *(Downar et al.,1977)* the underlying mechanisms have not yet been elucidated. In 1981 Corr et al. proposed that long chain acyl carnitines may act as potential progenitors of dysrhythmias during ischaemia based on observations of the effects of exogenous acyl carnitine on canine Purkinje fibres. They found that palmitoyl carnitine induced a concentration-dependent decrease in maximum diastolic potential, amplitude, maximum rate of rise of the action potential, and action potential duration that was reversible after a washout period. These changes were analogous to those observed in ischaemic tissue *in vivo (Downar et al.,1977)*. However, there are apparent discrepancies between studies depending on the type of

tissue used and possibly also the species involved. For example, palmitoyl carnitine has also been shown to increase the duration of the action potential without changing diastolic membrane potential in chick ventricular muscle *(Inoue & Pappano,1983)* whilst shortening duration and decreasing resting potential in guinea-pig papillary muscle *(Nakaya & Tohse,1986)*.

3. Enzyme Effects.

Over the past few decades long chain acyl carnitines have been shown to exert effects on a variety of enzymes, the observed action depending in many cases upon the concentration of drug used. For example, low concentrations of palmitoyl carnitine can stimulate $Ca^{2+}$-ATPase activity whilst higher concentrations are inhibitory *(Pitts et al.,1978; Adams et al.,1979)*. Similarly, this acyl carnitine exerts biphasic effects on the enzyme protein kinase C *(Katoh et al.,1981; Wise & Kuo, 1983)*. Many other enzyme inhibitory actions of palmitoyl carnitine have been documented including effects on the $Na^{+}/Ca^{2+}$ antiporter in sarcolemmal vesicles *(Lamers et al.,1984)*, cardiac $Na^{+}/K^{+}$-ATPase *(Adams et al.,1979)*, adenine nucleotide translocase *(Shug & Subramanian, 1987)* and adenyl cyclase *(Abe et al.,1984)*. However, many of these observations have been made using high concentrations of palmitoyl carnitine that, when applied to the whole heart, can induce myoglobin loss and contracture *(Hulsmann et al.,1985; Busselen et al.,1988)*. Therefore the results from these studies should be treated with caution when ascribing specific properties to this class of compound.

4. $\alpha$-adrenoceptors.

Increased $\alpha$-adrenoceptor numbers occur in the ischaemic myocardium of the cat and may contribute to the electrophysiological derangements observed under such conditions *(Corr et al.,1981)*. Using hypoxic adult canine myocytes as a model of ischaemia, these workers demonstrated one possible mechanism responsible for the increase in myocardial $\alpha_1$-adrenoceptors *(Heathers et al.,1987)*. Acute hypoxia produced a two- to threefold reversible increase in cell-surface $\alpha$-adrenoceptors

secondary to the sarcolemmal accumulation of long-chain acyl carnitines. Furthermore, inhibition of carnitine acyltransferase I, using sodium 2-[5-(4-chlorophenyl)-pentyl]-oxirane-2-carboxylate (POCA), prevented not only the accumulation of acyl carnitine but also the exposure of the $\alpha$-adrenoceptor *(Heathers et al.,1987)*. A similar finding has also been observed in the ischaemic rat heart *(Allely & Brown,1988)*. Therefore another detrimental role for long chain acyl carnitines may exist in myocardial ischaemia. Interestingly, there has been a report recently showing that palmitoyl carnitine is able to facilitate pressor responses to noradrenaline in the caudal artery of the rat *(Ugwu et al.,1987)* and since acyl carnitines can freely cross membranes *(Levitsky & Skulachev,1972)* it is possible that these compounds producd in the cardiac myocytes will have access to the coronary circulation potentially affecting vascular $\alpha_1$-adrenoceptors. Under conditions of ischaemia, therefore, acyl carnitines may contribute to the vasoconstriction induced by circulating catecholamines.

5. Acyl carnitines and disease.

Long chain acyl carnitine levels are elevated under such conditions as ischaemia *(Liedtke et al.,1978)*, diabetes *(Feuvray et al.,1979)*, peripheral vascular disease *(Hiatt et al.,1987)* and fasting *(Frohlich et al.,1978)*, however, their exact role in these situations and their potential to exert detrimental effects *in vivo* remains controversial. For example, despite the numerous effects of long chain acyl carnitines mentioned above, when examining the whole animal little is known about the local membrane concentration of these amphiphiles or the degree to which protein binding occurs, making extrapolation from *in vitro* data to the *in vivo* situation difficult. However, in recent years there has been interest in the potential therapeutic manipulation of acyl carnitine levels in disease states, primarily focussing on the prevention of accumulation of these fatty acid metabolites by inhibition of the enzyme carnitine acyl transferase 1 (CAT1).

Recently, several studies have demonstrated that CAT1 inhibitors, POCA *(Paulson et al.,1986)*, 2-tetraglycidic acid (TDGA) *(Hekimian & Feuvray,1985)* and

oxfenicine *(Molaparast-Saless et al.,1987)* exert protective effects on the ischaemic myocardium. Whether this action is due to prevention of long chain acyl carnitine or CoA accumulation is contentious since other factors may additionally be involved. For example, the change from fatty acid oxidation to that of carbohydrate with a resultant decrease in the oxygen consumption of the heart may also account for the beneficial effects of CAT1 inhibitors. Furthermore, etomoxir (ethyl 2-[-6-(4-chlorophenoxy)hexyl]oxirane-2-carboxylate), a more potent analogue of POCA, at low concentrations can inhibit accumulation of long chain acyl carnitines without exerting protective effects on the ischaemic heart, whilst 1000-fold higher concentrations do not prevent acyl carnitine accumulation in the heart but are protective *(Lopaschuk et al.,1988)*. These protective effects of etomoxir may be attributable to its ability to increase glucose utilisation in the myocardium *(Lopaschuk et al.,1989)* and for this reason this drug is currently being tested as an antidiabetic agent *(Eistetter & Wolf,1986)*. In conclusion, the potential of CAT1 inhibitors in the treatment of disease states is at present speculative, however, two major problems have first to be overcome, namely the irreversibility of many currently available inhibitors and also their ability to produce cardiomyopathy at high doses *(Spedding, 1990)*.

As mentioned previously, the detrimental role of long chain acyl carnitines in disease states remains contentious, at least until more concrete evidence can be obtained implicating these fatty acid metabolites as mediators of pathophysiological processes. However, both carnitine and its acyl derivatives have also been shown to have beneficial effects on the heart under certain conditions. So far much of the work has focussed on the protective effects of L-carnitine on the myocardium of both experimental animals and man *(Folts et al.,1978; Liedtke et al.,1979; Thomsen et al.,1979)* including certain studies demonstrating an ability of this compound to reverse the detrimental electrophysiological effects of long chain acyl carnitines on the heart *(Hayashi et al.,1981; Yokota et al.,1989)*. Similar salutary actions of short chain acyl carnitines such as propionyl($C_3$) and acetyl($C_2$) carnitines have also been documented *(Paulson et al.,1984; Matsui et al.,1985; Subramanian et al.,1987)*

along with their taurine amide derivatives *(Regitz et al.,1987)*. However, the precise involvement of carnitine and its derivatives remains unresolved, additionally complicated by studies demonstrating protective actions of long chain acyl carnitines, such as those of palmitoyl carnitine on ischaemia/reperfusion injury in rat heart *(Hulsmann et al.,1985)*.

## 2)VASCULAR SMOOTH MUSCLE.

### a) CONTRACTILE/RELAXANT MECHANISMS.

The main trigger for smooth muscle contraction is an elevation of the intracellular calcium concentration ($[Ca^{2+}]_i$). Not surprisingly, this is regulated by a large number of cellular processes, influencing the flux of calcium between three anatomical compartments; the extracellular space (ECS), cytoplasm and sarcoplasmic reticulum (SR) (for review see *van Breemen & Saida,1989*). Calcium influx into the cell from the ECS can be mediated i) by a passive leak across the plasma membrane, possibly as a consequence of non-specific disruption of the lipid bilayer, ii) via voltage-operated channels (VOCs) of which two types, "L" and "T", have been identified in smooth muscle *(Loirand et al.,1986),* and iii) via receptor-operated channels (ROCs) *(Bolton,1979).* Conversely, flow of calcium out of the cell is carried out by the $Ca^{2+}/Mg^{2+}$-ATPase pump and to a variable extent via the $Na^{+}/Ca^{2+}$ antiporter. Similarly, calcium movement occurs between the SR and the cytoplasm, efflux via i) passive leak, ii) inositol 1,4,5-trisphosphate ($IP_3$)-activated channels and iii) $Ca^{2+}$-activated channels, and entry into the SR driven by a $Ca^{2+}/Mg^{2+}$-ATPase pump *(Eggermont et al.,1988).*

At present there is some debate regarding the events responsible for the maintainance of contraction in smooth muscle. However, it is generally accepted that initiation of vascular contraction is due to a rise in intracellular calcium leading to an association of calmodulin (CaM) with myosin light chain kinase (MLCK), which then leads to the phosphorylation of myosin light chain and the formation of actin and myosin cross bridges resulting in tension development *(Dillon et al.,1981).* Prolonged exposure to a contractile agonist results in the maintainance of vascular smooth muscle in a sustained or 'tonic' contraction, however, in this state myosin light chain becomes dephosphorylated, ATP utilization and $O_2$ consumption fall, and $[Ca^{2+}]_i$ decreases to near basal levels. Consequently, this led to the hypothesis that a new type of long-lasting actin-myosin attachment could be formed i.e. 'latch bridges' formed between dephosphorylated myosin light chains and actin filaments.

However, to date there is no molecular evidence for the existence of such latch bridges. Another hypothesis has been advanced to explain the sustained phase of smooth muscle contraction, implicating other intracellular proteins such as caldesmon, gelsolin and filamin in tension maintainance, with a modulatory role for the ubiquitous enzyme protein kinase C *(Rasmussen et al., 1987)*.

b) ROLE OF THE ENDOTHELIUM.

The perception of the vascular endothelium has been radically altered over the past decade. It is now well established that the endothelial cell layer behaves as an organ, lining the entire vascular system and performing a plethora of functions, rather than merely providing a semi-permeable diffusion barrier. The endothelium exerts a modulatory influence on the coagulability and flow of blood in the vascular system, posseses synthetic activity e.g $PGI_2$ synthesis *(Weksler et al.,1985)* and also metabolises a variety of substances including angiotensin I, bradykinin and adenine nucleotides *(Ryan et al.,1976; Pearson & Gordon,1985)*. However, it was the seminal observations of Furchgott and Zawadzki in 1980 that delineated the importance of the endothelial cell layer in the modulation of vascular tone.

They first demonstrated the obligatory role of the endothelium in acetylcholine-induced relaxation of isolated arteries of the rabbit and suggested that some humoral factor(s), denoted endothelium-derived relaxant factor (EDRF), was responsible for this effect. Since then many substances have been identified that release EDRF including A23187 *(Zawadzki et al.,1980)*, bradykinin *(Cherry et al.,1981)*, thrombin *(De Mey et al.,1982)* and both saturated and unsaturated fatty acids *(Cherry et al.,1983; Furchgott et al.,1985)* whilst there has been much work directed toward identifying the chemical nature of EDRF, its endogenous precursor(s) and its mechanism of vascular relaxation (for review see *Furchgott, 1990*).

In recent years it has been suggested that EDRF is in fact nitric oxide (NO) on the basis of the selective manipulation of its effects by various agents and the

comparative chemical and biological properties of EDRF and NO *(Ignarro et al.,1987; Palmer et al.,1987; Furchgott et al.,1988)*. Some data exists suggesting that EDRF differs from NO *(Shikano et al.,1988)* and that other factors are involved in endothelium-dependent relaxation such as endothelium-dependent hyperpolarising factor (EDHF) *(Komori & Suzuki,1987; Feletou & Vanhoutte, 1988)*, however, the bulk of evidence to-date supports the identity of EDRF being nitric oxide.

EDRF is chemically unstable with a half-life ranging from 6s *(Griffith et al.,1984)* to 50s *(Forstermann et al.,1986)* and is probably broken down by superoxide anion ($\cdot O_2^-$) released simultaneously in order to elicit autocatalytic destruction of the mediator *(Gryglewski et al.,1986; Rubanyi & Vanhoutte,1986)*. It is thought to relax vascular smooth muscle by activation of soluble guanylate cyclase leading to accumulation of intracellular cyclic GMP *(Rapoport et al.,1983)*. Many substances that interfere with EDRF directly or its target enzyme guanylate cyclase, have been used to either attenuate or enhance its effects, for example, superoxide dismutase potentiates the effects of EDRF by preventing its inactivation *(Gryglewski et al.,1986)* and haemoglobin inhibits EDRF-mediated responses, probably by chemically binding EDRF *(Martin et al.,1986)*.

At present the source of nitric oxide in endothelial cells remains controversial. It has been suggested that L-arginine is the immediate precursor of NO, the formation being stereospecific and occuring from the terminal guanidino nitrogen atom(s) of the amino-acid *(Palmer et al.,1988a)*. Supportive of this is that $N^G$-monomethyl-L-arginine (L-NMMA), an L-arginine analogue, inhibits both the release of NO from aortic endothelial cells and the endothelium-dependent relaxation of rabbit aorta *(Palmer et al.,1988b)*. However, it has also been demonstrated that L-NMMA is a non-specific inhibitor of a variety of other vasodilators *(Thomas et al.,1989a)* and it has been suggested that EDRF may be derived from sources other than L-arginine such as an arginine-containing moiety *(Thomas & Ramwell,1988)*.

The majority of research so far investigating effects of EDRF has been carried out on large, conduit blood vessels whilst few studies have concentrated on a possible role for EDRF in the microvasculature. In 1985, Owen & Bevan demonstrated that in peripheral rabbit arteries the acetylcholine-induced endothelium-dependent vasodilation increased as the vascular size decreased. Since then several studies have proposed a role for EDRF in the modulation of vascular tone in resistance vessels of the coronary circulation *(Amezcua et al.,1989)*, kidney and mesenteric beds *(Bhardwaj & Moore,1988; Moore et al.,1990)*.

c) RESISTANCE & CONDUIT VESSELS.

Most studies investigating the mechanisms of vascular smooth muscle contraction so far have focussed on large, conduit arteries such as the aorta despite the obvious physiological importance of resistance vessels. However, it appears that significant differences exist between these two types of blood vessels *(Cauvin et al.,1984; Bevan et al.,1986)*.

Contractile agonists such as noradrenaline utilise both intracellular calcium and calcium influx from the extracellular space during activation of vascular smooth muscle *(Deth & Van Breemen,1974; Bolton,1979)* whilst high potassium-induced contractions are calcium influx-dependent *(Van Breemen,1969)*. It seems that variation exists between blood vessels in the degree to which $[Ca^{2+}]_i$ release and $Ca^{2+}$ influx contribute to agonist-induced tone *(Cauvin et al.,1983; Godfriand et al.,1986; Bevan et al.,1986)*. For example, there appears to be a lesser contribution of $Ca^{2+}$ release from the sarcoplasmic reticulum and a greater sensitivity to calcium antagonists when activated by noradrenaline as the arterial size diminishes. In addition, there seems to be a functional heterogeneity between the sarcoplasmic reticulum from different sized blood vessels. In rabbit aorta, caffeine- and noradrenaline-induced release of $^{45}Ca^{2+}$ into $Ca^{2+}$-free solution are similar, whilst in mesenteric artery marked differences exist in the patterns of SR $Ca^{2+}$-release *(Leijten & Van Breemen,1984; Saida & Van Breemen,1984; Leijten & Van Breemen,1986)*.

The importance of membrane depolarisation associated with receptor activation in vascular contraction also varies depending on the blood vessel and possibly species involved. For example, noradrenaline can activate the aorta without eliciting a change in membrane potential *(Cauvin et al.,1984)* whilst in smaller vessels the contraction to exogenous noradrenaline is accompanied by depolarisation *(von Loh & Bohr,1973; Cauvin et al.,1984)*. Cauvin et al. (1984) showed that in rabbit mesenteric resistance vessels this depolarisation is completely inhibited by diltiazem whereas that induced by 80mM $K^+$ is not, and suggested that it is the calcium ion entering through the ROCs that causes the membrane depolarisation rather than an initial depolarisation causing opening of VOCs. In addition, it has been proposed that in rat small mesenteric arteries the role of membrane potential in response to $\alpha$-adrenoceptor stimulation is not essential to but modulatory of the contractile response *(Mulvany et al.,1982)*.

d) ACYL CARNITINES & BLOOD VESSELS.

Although long chain acyl carnitines have been subject to investigation for several decades, there have been very few studies examining the effects of these compounds on blood vessels. In 1986, Bigaud & Spedding demonstrated that palmitoyl carnitine increases sensitivity to $Ca^{2+}$ in $K^+$-depolarised rat aorta. This same group have since produced evidence that palmitoyl carnitine can facilitate pressor responses to noradrenaline in rat tail artery *(Ugwu et al.,1987)* advancing the hypothesis that this acyl carnitine is an endogenous calcium channel activator *(Spedding & Mir,1987)*. However, the effects of palmitoyl carnitine on vascular tissue are likely to be complex. For example, depending on the concentration of drug used, palmitoyl carnitine can either elicit relaxations of precontracted rat aortae (<10μM) or produce direct contractile effects (>10μM), whilst also influencing the activity of exogenous endothelium-dependent vasodilators *(Dainty et al.,1990)*.

It is possible that long chain acyl carnitines may exert effects on the coronary circulation since millimolar concentrations of these compounds have been reported in the ischaemic heart *(Liedtke et al.,1978)* and as these amphiphiles can readily

cross lipid bilayers *(Levitsky & Skulachev,1972)* it is likely that they will have access to the coronary vessels. The initial aim of the present study was to examine the vascular effects of palmitoyl carnitine and related compounds, concentrating especially on resistance vessels e.g. coronary and mesenteric vascular beds, with a view to identifying any potential role for these lipid metabolites in the control of vascular tone. However, due to serendipitous discovery of novel vasodilator derivatives during the course of this research the work presented here is divided into two main sections; the vascular effects of the parent acyl carnitines, and the basic pharmacology of their novel, synthetic derivatives.

# CHAPTER 2.

# MATERIALS & METHODS.

## 1. ISOLATED LANGENDORFF PERFUSED HEART PREPARATION.

Hearts from male Wistar rats (University of Bath strain), weighing between 250-350g, were perfused by the Langendorff technique at a constant flow of 10 $mlmin^{-1}$ at 37°C with a modified Krebs-Henseleit solution of the following composition (mM): glucose 11.0; NaCl 118.0; $NaHCO_3$ 25.0; KCl 4.7; $KH_2PO_4$ 1.2; $CaCl_2$ 1.2; $MgSO_4$ 1.2, gassed with 95% $O_2$, 5% $CO_2$. Following a 15 minute stabilisation period, the perfusate was changed to one containing 3.2mM $K^+$ which raises coronary tone. Perfusion pressure changes were monitored with a Bell and Howell pressure transducer connected to a side-arm. Developed tension under a resting tension of 2g was recorded with a Devices isometric transducer attached to the apex of the heart; the signal from this transducer was also used to trigger an instantaneous rate meter. All recordings were made on a Gould 3000S recorder. Drug injections were made in volumes of less than 100µl via a side-arm situated close to the aortic valves. Figure 3 illustrates the perfusion apparatus used in these experiments.

## 2. $^{86}$RUBIDIUM EFFLUX MEASUREMENT.

Hearts were perfused with Krebs-Henseleit solution (5.9mM $K^+$) for a control period of 5 minutes, $^{86}Rb$ (0.2µCi/ml) was then added for 10 minutes after which time it was washed out with control Krebs-Henseleit solution. After a further period of 30 minutes, the hearts were perfused with a modified solution containing 3.2mM $K^+$ and the effluent collected 2 and 4 minutes later. After 5 minutes a standard dose of PCE (1-100nmoles) was added and further aliquots were collected for 15 minutes. 1 ml of perfusate was added to 4 mls of scintillation fluid (Optiphase 'safe') and counted in an LKB 1215 scintillation counter using an external standard channels ratio method for 10,000 counts or 10 minutes. At the end of the experimental period the heart was blotted with tissue paper, chopped into small pieces and left to dissolve in 5 mls of 1MNaOH. After 48 hours the dissolved heart was neutralised with 5 mls of 1M HCl to prevent chemiluminesence, and the decay during this period allowed for. Finally, 1 ml of the neutralised material was added to

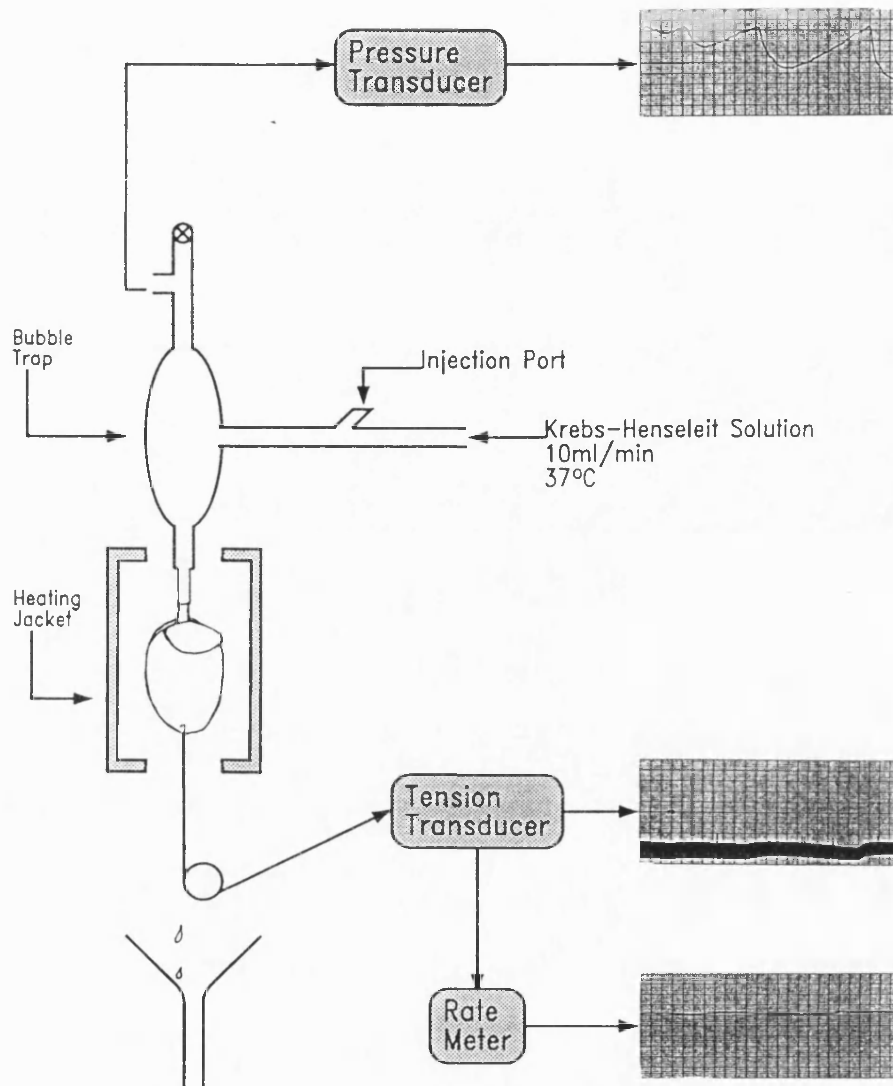



Figure 3. Diagram of the apparatus used for the isolated Langendorff perfused heart preparation.

4 mls of the scintillation fluid and counted.

## 3. ISOLATED PERFUSED MESENTERIC VASCULAR BED PREPARATION.

The superior mesenteric artery was cannulated, the middle colic artery ligated and the mesenteric vascular bed removed by careful dissection from male Wistar rats (University of Bath strain) weighing between 250-350g. Perfusion of the bed was carried out at a constant flow rate of 4 $mlmin^{-1}$ at 37°C with standard Krebs-Henseleit solution (5.9mM $K^+$) gassed with 95% $O_2$, 5% $CO_2$. Changes in perfusion pressure were monitored in a similar manner to that described above for the Langendorff heart preparation. Following dissection, tissues were left for an equilibration period of 30-40 minutes before drugs were added; injections of drugs (volume $<100\mu l$) were made directly into the perfusate at a site close to the cannula.

In certain experiments the vascular tone was elevated artificially by inclusion of constrictor agents into the perfusion fluid, or by elevation of the potassium concentration of the Krebs-Henseleit solution. In the latter case, the sodium chloride in the solution was reduced accordingly to maintain osmolarity.

Additionally in some experiments, the vascular beds were perfused with a modified, calcium-free Krebs-Henseleit solution containing elevated potassium (110mM; NaCl reduced to maintain osmolarity) for an initial equilibration period of 30 minutes. Following this calcium chloride (0.1-10mM) was added cumulatively to the perfusion solution, the preparation allowed to recover in calcium-free solution for 40 minutes, and the addition of calcium chloride repeated. However, when assessing the effects of a drug on the contractile response to calcium, the drug was included in the perfusion solution for 20 minutes prior to the further addition of calcium.

* Wherever required, de-endothelialisation was achieved by rubbing the intimal surface of the aorta with a cotton bud.

4. THORACIC AORTA PREPARATION.

Segments of thoracic aorta of about 0.2cm length were prepared either as rings or as transverse strips, opened out along their longitudinal axes, and mounted for isometric tension recording under a resting tension of 2g in a tissue bath containing Krebs-Henseleit solution (5.9mM $K^+$) gassed with 95% $O_2$, 5% $CO_2$ at 37°C. Tissues were left for an equilibration period of 30-40 minutes before commencing the experiment. *

5. TAENIA-COLI PREPARATION.

Taenia preparations from the caecum of male guinea-pigs (350-450g) were set up in 10ml isolated organ baths containing standard Krebs-Henseleit solution (5.9mM $K^+$) gassed with 95% $O_2$, 5% $CO_2$ at 37°C. Contractions were measured using a Devices isometric tension transducer connected to a Lectromed Multitrace 2 chart recorder. All preparations were mounted for recording under a resting tension of 1g and initially left to equilibrate for 30 minutes before changing the bath solution to a modified, calcium-free Krebs-Henseleit solution for a further 30 minutes. The effects of a drug on the contractile reponse to cumulatively added calcium chloride were assessed as described previously for the mesenteric vascular bed preparation (*see above*).

6. ELECTROPHYSIOLOGY

a) Guinea-pig Papillary Muscle.

All dissections were made in well oxygenated (95% $O_2$, 5% $CO_2$) physiological salt solution (PSS) of the following composition (mM): NaCl 130.0; KCl 5.6; $NaHCO_3$ 25.0; $MgCl_2$ 0.5; $NaH_2PO_4$ 0.6; glucose 11.0; $CaCl_2$ 2.16. Guinea-pigs were killed by stunning followed by cervical dislocation. Hearts were rapidly removed into well oxygenated PSS (room temperature) and the papillary muscles dissected free from the ventricles. These were then pinned out on Sylgard (Dow Corning) in a tissue bath (volume 1.5ml) perfused (13 ml/min) with PSS at 37°C.

All tissues were left to equilibriate for 1-3 hours. Stimulation of papillary muscles was applied from an isolated stimulator (Digitimer DS2) via fine stainless steel pins placed close to one end of the preparation. Square pulses (0.05-1ms; twice threshold voltage) were used to elicit action potentials. Intracellular recordings were made using glass microelectrodes of resistance 10-30 megohms. Records were amplified using a high impedance (1012 ohms) amplifier (Axoclamp-2) with capacity compensation. Action potentials (APs) were displayed on an oscilloscope (Gould OS4000) and analysed on-line by computer (Motorola 68000/Disc.Biol. on line-system) to give standard AP parameters i.e. resting potential, AP amplitude, maximum upstroke velocity (dv/dt max) and times to 50% and 90% repolarisations ($APD_{50}$ and $APD_{90}$, respectively).

b) Guinea-pig Taenia-coli and Rabbit Ear Artery.

For guinea-pig taenia-coli and rabbit ear artery preparations similar equipment and protocol were employed for the measurement of resting membrane potentials with the following exceptions. The preparations were not electrically stimulated during the experimental period and higher resistance glass electrodes were required to impale the tissues (50-80 megohms).

## 7. *IN VIVO* ANAESTHETISED RAT PREPARATION.

Male Wistar rats (University of Bath strain), weighing 250-350g were anaesthetised using an intraperitoneal injection of sodium pentobarbitone (Sagatal, May & Baker) 60mgkg$^{-1}$ body weight. A tracheal cannula was inserted and artificial respiration was maintained (ventilation rate 80min$^{-1}$, stroke volume 10mlkg$^{-1}$) with a Miniature Starling (Bioscience) pump. Systemic arterial blood pressure was recorded from the left common carotid artery using a physiological pressure transducer (Gould P23 ID) coupled to a pressure processor amplifier (Gould 13-4615-52) via a polythene cannula filled with heparinised saline (50Uml$^{-1}$). The signal from the pressure transducer was also used to trigger an instantaneous rate meter for monitoring of heart rate throughout the experimental period. All

recordings were made using a Gould 3000S thermal chart recorder. A saline-filled polythene cannula was also inserted into the left jugular vein for administration of drugs. Rectal temperature was maintained at 37°C by means of a heating lamp.

8) STATISTICAL ANALYSIS.

Results are expressed as the mean ± standard error of the mean (s.e.mean).

Statistical differences between means were assessed using either a paired or a non-paired Student's t-test, where applicable.

9) MATERIALS.

The following compounds were used; (±) palmitoyl carnitine, (±) stearoyl carnitine, (±) myristoyl carnitine, palmitic acid, palmitoyl lysophosphatidylcholine, carnitine, phenylephrine, noradrenaline, angiotensin II, indomethacin, superoxide dismutase, L-NG-nitro arginine, atropine, adenosine, adenosine deaminase, mepacrine, bradykinin, sodium nitroprusside, glyceryl trinitrate, papaverine (*Sigma*), cromakalim, BRL38227 (*Beechams*), endothelin, D-Arg$^{0}$[Hyp$^{3}$,Thi$^{5,8}$,D-Phe$^{7}$]-bradykinin (*Nova*), glibenclamide (*Hoechst*), flurbiprofen (*Boots*), BW755C (*Wellcome*), methylene blue (*Fisons*), iloprost (*Schering*), clonidine (*Gibbs*), isoprenaline (*Pharmax*), phentolamine (*Ciba*), amyl nitrate (*BDH*), and verapamil (*Abbot*).

All novel compounds were synthesised by Dr. W.B.Wathey (*University of Bath*).

Drugs were prepared in distilled water, except cromakalim, BRL38227, glibenclamide and palmitic acid, which were first dissolved in ethanol and then diluted in distilled water; at the final concentration used the ethanol was without effect on the coronary circulation.

# CHAPTER 3.

## PHARMACOLOGY OF LONG CHAIN ACYL CARNITINES.

## 1)HEART.

### a) CONTROL RESPONSES.

Bolus injections of palmitoyl carnitine elicited large dose-related vasoconstrictions of the coronary vascular bed (figs 4 & 5) and at the highest doses used (30-100nmoles) small vasodilations were occasionally observed. This acyl carnitine also produced a cumulative, irreversible depression of myocardial contractility with no concomitant effect on heart rate. Other long chain acyl carnitines examined, myristoyl($C_{14}$) and stearoyl($C_{18}$), exhibited a similar profile of action (fig.6).

To assess whether these effects were specific, other amphiphilic compounds were investigated (fig.7). Palmitic acid (1-30nmoles) elicited biphasic responses consisting of a fast, initial vasoconstrictor phase followed by a later, variable vasodilator component. Palmitoyl lysophosphatidylcholine (LPC) (10-80nmoles), however, produced smaller vasoconstrictions of the coronary vessels than the long chain acyl carnitines, whilst carnitine (50-250nmoles) was without effect on vascular tone ($n$=3).

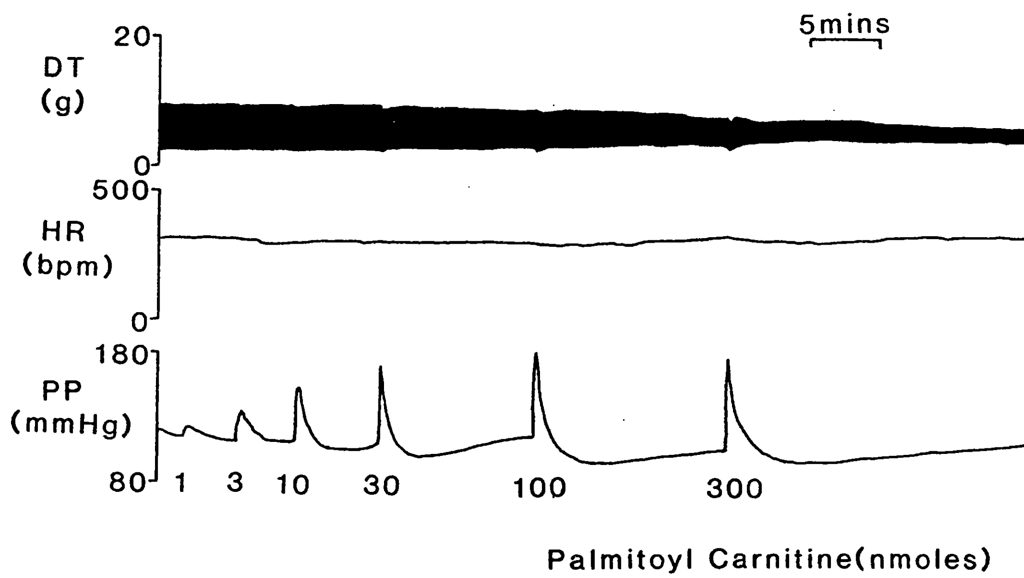



Figure 4. Typical trace showing the effects of palmitoyl carnitine (1-300nmoles) on developed tension (DT), heart rate (HR) and perfusion pressure (PP) in the isolated Langendorff perfused rat heart.

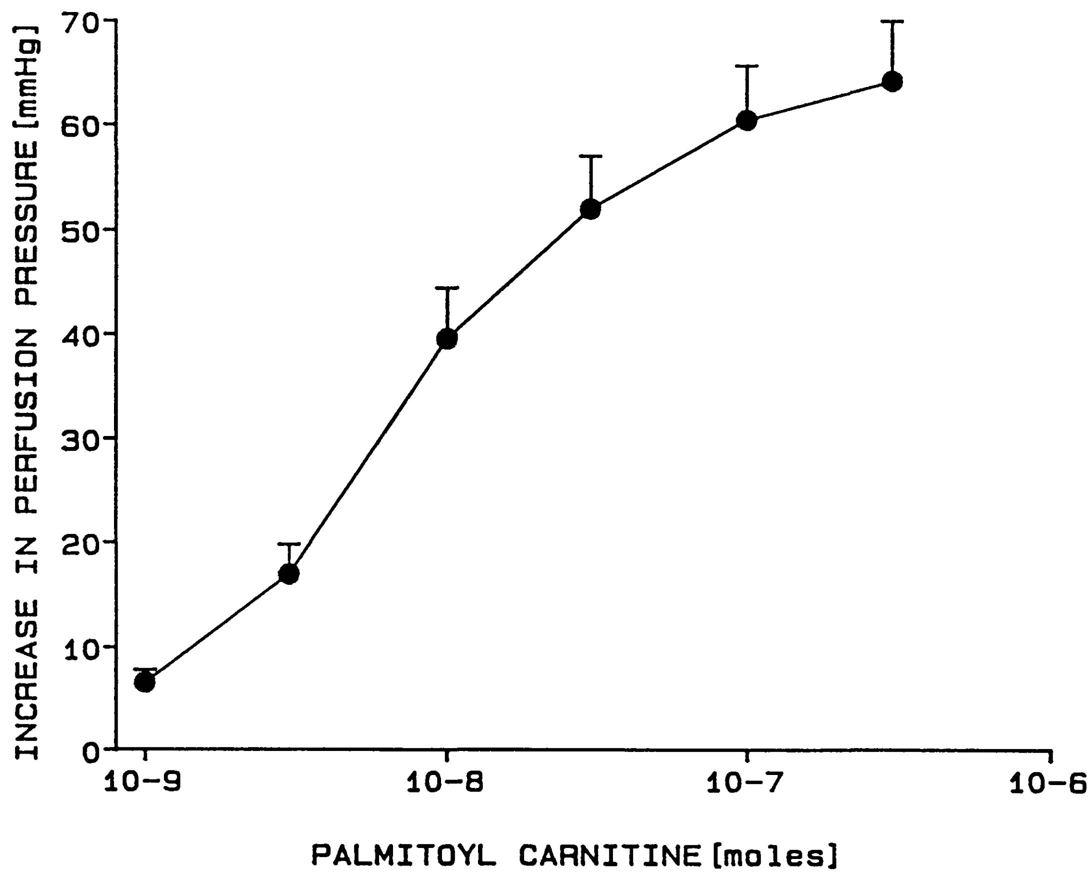



Figure 5. Effects of palmitoyl carnitine on perfusion pressure in the isolated perfused rat heart. Vertical bars represent s.e.mean ($n$=4).

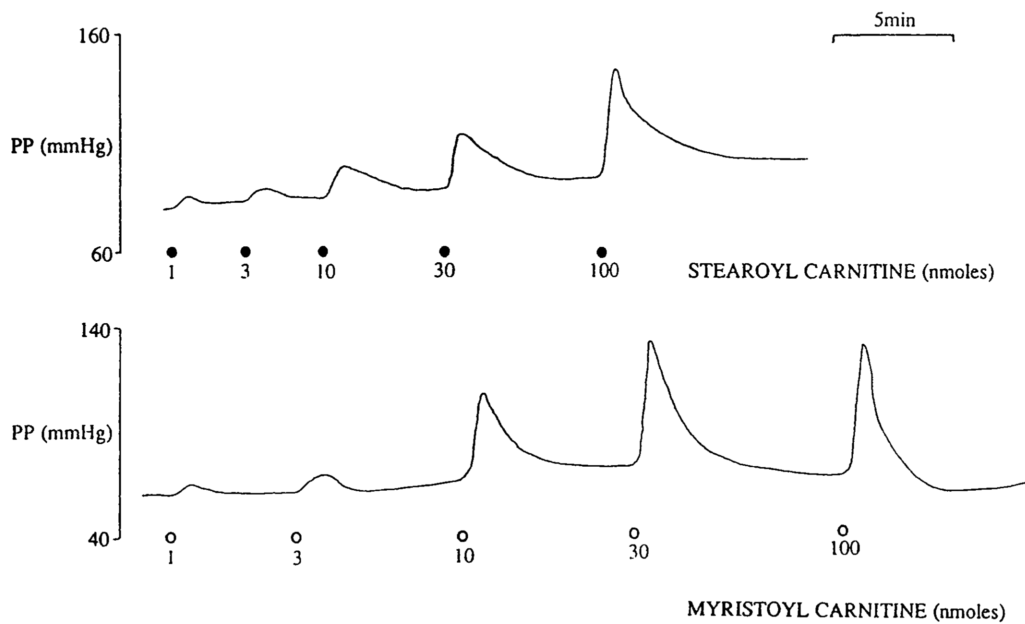
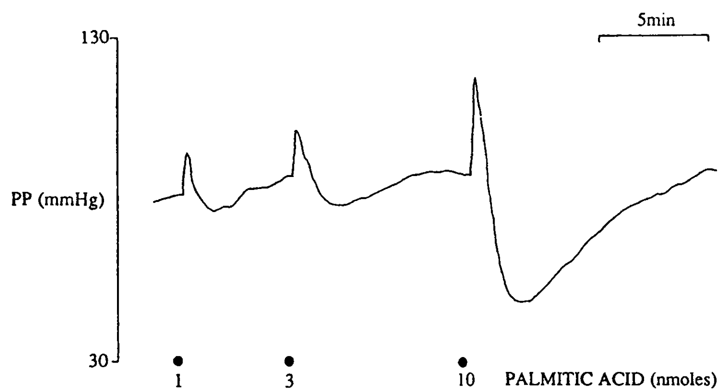



Figure 6. Typical traces showing the effects of stearoyl ($C_{18}$) and myristoyl ($C_{14}$) carnitine on perfusion pressure (PP) in the isolated perfused rat heart (*n*=4).



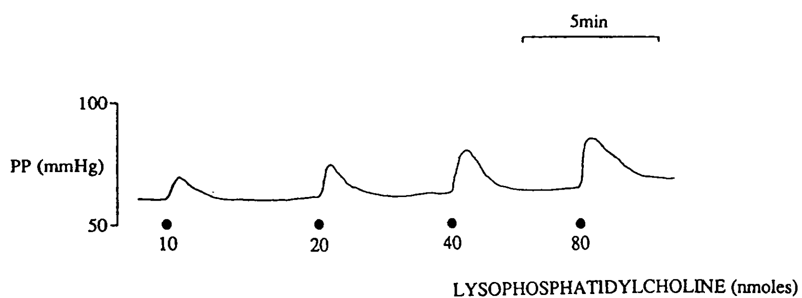




Figure 7. Typical traces showing the effects of palmitic acid (*upper*) and lysophosphatidylcholine (LPC) (*lower*) on perfusion pressure (PP) in the isolated perfused rat heart ($n$=4).

## 2)MESENTERIC VASCULAR BED.

### a) CONTROL RESPONSES.

In this vascular bed bolus injections of palmitoyl carnitine caused small dose-related increases in perfusion pressure (10-300nmoles) (fig.8). At higher doses there was a sustained rise in the resting tone of the mesenteric vessels (data not shown) and consequently any further experiments that required repeated dose-responses to the drug were restricted to lower doses of palmitoyl carnitine i.e. 10-300nmoles. In addition, the compound was poorly soluble in distilled water at higher concentrations (>$10^{-2}$M).

### b) INTERACTIONS WITH VARIOUS VASOACTIVE AGENTS.

Long chain acyl carnitines have been reported to increase myocardial $\alpha$-adrenoceptor numbers *(Heathers, et al., 1987)*, whilst their actions on vascular tissues have so far received little attention. Consequently, it was decided to investigate any possible interactions of palmitoyl carnitine with $\alpha$-adrenoceptor agonists, such as phenylephrine and noradrenaline, using the perfused mesenteric vascular bed. Control dose-responses to palmitoyl carnitine were obtained and the tissue allowed to recover for 15 minutes at which time a pharmacological agent was introduced into the perfusion medium. A further 15 minutes later the dose-response to palmitoyl carnitine was repeated in the presence of the perfused agent. All responses are expressed as a percentage of the peak vasoconstriction obtained in the initial control dose-response to palmitoyl carnitine. Repeated additions of palmitoyl carnitine to the mesenteric vascular bed, in the absence of any other pharmacological agent, induced tachyphylaxis (fig.9) and consequently when assessing the effect of another drug on this vasoconstrictor action time-matched controls were employed.

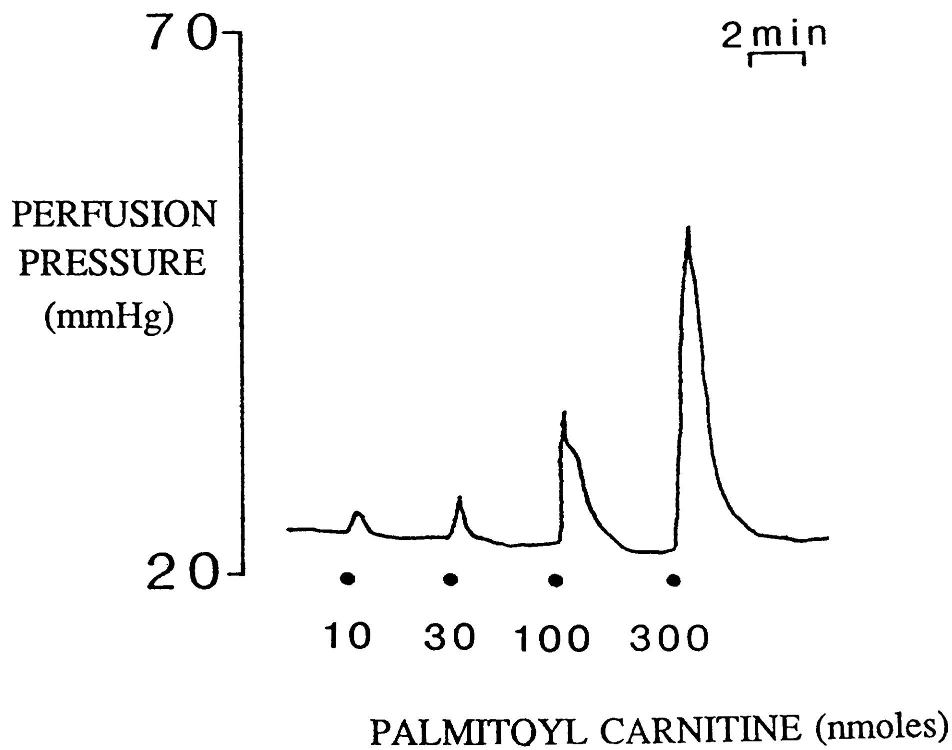



Figure 8. Typical trace showing the effects of palmitoyl carnitine (10-300nmoles) on perfusion pressure in the isolated perfused mesenteric vascular bed of the rat.

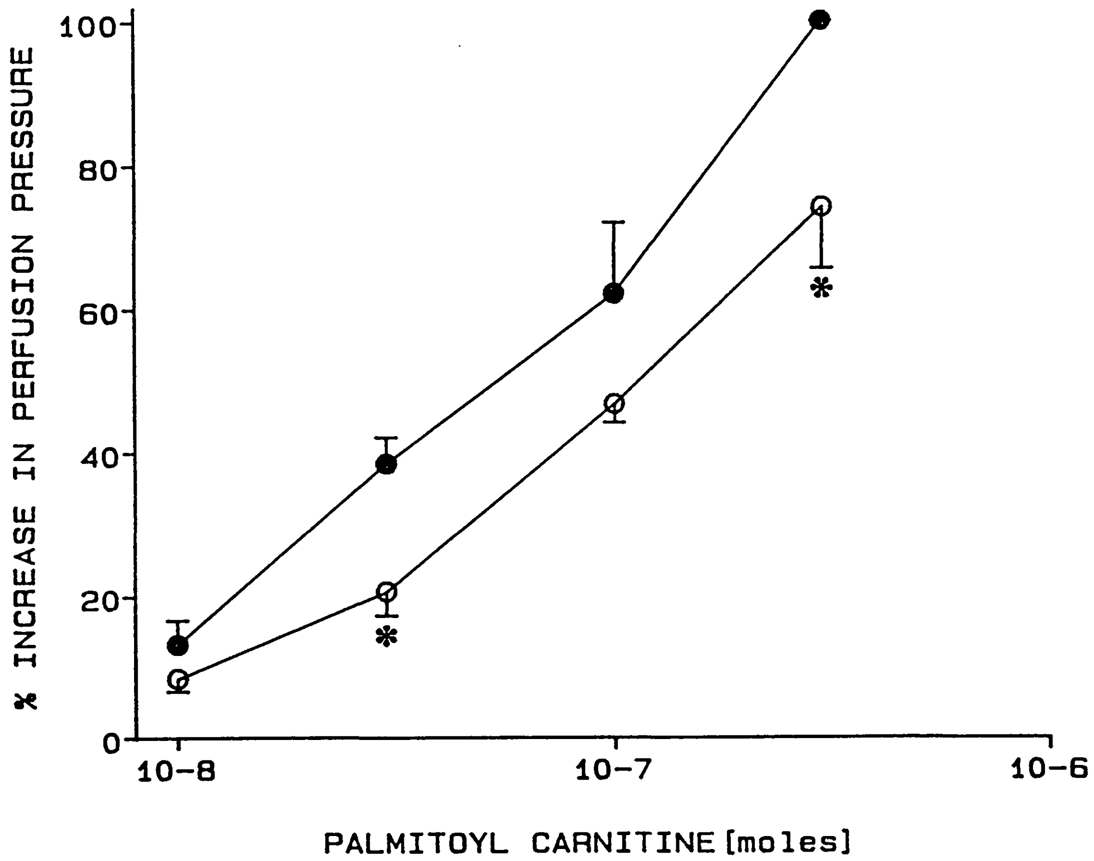



Figure 9. Effects of repeated administration of palmitoyl carnitine (10-300nnoles) on perfusion pressure in the isolated perfused mesenteric vascular bed of the rat, shown as control (•) and 2nd dose-response (o) performed 15 minutes later. Vertical bars represent s.e.mean ($n$=9, *p<0.05) (100%=18.8±2.7mmHg).

Responses of the mesenteric vascular bed to perfused potassium, noradrenaline and phenylephrine were obtained to determine submaximal concentrations of agonist required to precontract tissues when examining interactions with palmitoyl carnitine (data not included).

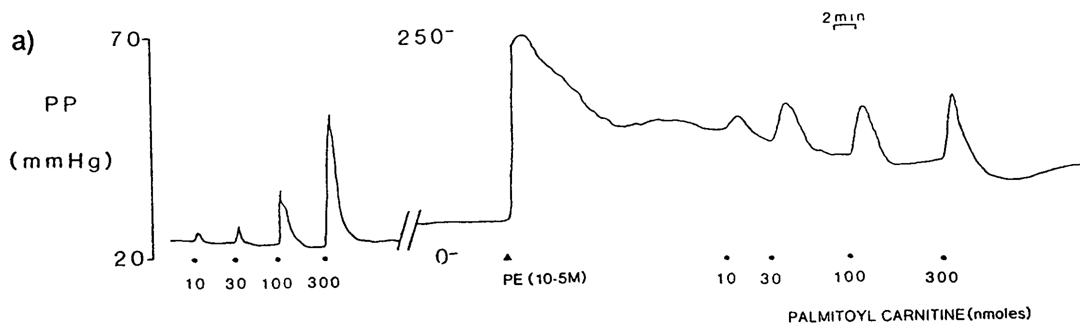
1. Adrenoceptor agonists.

Figure 10 shows the effect of the $\alpha_1$-adrenoceptor selective agonist phenylephrine on the vasoconstrictor action of palmitoyl carnitine in the mesenteric vascular bed. On addition of phenylephrine ($10^{-5}$M) to the perfusion medium, a concentration that produced 70-80% maximal contractile response, the perfusion pressure rose sharply from a basal level of 33.0±2.4mmHg, reached a peak several minutes later, and then declined to a plateau of 145.2±4.6mmHg. The vasoconstrictor effect of palmitoyl carnitine was greatly potentiated in the presence of phenylephrine, over a 400% increase being seen at the highest dose used, whilst a smaller late vasodilator component also became apparent although this varied in magnitude.

The perfusion presssure was affected in a similar manner by addition of noradrenaline (7µM) to the preparation as it was to phenylephrine; in this case the basal tone of the vascular bed was raised from a resting level of 31.5±1.1mmHg to 141.5±3.1mmHg. Likewise, a marked potentiation of the vasoconstriction produced by palmitoyl carnitine occurred in the presence of noradrenaline, a non-selective adrenoceptor agonist (fig.11).

The $\alpha_2$-agonist, clonidine, was also used to investigate whether a similar interaction occurred between palmitoyl carnitine and $\alpha_2$-adrenoceptor-mediated responses. Clonidine ($10^{-6}$M) raised basal tone in the preparation to 71.0±5.8mmHg, and under these conditions the vasoconstrictor responses to palmitoyl carnitine were also greatly potentiated (fig.12).

a)




b)

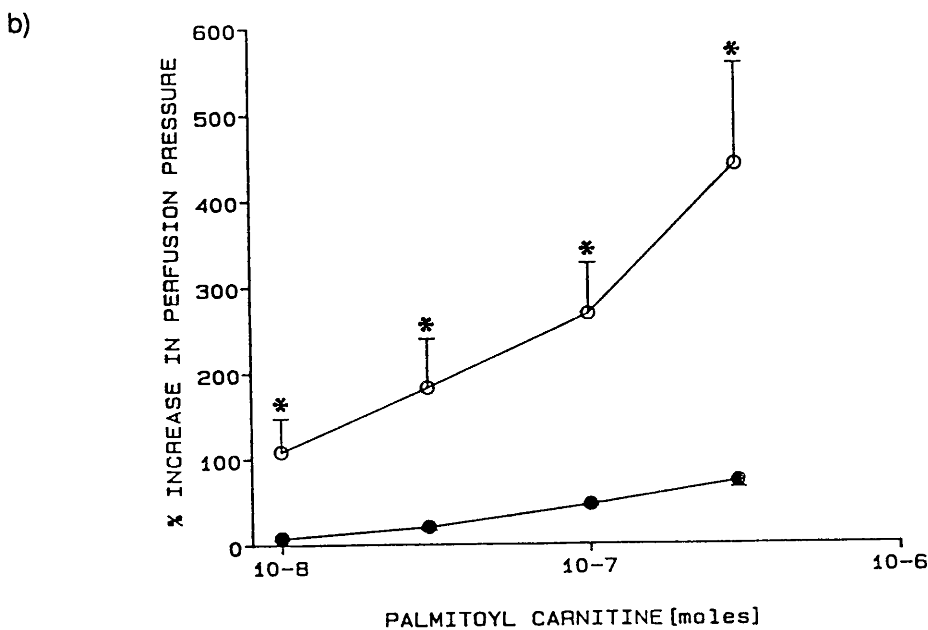



Figure 10. a) Typical trace showing the effects of palmitoyl carnitine (10-300nmoles) on perfusion pressure (PP) in the isolated perfused mesenteric vascular bed of the rat precontracted with phenylephrine (10μM). b) Effects of palmitoyl carnitine on perfusion pressure in phenylephrine-precontracted beds (o) (100%= 15.5±2.6mmHg, *n*=9) compared with those in time-matched control preparations (•) (100%=18.8±2.7mmHg , *n*=9). Vertical bars represent s.e.mean (*p<0.05).

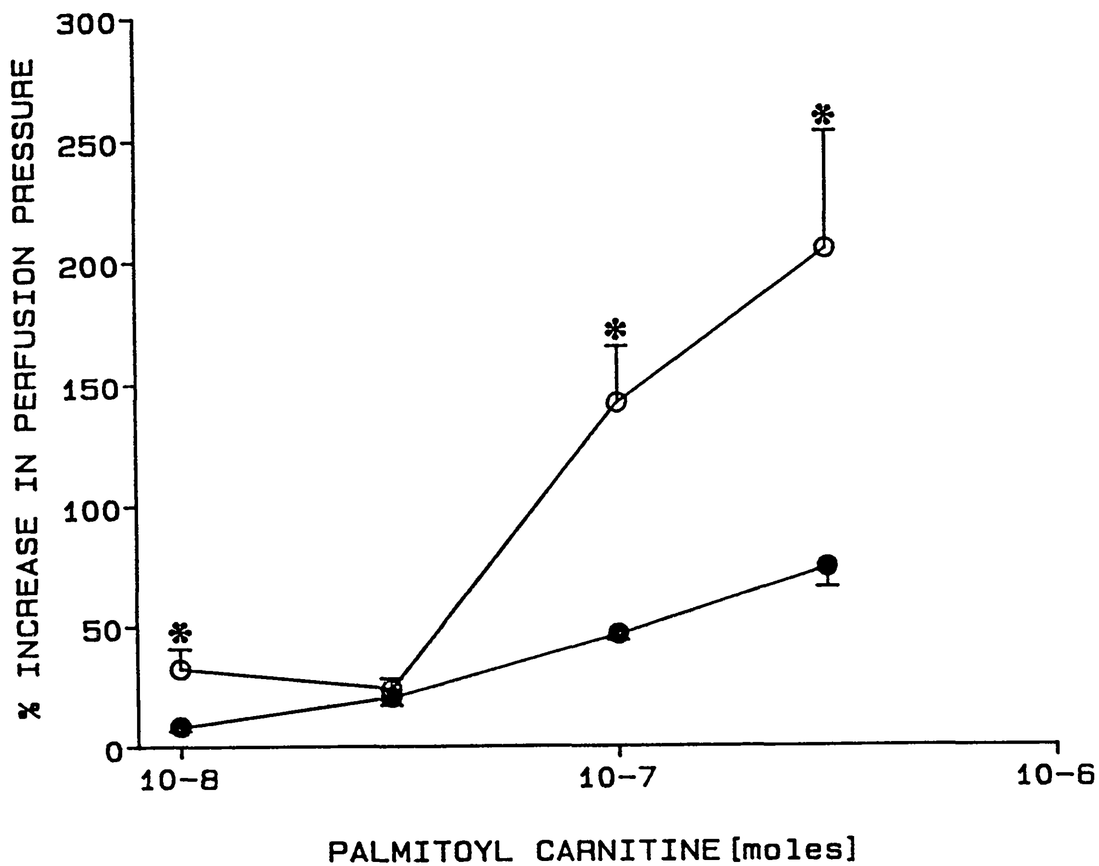



Figure 11. Effects of palmitoyl carnitine (10-300nmoles) on perfusion pressure in isolated perfused mesenteric vascular beds of the rat precontracted with noradrenaline (7μM) (o) (100% 29.0±0.6mmg, *n*=4) compared with those in time-matched control preparations (•) (100%=18.8±2.7mmHg, *n*=9). Vertical bars represent s.e.mean (*p<0.05).

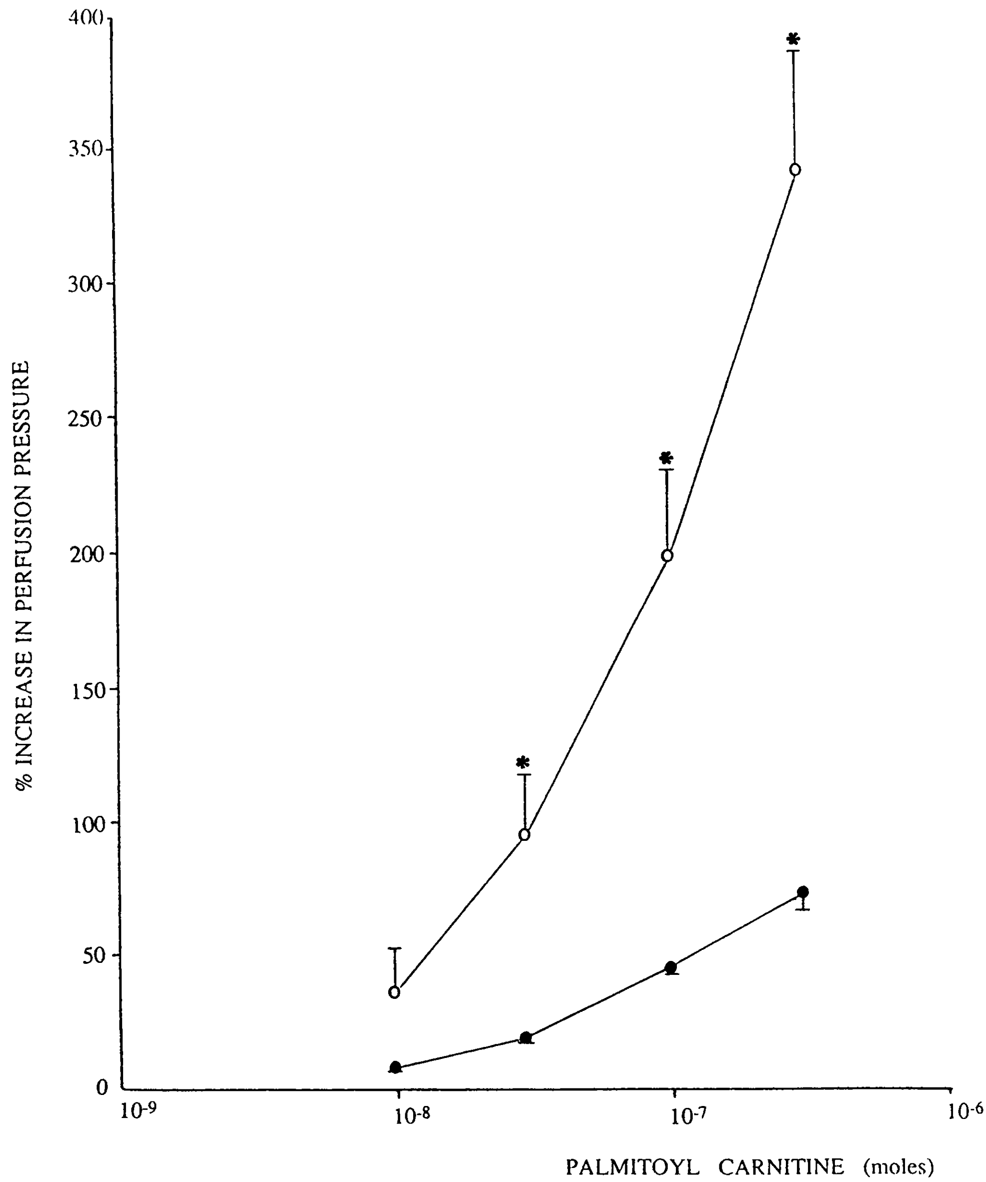



Figure 12. Effects of palmitoyl carnitine (10-300nmoles) on perfusion pressure in isolated perfused mesenteric vascular beds of the rat precontracted with clonidine (1μM) (o) (100%=18.2±2.3mmHg, $n$=4) compared with those in time-matched control preparations (●) (100%=18.8±2.7mmHg, $n$=9). Vertical bars represent s.e.mean (*$p<0.05$).

Finally, to see whether there might be some interaction of palmitoyl carnitine with $\beta$-adrenoceptors, some preparations were perfused with isoprenaline ($10^{-5}$M) using the same protocol as before. Isoprenaline affected neither the basal tone of the preparation nor altered the response to palmitoyl carnitine (fig.13).

In order to assess whether these potentiations were specific and not merely a result of raising the basal tone of the preparation, the effects of other vasoconstrictor agents on the action of palmitoyl carnitine were investigated.

2. Potassium.

Perfusion of the vascular bed with a modified Krebs-Henseleit solution containing 65mM potassium (sodium chloride reduced to maintain osmolarity) induced an increase of tone in the preparation from a resting value of 34.6±1.7mmHg to 88.4±2.6mmHg. Figure 14 shows that at this potassium concentration there was no potentiation of the vasoconstrictor response to palmitoyl carnitine. In contrast to this, the situation in the presence of a higher potassium concentration was more complicated (fig.15). Basal perfusion pressure in the vascular bed was raised from 32.6±3.1mmHg to 159.4±10.0mmHg by increasing the level of potassium to 110mM in the perfusion medium. Under these conditions the responses to palmitoyl carnitine varied; at lower doses (10-30nmoles) 50% of the preparations exhibited vasoconstrictions and 50% vasodilations, a dose of 100nmoles elicited vasoconstriction in 75% of tissues, whilst at the highest dose used (300nmoles) there was always a vasoconstrictor response of the mesenteric vessels to palmitoyl carnitine (fig.15).

3. Endothelin.

Endothelin, a potent vasoconstrictor peptide, was also used to raise vascular tone in the mesenteric bed (fig.16). Perfusion of this drug increased basal pressure from 36.8±2.3mmHg to 150.4±12.0mmHg although, in contrast to previous agents used e.g. phenylephrine, noradrenaline and potassium, endothelin induced a much

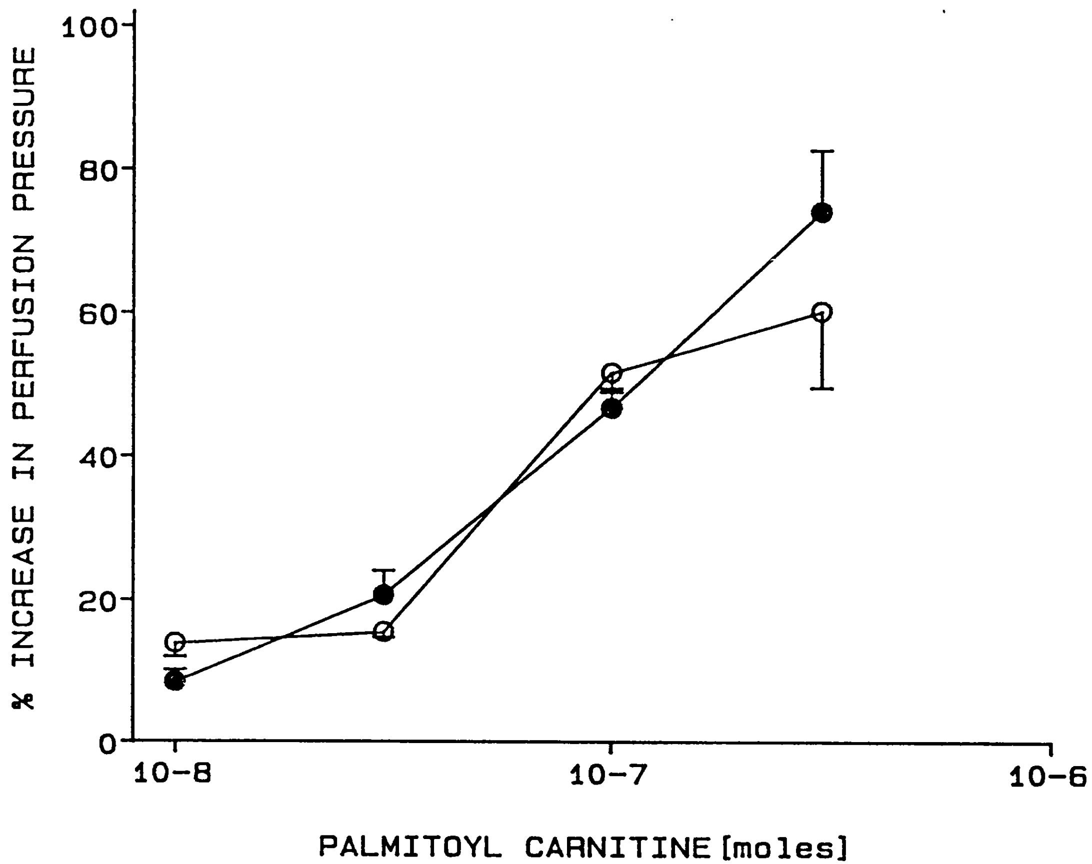



Figure 13. Effects of palmitoyl carnitine (10-300nmoles) on perfusion pressure in isolated mesenteric vascular beds of the rat in the presence of isoprenaline (10µM) (o) (100%=27.6±7.2mmHg, *n*=3) compared with those in time-matched control preparations (●) (100%=18.8±2.7mmHg, *n*=9). Vertical bars represent s.e.mean (*p<0.05).

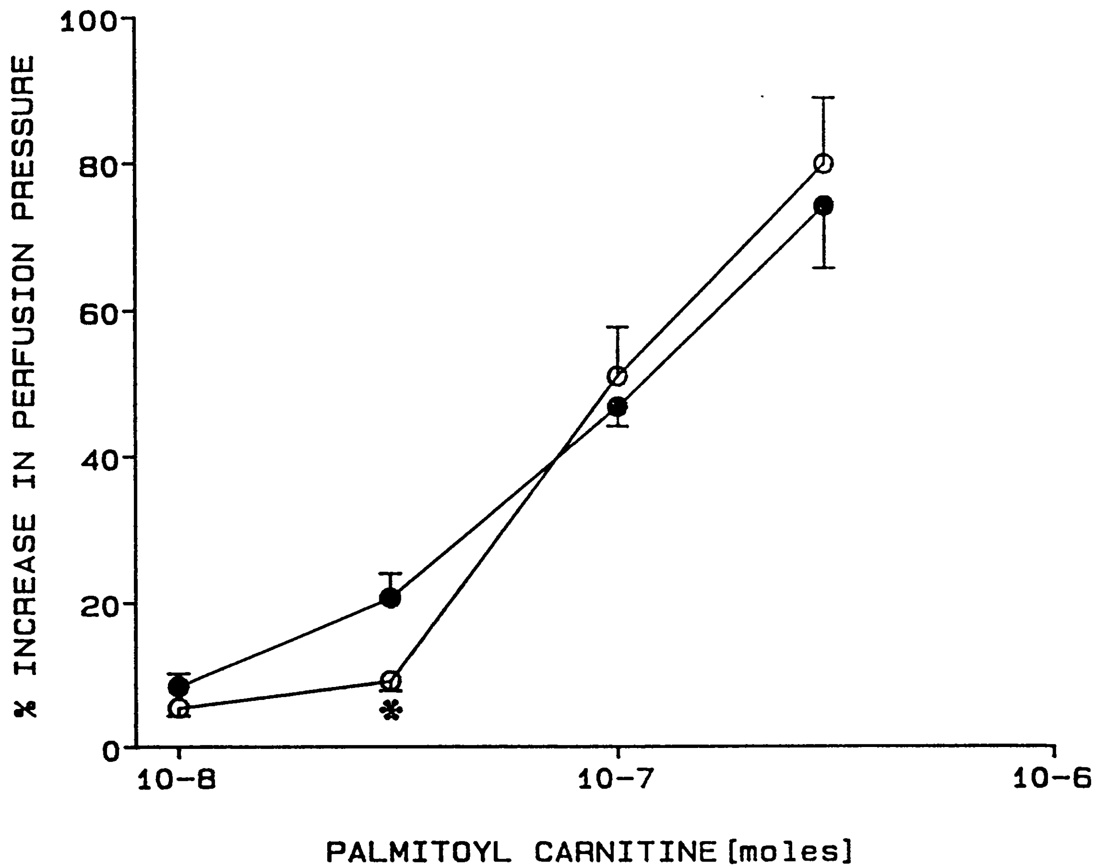



Figure 14. Effects of palmitoyl carnitine (10-300nmoles) on perfusion pressure in isolated mesenteric vascular beds of the rat precontracted with high potassium (65mM) (o, 100%=29.2±4.4mmHg, *n*=5) compared with those in time-matched control preparations (•) (100%=18.8±2.7mmHg, *n*=9). Vertical bars represent s.e.mean (*p<0.05).

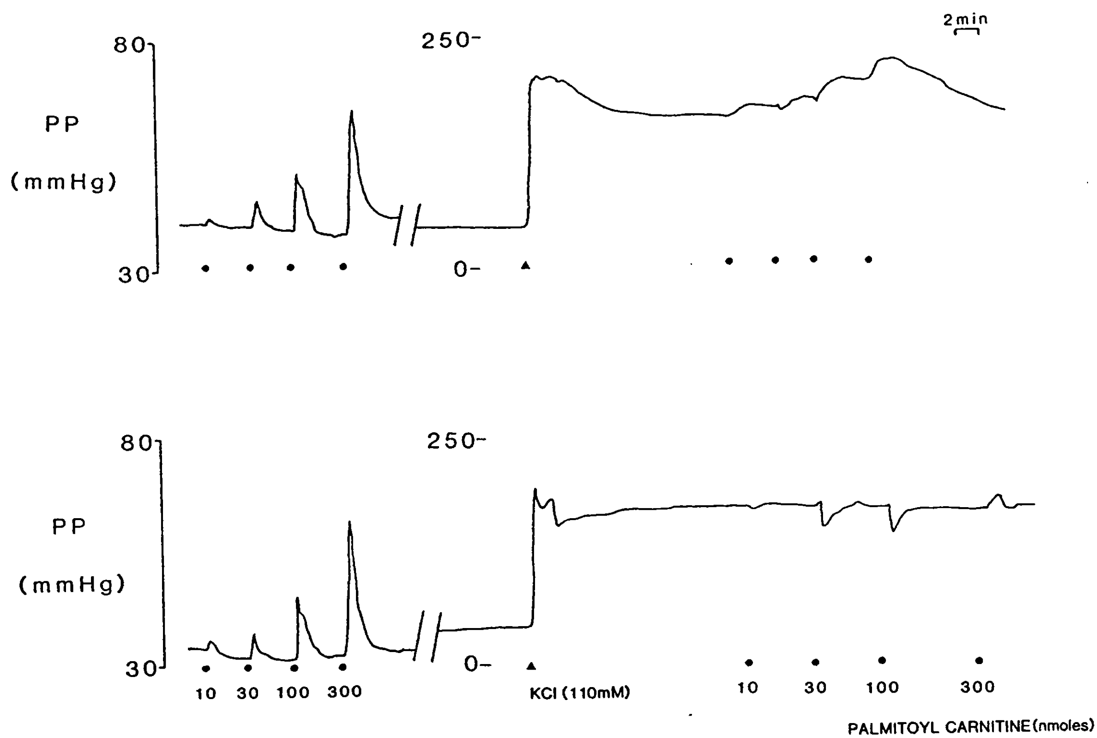



Figure 15. Sample traces illustrating the variability in response to palmitoyl carnitine (10-300nmoles) of perfusion pressure (PP) in isolated perfused mesenteric vascular beds precontracted with high potassium (110mM) ($n$=8).

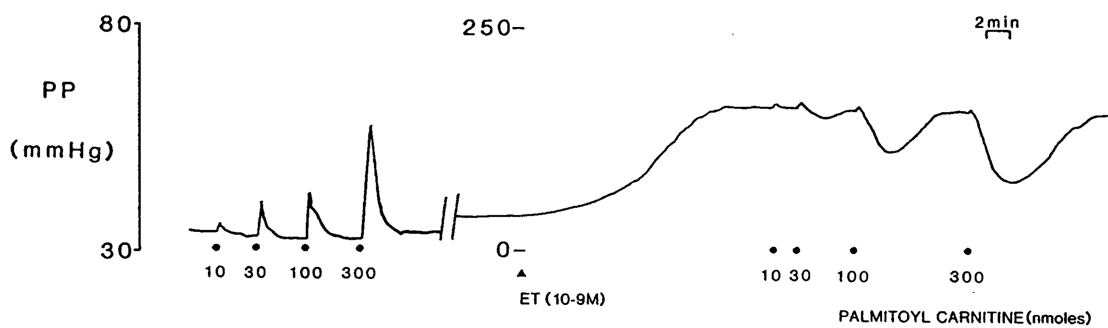



Figure 16. Typical trace showing the effects of palmitoyl carnitine (10-300nmoles) on perfusion pressure (PP) in isolated perfused mesenteric vascular beds of the rat precontracted with endothelin (1nM).

slower rise in perfusion pressure. In the presence of endothelin ($10^{-9}$M), palmitoyl carnitine now elicited dose-related dilations of the mesenteric vessels (figs.16 & 17a) which showed recovery at all doses tested. In some preparations these dilations were preceded by small vasoconstrictions, however, these were not significantly greater than control values (fig.17b).

4. Angiotensin.

Another potent vasoconstrictor peptide, angiotensin II, was also investigated for possible interaction with palmitoyl carnitine. However, several different batches of drug tested were without effect on perfusion pressure in the isolated mesenteric vascular bed of the rat at concentrations of up to 10µM (*n*=6). Figure 18 shows the effect of angiotensin II on the vasoconstrictor action of palmitoyl carnitine; no potentiation of the response was observed.

c) EFFECT OF PHENTOLAMINE ON POTENTIATION OF VASCULAR RESPONSE TO PALMITOYL CARNITINE.

Having established that an interaction existed between palmitoyl carnitine and $\alpha$-adrenoceptor-mediated responses, the mechanism underlying this effect was investigated by examining the action of the competitive $\alpha$-adrenoceptor antagonist phentolamine, on the potentiation of the vasoconstrictor action of palmitoyl carnitine in the presence of phenylephrine. A similar protocol was adopted to previous experiments, except that following a plateau response to phenylephrine, phentolamine ($10^{-7}$M), a concentration that maximally inhibited the contractile response to phenylephrine (10µM) (*n*=4, data not shown), was introduced into the perfusion medium for 15 minutes. In the presence of phentolamine the basal perfusion pressure of the mesenteric vascular bed was reduced from 147±12.6mmHg to 38.5±8.2mmHg, and under these conditions the vasoconstriction elicited by palmitoyl carnitine was no longer potentiated (fig.19).

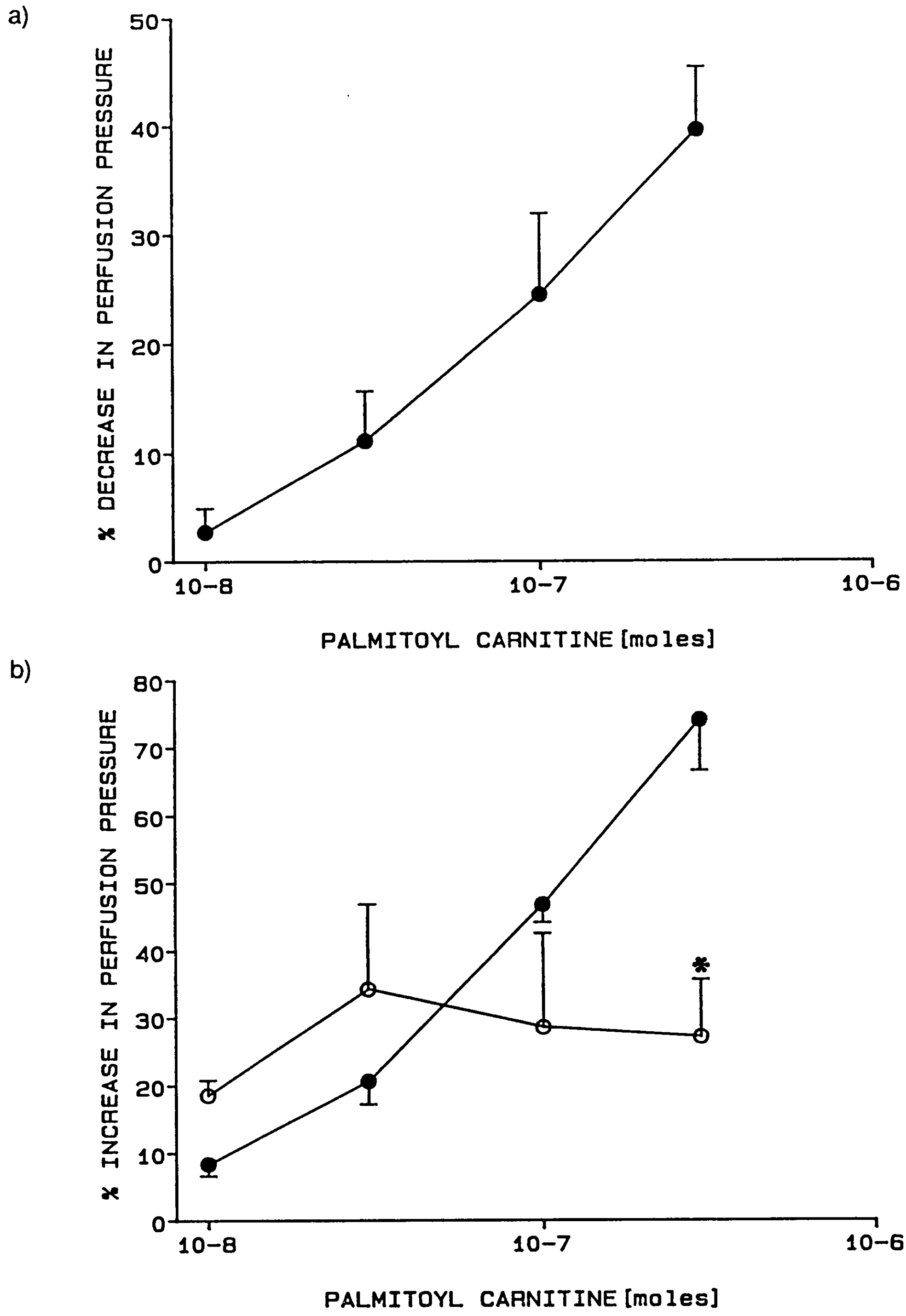



Figure 17. a) Vasodilator effects of palmitoyl carnitine (10-300nmoles) expressed as % fall in perfusion pressure of induced vascular tone, in isolated perfused mesenteric vascular beds of the rat precontracted with endothelin (*n*=5). b) Constrictor effects of palmitoyl carnitine (10-300nmoles) in endothelin-precontracted mesenteric beds (o) (100%=20.4±5.2mmHg, *n*=5) compared with those in time-matched control preparations (•) (100%=18.8±2.7mmHg, *n*=9). Vertical bars represent s.e.mean (*p<0.05).

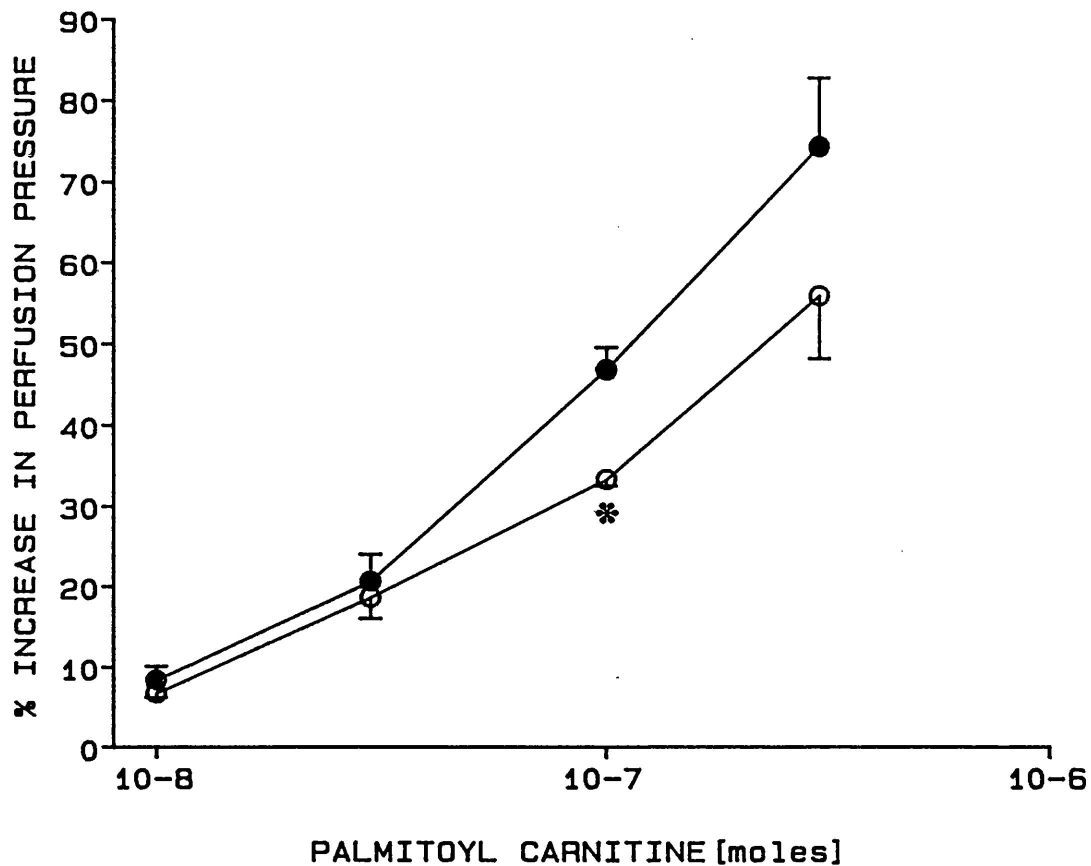



Figure 18. Effects of palmitoyl carnitine (10-300nmoles) on perfusion pressure in isolated perfused mesenteric vascular beds of the rat in the presence of angiotensin II (10nM) (o) (100%=18.5±4.0mmHg, $n$=4) compared with those in time-matched control preparations (•) (100%=18.8±2.7mmHg, $n$=9). Vertical bars represent s.e.mean (*$p<0.05$).

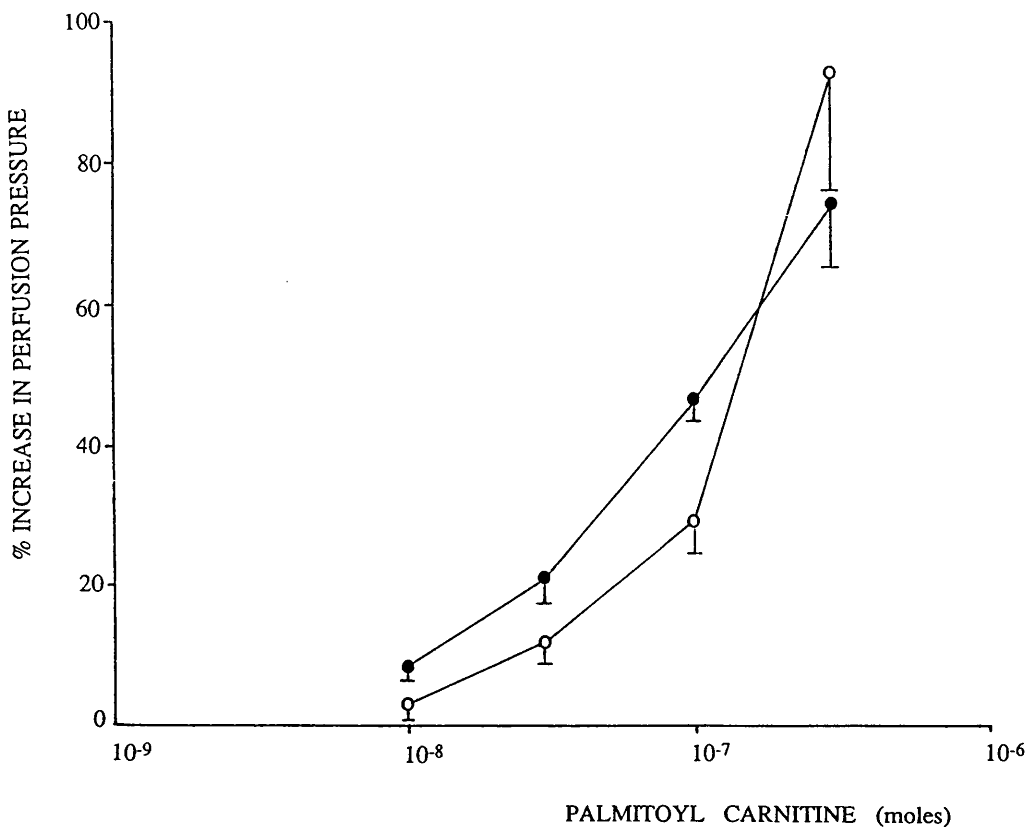



Figure 19. Effects of palmitoyl carnitine (10-300nmoles) on perfusion pressure in isolated perfused mesenteric vascular beds of the rat precontracted with phenylephrine (10μM) and subsequently exposed to phentolamine ($10^{-7}$M) (o) (100%= 13.8±1.9mmHg, *n*=4) compared with time-matched control preparations (•) (100% =18.8±2.7mmHg, *n*=9). Vertical bars represent s.e.mean (*$p<0.05$).

d) EFFECT OF ANTAGONISTS ON THE VASCULAR RESPONSE TO PALMITOYL CARNITINE.

In order to investigate the mechanism of palmitoyl carnitine-induced vasoconstriction in the mesenteric vascular bed, the use of specific blocking agents was employed. The effects of phentolamine and verapamil were examined to assess the possible involvement of $\alpha$-adrenoceptor or calcium channel activation, respectively, in the action of palmitoyl carnitine. The protocol was the same as that used to study agonist interactions (see above) with the antagonists being in contact with the tissue for 10 minutes prior to repeating the dose-response to palmitoyl carnitine.

Figure 20a shows the effect of phentolamine on vasoconstrictor responses to bolus injections of phenylephrine in the mesenteric vascular bed. There is a parallel, rightward shift in the dose-response curve with increasing concentrations of phentolamine, as would be expected with this competitive $\alpha$-adrenoceptor antagonist; the concentration chosen for subsequent experiments was $10^{-8}$M. At this concentration there was no effect on the vasoconstrictor activity of palmitoyl carnitine (fig.20b).

Verapamil (0.5 & 5.0 µM) was perfused through the vascular bed and at neither concentration was there any blockade of the response to palmitoyl carnitine (fig.21).

e) EFFECT OF PERFUSED PALMITOYL CARNITINE ON RESPONSES TO PHENYLEPHRINE.

To further examine the interaction of palmitoyl carnitine with phenylephrine, the effect of perfused acyl carnitine on the vascular response to bolus injections of phenylephrine was investigated in the mesenteric vascular bed of the rat.

Dose-response curves to phenylephrine were constructed with time allowing 30 minutes washout period between each curve. A typical trace of the vasoconstriction produced by phenylephrine is illustrated in figure 22a. Sensitivity of the mesenteric

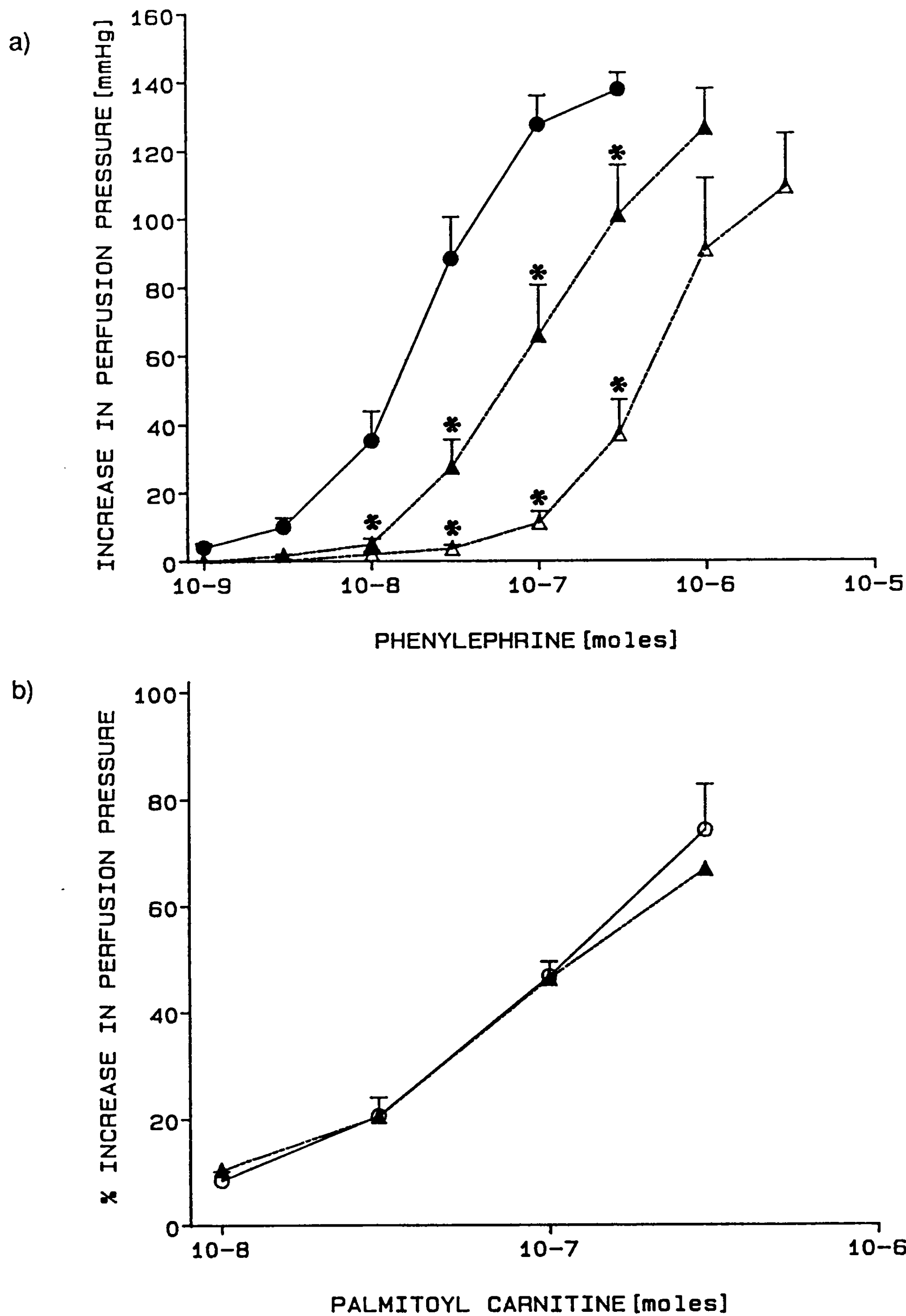



Figure 20. a) Control effects of phenylephrine (1-300nmoles) (●, *n*=4) on perfusion pressure and the antagonism of its constrictor action by phentolamine ($10^{-8}$M) (▲, *n*=4) & $10^{-7}$M (△, *n*=4) in the isolated perfused mesenteric vascular bed of the rat (*p<0.05). b) Effects of palmitoyl carnitine (10-300nmoles) on perfusion pressure in the mesenteric vascular bed of the rat in the presence of phentolamine ($10^{-8}$M) (▲, 100%=27.0±1.8mmHg, *n*=4) compared with those in time-matched control preparations (o) (100%=18.8±2.7mmHg, *n*=9) (*p<0.05).

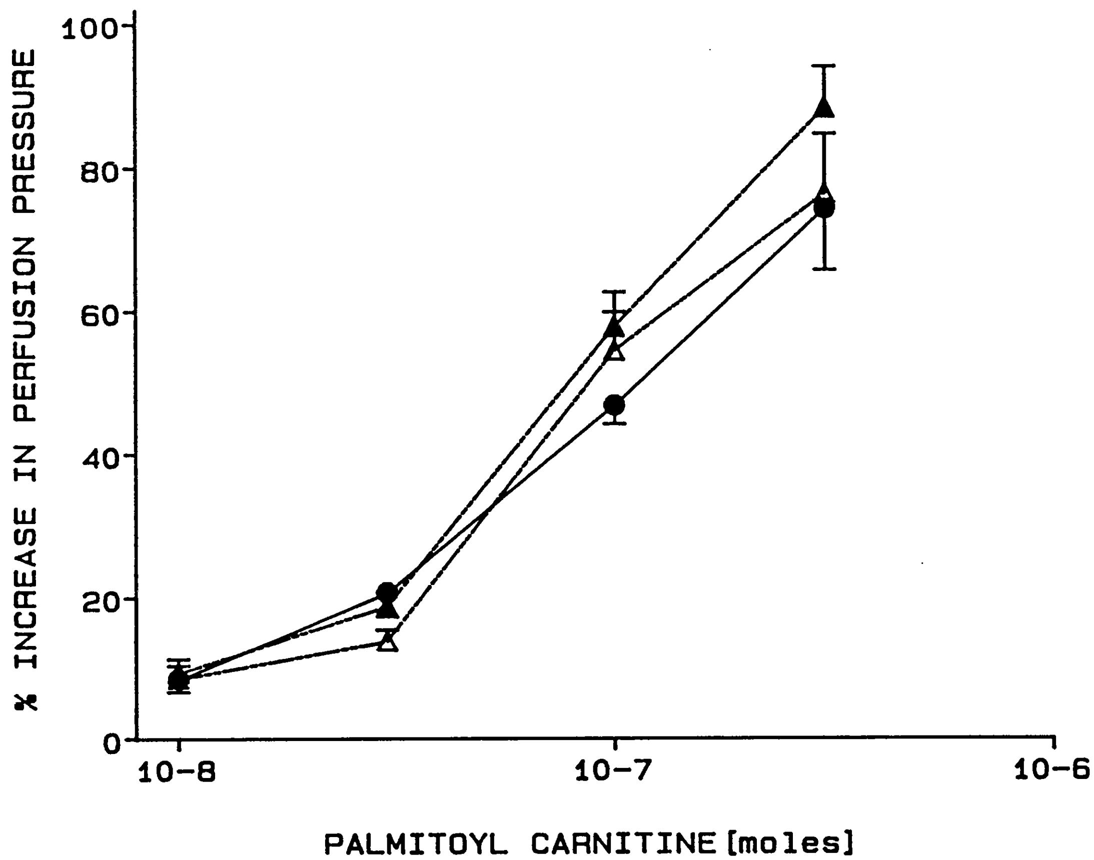
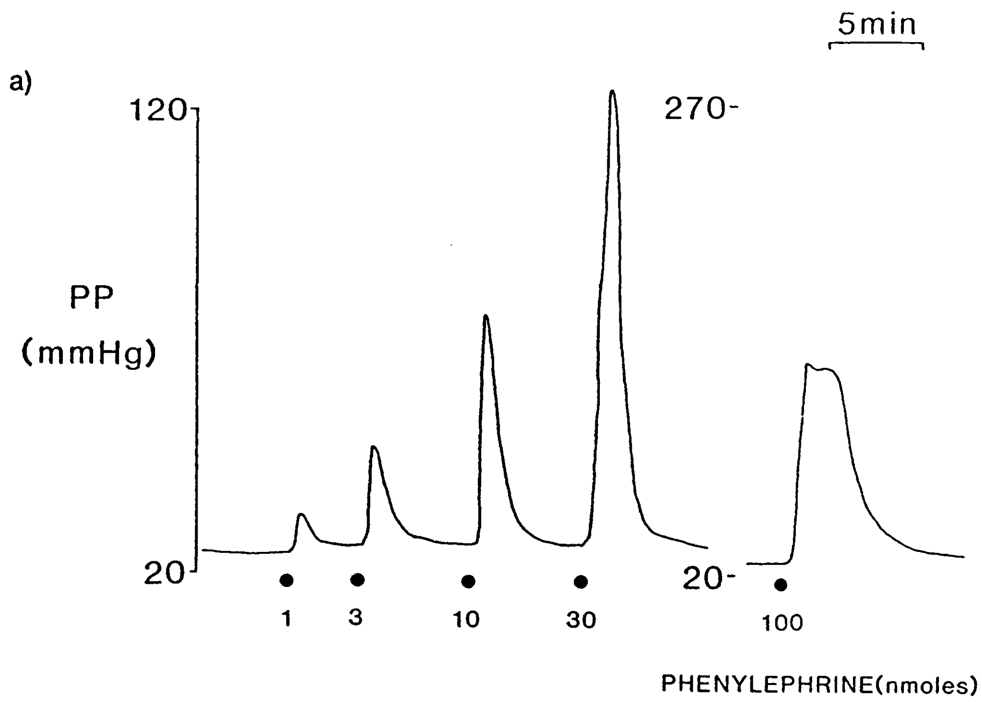



Figure 21. Effects of palmitoyl carnitine (10-300nmoles) on perfusion pressure in the isolated perfused mesenteric vascular bed of the rat in the presence of verapamil 0.5μM (▲) & 5.0μM (△) (100%=28.5±2.4mmHg, *n*=8) compared with those in time-matched control preparations (●, *n*=9) (*p<0.05).



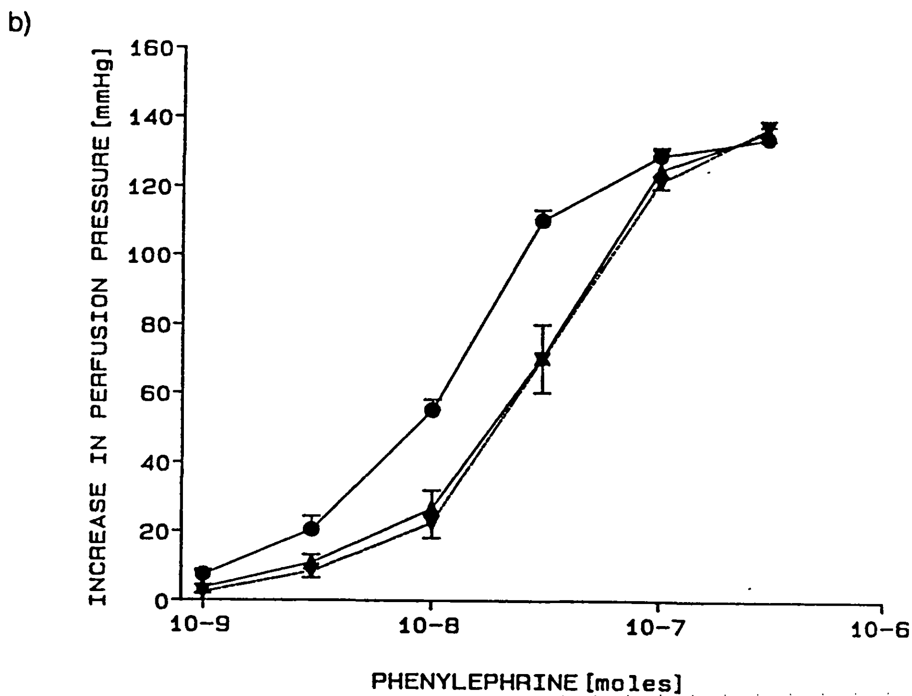




Figure 22. a) Typical trace showing the effects of bolus injections of phenylephrine (1-100nmoles) on perfusion pressure (PP) in the isolated mesenteric vascular bed of the rat. b) Effects of repeated administration of phenylephrine (1-300nmoles) on perfusion pressure in the mesenteric vascular bed of the rat, shown as control (●), 2nd (▲) & 3rd (▼) dose-response curves, performed with 30 minute intervals ($n$=4).

vessels to phenylephrine varied with time (fig.22b) so any subsequent experiments investigating possible interaction with palmitoyl carnitine, involved comparison with time-matched controls.

The effect of perfused palmitoyl carnitine on the vasoconstrictor action of phenylephrine is shown in figure 23. The response to phenylephrine was not altered by $10^{-6}$M palmitoyl carnitine, however, a ten-fold increase in acyl carnitine concentration resulted in a depression of the vasoconstriction at higher doses (30-300nmoles) and a significantly reduced maximal response to phenylephrine.

f) EFFECT OF LYSOPHOSPHATIDYLCHOLINE IN THE MESENTERIC VASCULAR BED.

The action of palmitoyl lysophosphatidylcholine (LPC), a structurally related amphiphile to palmitoyl carnitine, was also investigated in the perfused mesenteric vascular bed. This compound (10-300nmoles) elicited small rises in perfusion pressure when applied as bolus injections to the quiescent bed (fig.24) similar to those observed with palmitoyl carnitine.

Using the previously described protocol, potential interactions between LPC and two vasoactive compounds, phenylephrine and endothelin, were examined. Phenylephrine ($10^{-5}$M) raised the perfusion pressure from a basal level of 37.2±3.5mmHg to 111.2±21.7mmHg (fig.24), and in the presence of this drug, LPC induced large biphasic responses; there were now dose-related vasoconstrictions followed by slower, more sustained relaxations of the mesenteric vasculature (fig.24). A similar profile of response was exhibited when vascular tone was raised using endothelin ($10^{-9}$M) (fig.24). The peptide, endothelin, induced a much slower rise in perfusion pressure than did phenylephrine, increasing tone from 34.0±3.3mmHg to 89.2±13.7mmHg, and biphasic responses were also observed to bolus injections of LPC in the presence of this agent. Both pressor agents caused a marked potentiation of the vasoconstrictor action of LPC (30-300nmoles) and there was no appreciable difference between these effects (fig.25).

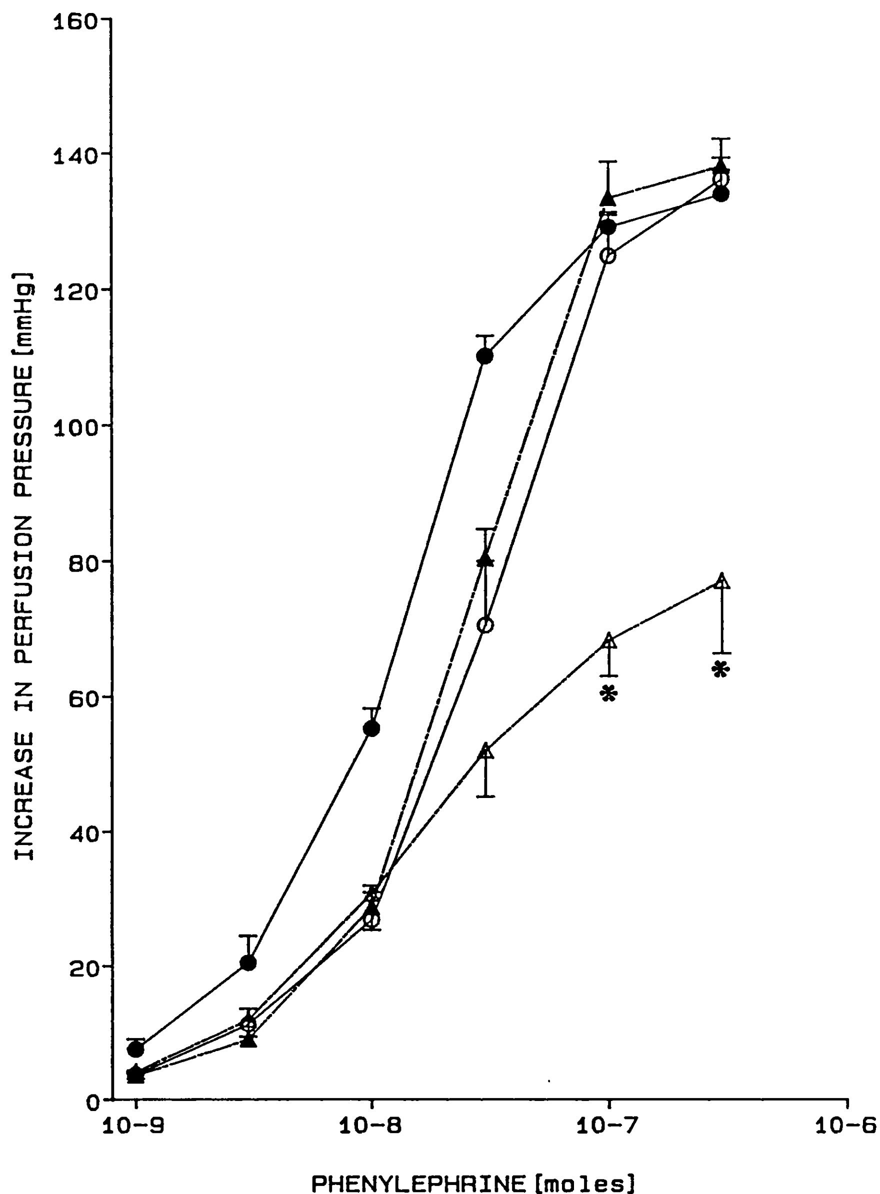



Figure 23. Effects of palmitoyl carnitine on the vasoconstrictor action of phenylephrine (1-300nmoles) in the isolated perfused mesenteric vascular bed of the rat. Constrictor responses to phenylephrine in the presence of $10^{-6}$M (▲, *n*=3) and $10^{-5}$M (Δ,*n*=3) palmitoyl carnitine, included in the perfusion medium 15 minutes after the initial dose-response curve (●, *n*=4), are compared with time-matched controls (o, *n*=4) (*$p<0.05$).

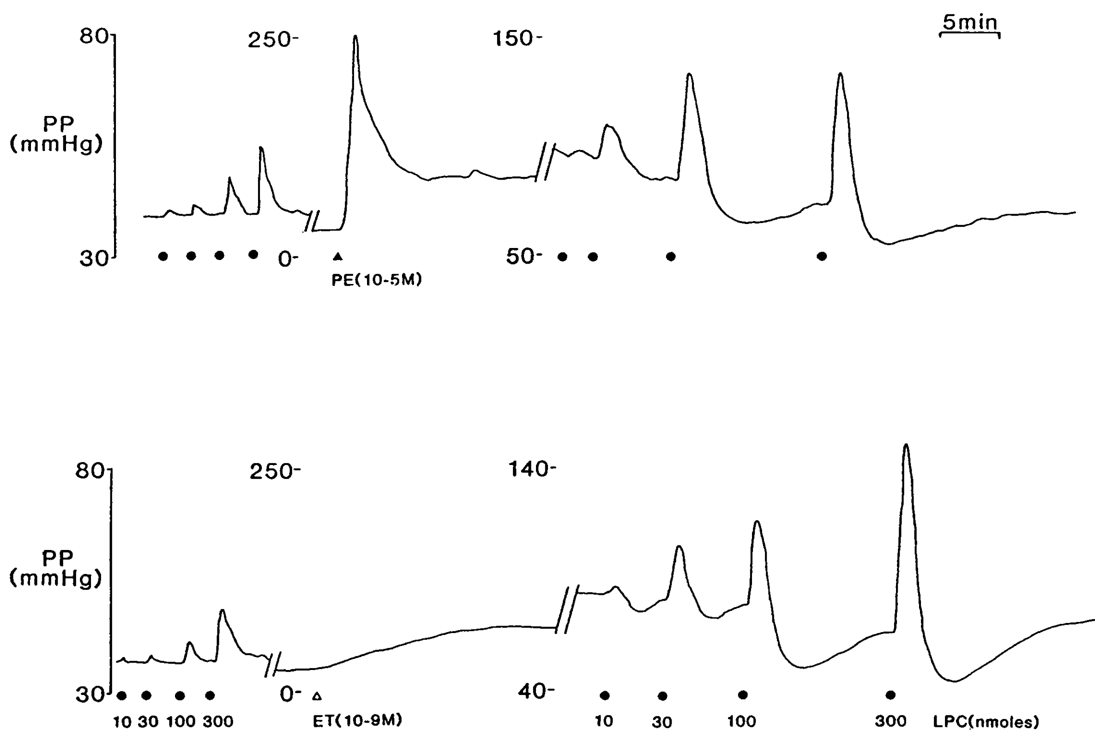



Figure 24. Typical traces showing the effects of lysophosphatidylcholine (LPC) (●, 10-300nmoles) on perfusion pressure (PP) in isolated perfused mesenteric vascular beds of the rat precontracted with phenylephrine (10μM) (*n*=4) (*upper trace*) and endothelin (*n*=4) (*lower trace*).

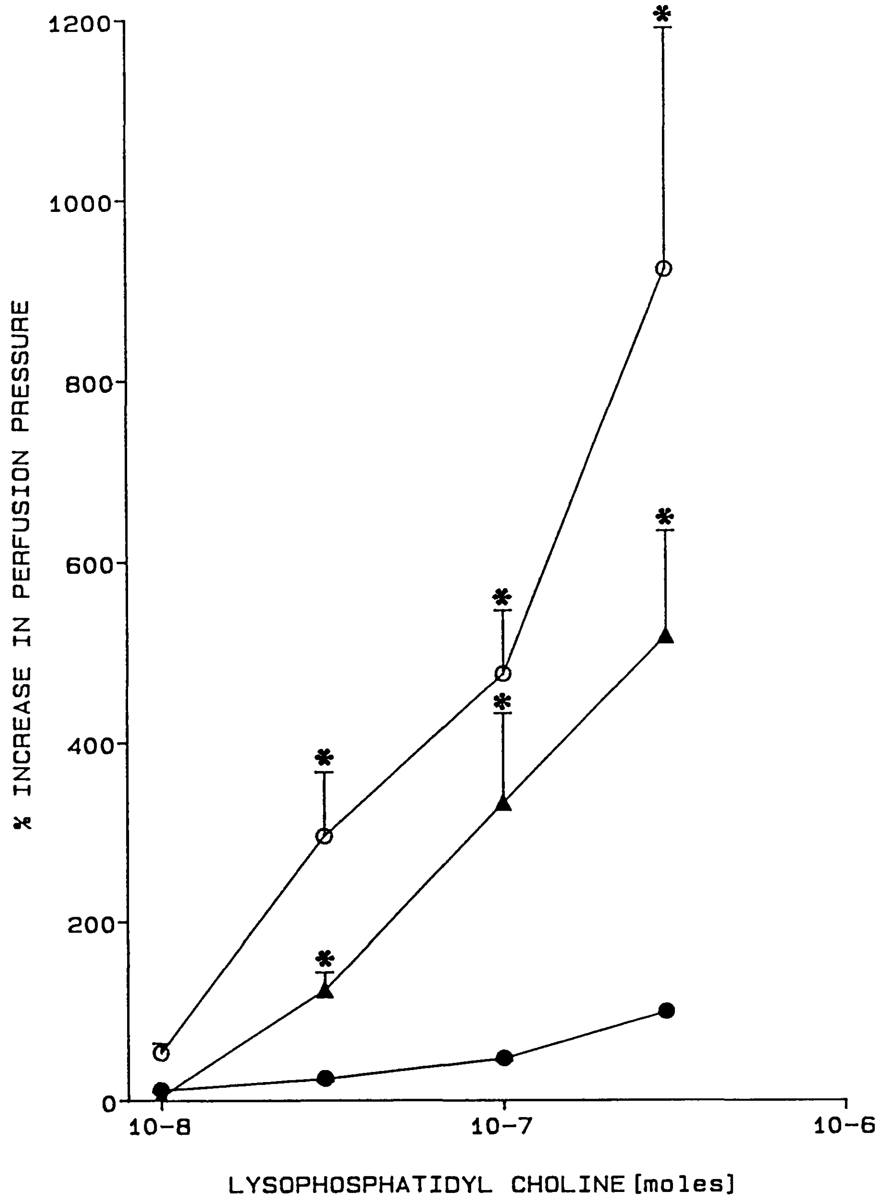



Figure 25. Effects of lysophosphatidylcholine (10-300nmoles) on perfusion pressure in the isolated perfused mesenteric vascular bed of the rat precontracted with either phenylephrine (10μM) (▲, $n$=4) or with endothelin (1nM) (o, $n$=4) compared with control values (●) (100% = 10.1±1.8mmHg, $n$=8) (*$p<0.05$).

### 3)THORACIC AORTA.

a) CONTROL RESPONSES.

Following experiments carried out in fine resistance vessels i.e. coronary and mesenteric vascular beds, the effects of palmitoyl carnitine on a larger blood vessel, the thoracic aorta, were investigated. Palmitoyl carnitine (1-64µM) had no effect on quiescent transverse aortic strips and at the higher concentrations tension recording was impaired by foaming of the organ bath due to the compound's detergent properties (data not shown, *n*=4).

b) INTERACTIONS WITH VARIOUS VASOACTIVE AGENTS.

Due to the lack of action of palmitoyl carnitine on the quiescent aorta, the effect of raising the basal tone of the preparation, to perhaps manifest some relaxant activity of the compound, was investigated. Initially this was performed using $10^{-7}$M phenylephrine, a concentration that produced a 70-80% maximal contractile response in the tissue. Following a stable plateau rise in tension to phenylephrine, the integrity of the endothelium was tested by addition of acetylcholine (1µM); less than 80% relaxation of the aortic strip was taken to be indicative of endothelial cell damage and these preparations rejected. The tissues were then washed and, following a period of recovery, were recontracted with phenylephrine.

Palmitoyl carnitine caused a gradual relaxation of the aorta in those tissues that possessed an intact endothelium (fig.26). Again, higher concentrations of the drug produced a foaming of the organ bath impairing accurate assessment of its action. In some tissues the endothelium was mechanically removed, and this significantly attenuated the relaxant effect of palmitoyl carnitine (fig.26). In addition, control preparations were set up in which phenylephrine was in contact with the tissues for the duration of the experimental period in the absence of palmitoyl carnitine; under these conditions the plateau contraction to phenylephrine was maintained.

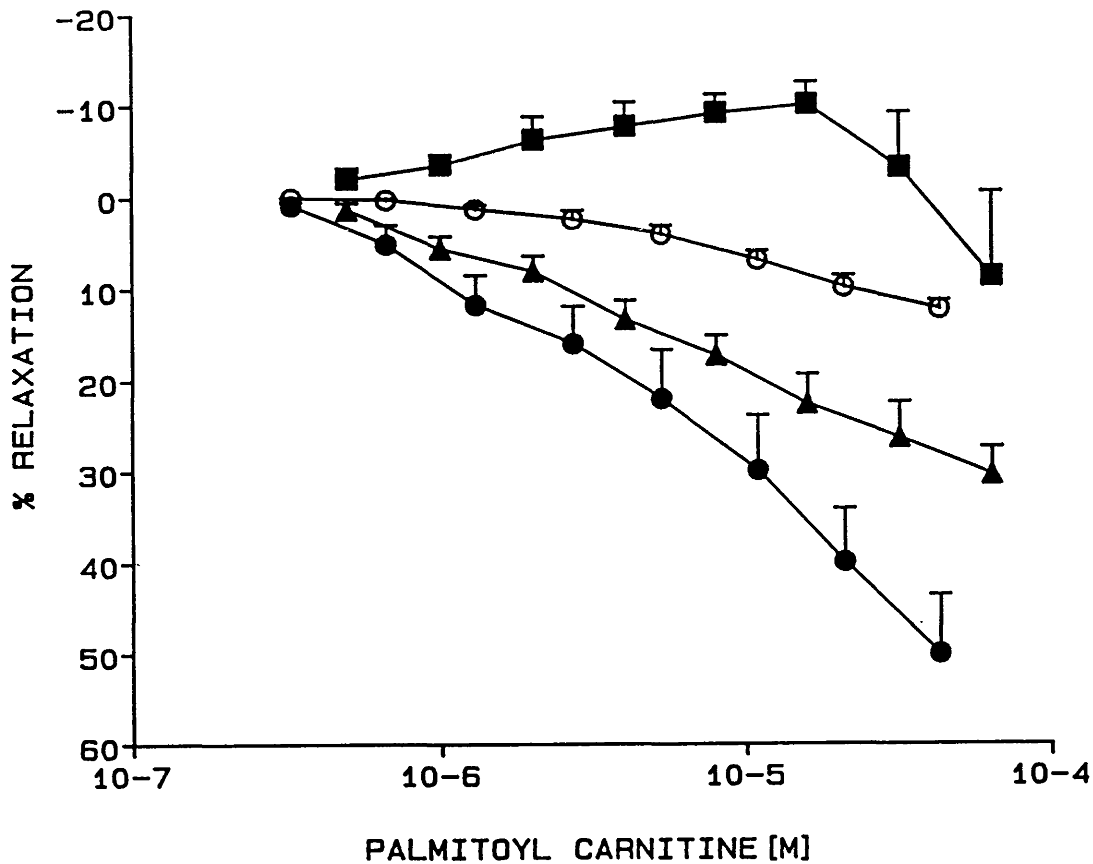



Figure 26. Relaxant effects of palmitoyl carnitine (0.3-64µM) on rat aorta precontracted with phenylephrine ($10^{-7}$M) either with endothelium (●, 100%=0.75±0.02g (*n*=7)) or without endothelium (o, 100%=0.88±0.06g (*n*=7)), with endothelin (1nM) (■, 100%=0.38±0.07g (*n*=4), or with high potassium (110mM) (▲, 100%=0.73±0.09g (*n*=4)).

High potassium (110mM) was also used to raise tone in aortic preparations and in this instance tissues were prepared as rings. The rise in tension elicited by potassium was comparable to that of phenylephrine, however, palmitoyl carnitine did not relax the tissues to such an extent in the presence of this agent (fig.26 & 27). However, in the presence of high potassium the integrity of the endothelium was difficult to assess as relaxation to acetylcholine is virtually abolished *(Taylor et al.,1988).*

In certain preparations vascular tone was raised using endothelin-1 ($10^{-9}$M), and in these tissues there was no appreciable relaxation produced by palmitoyl carnitine (fig.26).

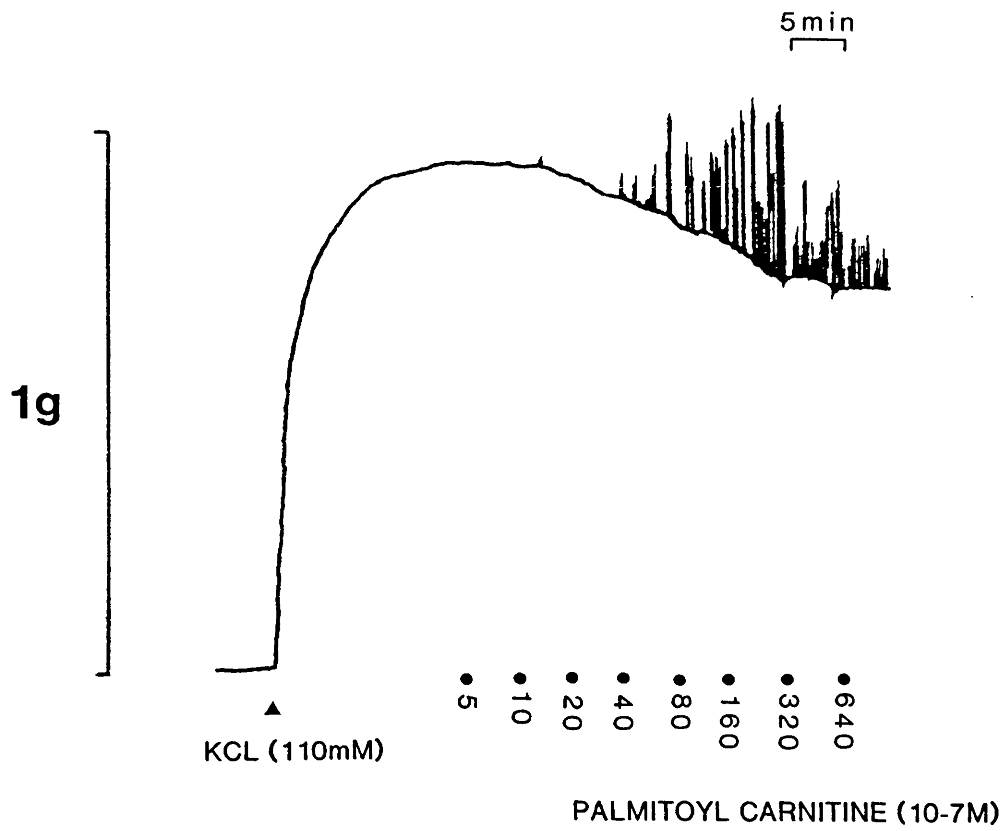



Figure 27. Typical trace showing the effects of palmitoyl carnitine (0.5-64µM) on tension in rat aorta precontracted with high potassium (110mM) ($n$=4).

# CHAPTER 4.

## DISCUSSION.

It has been suggested that long chain acyl carnitines can act as endogenous mediators of myocardial damage during ischaemia in view of their accumulation under such conditions *(Liedtke et al., 1978; Corr et al., 1984)* and ability to produce effects similar to those observed during ischaemia *in vivo* when applied exogenously to tissues *in vitro (Corr et al., 1984)*. The detrimental effects of acyl carnitines on the heart are confirmed by the present study in which palmitoyl carnitine caused an irreversible depression of contractility in the Langendorff perfused rat heart when administered as bolus injections. However, conflicting reports exist regarding the action of palmitoyl carnitine and related compounds on the myocardium. For example, a positive inotropic action of palmitoyl carnitine has been demonstrated in avian ventricular muscle *(Inoue & Pappano, 1983)* and in chick myocytes *(Patmore et al., 1989)*, whilst other workers have reported opposite effects on contractility in perfused hearts *(Hulsmann et al., 1985; Busselen et al., 1988)*. This large variation in reported effects of long chain acyl carnitines on myocardial contractility may have arisen due to differences in the concentration of drug used *(Nakaya & Tohse, 1986)*, mode of application, experimental conditions or perhaps more importantly the particular tissues investigated. In this present study, however, the putative detrimental role of long chain acyl carnitines in the myocardium is supported.

In contrast to the many reports regarding the action of long chain acyl carnitines on isolated cells, subcellular fractions or purified enzyme systems, there have been few studies examining the effects of such compounds on intact blood vessels. In the isolated perfused Langendorff heart palmitoyl, myristoyl and stearoyl carnitines exerted a predominantly vasoconstrictor action on the coronary circulation, whereas carnitine itself was without effect on the coronary vessels, suggesting that a fatty acid chain is required for the vascular activity of the molecule. In other vascular preparations examined, palmitoyl carnitine elicited primarily a vasoconstrictor response, however, a certain degree of relaxant activity was observed with this compound in situations where vascular tone had been artificially raised, for example, in endothelin-precontracted mesenteric vascular beds. The few existing reports regarding the action of acyl carnitines on blood

vessels appear essentially to be in accord with the present observations of the constrictor effects of these compounds. For example, Bigaud and Spedding (1986) observed that palmitoyl carnitine contracted rat aorta, whilst other studies have demonstrated that low concentrations of palmitoyl carnitine facilitated pressor responses to noradrenaline in the rat caudal artery *(Ugwu et al., 1987)* and that preincubation with the carnitine acyl transferase inhibitor POCA (sodium 2[5-(4-chlorophenyl)-pentyl]oxirane-2-carboxylate) prevented the hypoxia-induced augmentation of contractions to KCl in rat portal vein *(Fasehun et al., 1987)*. In the present study, however, palmitoyl carnitine was without effect on the quiescent aorta, a discrepancy with the above study that may have arisen due to differences in experimental conditions. For example, Bigaud and Spedding (1986) used a Krebs-Henseleit bathing solution containing 12mM $K^+$ in contrast to the lower concentration of 5.9mM $K^+$ used here, whilst also applying higher concentrations of the acyl carnitine.

Another feature of the vascular action of palmitoyl carnitine was that the magnitude of the vasoconstrictor response varied according to the blood vessels on which the drug was acting; it produced a powerful coronary vasoconstriction, a weaker effect on the mesenteric vessels and no effect on the quiescent aorta. This may therefore reflect a degree of vascular selectivity of the drug for fine resistance vessels, however, these results may also arise as a consequence of differences in mode or rate of delivery of compound to the tissues.

Two fundamental questions arise from the preceding observations; how does palmitoyl carnitine produce its vascular effects and why does such variation exist in its action with regard to different blood vessels? Palmitoyl carnitine is an amphiphile, a molecule that contains both hydrophobic and hydrophilic moieties, a long palmitoyl chain and the charged quaternary nitrogen and carboxyl groups, respectively, and previously it has been suggested that the biological effects of these fatty acid derivatives are attributable to this amphiphilic property *(Idell-Wenger et al., 1978; Katz & Messineo, 1981)*. Amphiphiles at low concentrations exist as

monomers and can insert into the hydrophobic environment of the lipid membrane thereby influencing fluidity *(Watanabe et al., 1989)*, whilst at higher concentrations, >13μM, this class of compound can aggregate into micelles *(Piper et al.,1984)*, in which the hydrophilic regions of the molecule remain in contact with the aqueous medium and the lipophilic portions are clustered in a hydrophobic core. In these various states amphiphiles can physically disrupt the lipid membrane via a non-specific "detergent" action. In addition, it has been suggested that as a consequence of such incorporation into membranes amphiphiles may displace $Ca^{2+}$ ions from negatively charged binding sites on the membrane phospholipids *(Katz & Messineo, 1981; Philipson et al., 1985)* and modulate the function of integral membrane proteins by influencing the local lipid environment *(Helenius & Simons, 1975; Sanderman, 1978)*. Furthermore, there have been a large number of studies carried out regarding the actions of long chain acyl carnitines on various enzymes including $Na^+/K^+$-ATPase *(Adams et al., 1979; Owens et al., 1982)*, sarcoplasmic reticulum $Ca^{2+}$-ATPase *(Pitts et al., 1978; Adams et al., 1979)* and protein kinase C *(Katoh et al., 1981)*, and it has been suggested that many of these effects may simply be due to non-specific membrane disruption allowing increased permeability to both $Na^+$ and $Ca^{2+}$ ions *(Lamers et al., 1984)*.

Can such non-specific amphiphilic actions, therefore, be responsible for the effects observed with palmitoyl carnitine in this present study? Certainly membrane disruption allowing leakage of ions across the bilayer would tend to cause an influx of extracellular calcium ions resulting in constriction of blood vessels, as seen in the coronary and mesenteric vascular beds, however, palmitoyl carnitine should have exerted a similar constrictor action on the rat aorta as well. In addition, this acyl carnitine was also capable of eliciting relaxations of vascular tissue under certain conditions, suggesting a more complex profile of activity than that resulting purely from the compound's amphiphilic nature. Moreover, another amphiphile, palmitic acid, has been shown to dilate the coronary circulation *(Hulsmann, 1976)* and in this present study displayed effects distinct from those of its corresponding carnitine derivative. Several workers have also concluded that non-specific detergent effects

of palmitoyl carnitine are insufficient to account for its actions in certain experimental conditions, including effects on mitochondrial disruption following ischaemia *(Piper et al., 1984)* and interactions with calcium channels *(Spedding & Mir, 1987)*.

It appears likely, therefore, that there are more specific mechanisms underlying the effects of long chain acyl carnitines, and there are many possible targets for the action of palmitoyl carnitine including membrane calcium channels, intracellular calcium movement, second messenger systems, enzymes and contractile proteins.

In recent years it has been suggested that palmitoyl carnitine may be an endogenous activator of calcium channels *(Spedding & Mir, 1987)*. Previously it has been shown that amphiphiles can affect calcium binding *(Philipson et al., 1985)* and that palmitoyl carnitine increases the calcium current in avian ventricular muscle via an effect on membrane surface charge *(Innoue & Pappano, 1983)*. In 1987, Spedding and Mir demonstrated that this acyl carnitine resembled Bay K8644, the calcium channel activator, in its contractile effects on $K^+$-depolarised taenia coli preparations and in its selective displacement of tritiated calcium antagonists in binding studies using rat brain, although differences in potency between these two compounds were apparent. They suggested that palmitoyl carnitine was interacting either directly with the voltage sensitive channel (VOC) or with tightly associated lipids *(Spedding & Mir, 1987)*, however, they also expressed the need for caution in interpretation of results due to the amphiphilicity of the compound. Whether a specific activation of voltage sensitive calcium channels or a decrease in surface charge, leading to movement of extracellular calcium into the cell, the resultant action of palmitoyl carnitine in blood vessels would be to elicit vasoconstriction, an effect observed in the present study in both mesenteric and coronary vascular beds. The involvement of VOCs in the action of palmitoyl carnitine in the mesenteric vascular bed of the rat, however, appears doubtful in the present study since its vasoconstrictor action was not attenuated by verapamil, at a concentration known to inhibit contractions to high potassium in this preparation *(Kondo et al., 1980)*.

Another possible explanation of the vasoconstrictor action of palmitoyl carnitine may be that of specific receptor activation. As endogenous acyl carnitine preferentially accumulates in the sarcolemma *(Knabb et al., 1986)* it is possible that specific binding sites may exist on the cell membrane linked to ionic channels or to second messenger processes. Previously it has been suggested that many constrictor agents stimulate influx of extracellular calcium into the cell via common "receptor-operated" calcium channels (ROCs) that are distinct from VOCs *(Bolton, 1979; van Breemen et al., 1979)*. There have been reports showing that $^{45}Ca^{2+}$ entry into cultured aortic smooth muscle cells upon agonist stimulation occurs via pathways that are insensitive to dihydropyridines *(Wallnofer et al., 1987; Zschauer et al., 1987)* although the mechanisms and second messengers involved in mediating this effect are still unclear. It is possible, therefore, that palmitoyl carnitine may activate specific receptors linked to ROCs, thereby stimulating calcium entry into the cell and causing vasoconstriction, however, this action might also have been expected to be affected by verapamil in the present study as this calcium antagonist attenuates the response to noradrenaline in the rat mesenteric artery *(Kondo et al., 1980)*.

Palmitoyl carnitine can freely cross lipid membranes *(Levitsky & Skulachev, 1972)* and may therefore have an intracellular mode of action, perhaps causing release of calcium from stores in a manner similar to caffeine *(Leijten & van Breemen, 1984; Kanaide et al, 1987)*, inhibition of calcium sequestration, or maybe affecting intracellular second messenger systems. Contractile agonists such as noradrenaline have been shown to increase cellular levels of inositol triphosphate ($IP_3$), derived from membrane phospholipid hydrolysis, which causes mobilisation of intracellular calcium from sarcoplasmic reticulum *(Berridge & Irvine, 1984)* and it is possible that palmitoyl carnitine may exert its vasoconstrictor action in a similar manner. However, it appears more likely that palmitoyl carnitine would interact with pathways affected by the other product of phosphoinositide hydrolysis, diacylglycerol (DAG), in view of the structural similarities between the two compounds. DAG is an activator of protein kinase C *(Nishizuka, 1984)*, an enzyme

which phosphorylates a number of intracellular proteins including myosin light chain kinase (MLCK) *(Ikebe et al., 1985)*, smooth muscle myosin light chain *(Endo et al., 1982)* and the $\alpha$-adrenoceptor *(Sibley et al., 1984)*, and additionally has recently been postulated to increase the calcium sensitivity of the myofilaments *(Kamm & Stull, 1989; Sugiura et al., 1989)*. However, conflicting reports have appeared regarding the action of palmitoyl carnitine on protein kinase C, for example, palmitoyl carnitine has been shown to inhibit the purified enzyme from bovine heart *(Wise & Kuo, 1983)* whilst causing activation in rat mast cells *(Grosman, 1988)*. Phorbol esters such as phorbol 12,13-dibutyrate (PDB), which activate protein kinase C, have been shown to exhibit contractile effects on isolated blood vessels *(Danthuluri & Deth, 1984; Rasmussen et al., 1984)* and therefore it is possible that the vasoconstrictor action of palmitoyl carnitine observed in the present study may be explained by such a mechanism. There are many further possibilities to explain the vasoconstrictor action of palmitoyl carnitine including effects on intermediates in the contractile process such as calmodulin, although aliphatic amphiphiles have been shown to exert an inhibitory action on calmodulin-activated processes *(Isomaa et al., 1989)*, or direct effects on smooth muscle contractile proteins.

The apparent selectivity in the effects of palmitoyl carnitine with respect to blood vessels may be due to differences in the mechanism(s) involved in the contractile proccesses of different vessels or perhaps may arise as a consequence of the mode of application of the drug. Firstly, there are known to be differences in the involvement of intracellular calcium release in response to certain contractile agents e.g. noradrenaline, between fine resistance vessels, as contained in the mesenteric and coronary vascular beds, and large conductance vessels such as the thoracic aorta *(van Breemen & Siegel, 1980; Cauvin et al., 1982)*. Cauvin et al. (1982) demonstrated that the contribution of calcium release from intracellular sites to noradrenaline-mediated activation decreases while that of calcium influx increases, when moving from conduit to resistance vessels. Thus differences in the effects of palmitoyl carnitine, either directly or indirectly, on calcium influx via potential-

insensitive pathways may account for its seemingly more profound action on fine resistance vessels. Alternatively, there may be differences in sensitivity of intracellular enzymic pathways or second messenger systems to palmitoyl carnitine between conduit and resistance vessels, or perhaps variation in the contractile proteins involved in the development of vascular tone. For example, major proteins such as caldesmon, gelsolin, filamin and desmin are abundant in smooth muscle and may play an important role in contraction *(Rasmussen et al., 1987)* as well as the more widely recognised calmodulin and myosin light chain kinase. However, the apparent selectivity in the effects of the acyl carnitine may alternatively be due simply to a difference in the mode of application of the drug. For example, the flow rate used in the present study for perfusion of the coronary vascular bed was greater than that for the mesenteric vascular bed, potentially allowing a higher concentration of drug to arrive at the tissue when administered as a bolus injection into the perfusion stream. In contrast, palmitoyl carnitine was applied to aortic tissue as cumulative additions to the organ bath, exposing the tissue to steadily increasing concentrations of drug.

Although the predominant action of palmitoyl carnitine is that of vasoconstriction, a certain degree of vascular relaxant activity was associated with this drug under conditions of artificially elevated vascular tone e.g. in the coronary circulation in which tone had been increased using modified Krebs-Henseleit solution containing 3.2mM $K^+$, in mesenteric vascular beds perfused with various agents such as endothelin, and in certain precontracted aortae. Palmitoyl carnitine has previously been shown to exert biphasic effects on many enzymes including $Na^+/K^+$-ATPase and $Ca^{2+}$-ATPase *(Adams et al., 1979)*, the effects being dependent on the concentration of drug used, and it is not surprising therefore that both contractile and relaxant actions were observed in the present vascular studies. However, there appears to be a great deal of variation in the response elicited by palmitoyl carnitine, depending on both the vascular preparation used and the agent used to precontract the tissue. For example, large relaxations of the mesenteric vascular bed were caused by palmitoyl carnitine in the presence of endothelin,

whereas it did not relax endothelin-precontracted aortae. Furthermore, if high concentrations of potassium were used to precontract the mesenteric vascular bed, the response to palmitoyl carnitine varied within a single preparation and also between tissues, the compound eliciting a seemingly random mixture of vasoconstrictions and dilations. This latter effect may perhaps have been due to differences in the integrity of the mesenteric vascular endothelial cell layer, or many other factors such as variation in the lipid content of cell membranes between animals.

The most striking relaxant effect of palmitoyl carnitine was observed in mesenteric vascular beds that had been precontracted with the peptide endothelin. This may indicate a latent vasodilator property of palmitoyl carnitine manifested as a result of the increase in resting tone of the preparation, or may indicate a more specific interference with some aspect of the mechanism whereby endothelin maintains vascular contraction. It is possible that these relaxations of endothelin-precontracted mesenteric beds may be in part a result of protein kinase C inhibition, since a similar inhibition of endothelin action has been demonstrated using H7, a PKC inhibitor *(Sagiura et al., 1989)*, and palmitoyl carnitine can also inhibit this enzyme *(Katoh et al., 1981; Wise & Kuo, 1983)*. However, there are many potential ways in which this compound could interfere with the sustained contraction elicited by endothelin, including effects on intracellular calcium movement such as inhibition of release from stores. Another instance where relaxant effects of palmitoyl carnitine were observed in the present study was in aortae precontracted with phenylephrine or high potassium, an effect that appeared to be attenuated in the absence of an intact endothelial cell layer. Consequently, it is possible that palmitoyl carnitine may possess the ability to stimulate release of endothelium-derived relaxant factor (EDRF), in a manner similar to a number of vasoactive compounds including acetylcholine, bradykinin and free fatty acids *(Furchgott, 1984)*, which would then act on the aortic smooth muscle to produce vascular relaxation. However, there is at present no additional evidence to support this hypothesis. In fact, certain data cast doubt on the involvement of the endothelium in the action of

palmitoyl carnitine; firstly, acetylcholine, which causes release of EDRF, produces a rapid, smooth relaxation of phenylephrine-precontracted aortae unlike the slower, less well-defined response to palmitoyl carnitine, and secondly, relaxation to acetylcholine is impaired in tissues precontracted with high potassium *(Taylor et al., 1988)*, whereas the acyl carnitine still produced a certain degree of vasorelaxation under these conditions.

Palmitoyl carnitine elicited a small vasodilator effect in mesenteric vascular beds precontracted with phenylephrine, however, the predominant effect was a large potentiation of its vasoconstrictor action. Previously it has been demonstrated that long chain acyl carnitines can produce an increase in myocardial $\alpha$-adrenoceptor numbers *(Heathers et al., 1987)* and as these compounds readily cross membranes *(Levitsky and Skulachev, 1972)*, it is likely that they will have access to the coronary circulation and therefore possibly exert similar effects on vascular tissue. The vasoconstrictor action of palmitoyl carnitine, when administered as bolus injections to the mesenteric vessels, was greatly potentiated in the presence of agonists of both $\alpha_1$- and $\alpha_2$-adrenoceptors, an effect that appeared specific and not merely the result of increased tone in the preparation. This potentiation in the presence of clonidine, however, may have been mediated via effects at $\alpha_1$-adrenoceptors since the mesenteric vascular bed of the rat is thought to be virtually devoid of post-synaptic $\alpha_2$-receptors *(Eikenburg, 1984; Yamamoto et al., 1984; Nichols & Hiley, 1985)*. A facilitation of pressor responses to noradrenaline by palmitoyl carnitine has been reported in the rat caudal artery *(Ugwu et al., 1987)*, which was partly attributable to facilitation of calcium channel function. However, in the present study, the potentiation of constrictor responses to palmitoyl carnitine in the presence of $\alpha$-agonists is unlikely to be a result of direct adrenoceptor stimulation by the acyl carnitine since the action of palmitoyl carnitine on the mesenteric vasculature, in the absence of any pharmacological agent, was not attenuated by verapamil or phentolamine suggesting a site(s) of action distinct from membrane $\alpha$-adrenoceptors or voltage-dependent calcium channels. The possibility arises therefore that this long chain acyl carnitine may have an alternative mode of action,

such as a facilitation of receptor coupling to or a direct interaction with second messenger processes, ultimately affecting intracellular calcium movement and resultant contractile processes.

However, in contrast to the large potentiation of vasoconstrictor responses to palmitoyl carnitine in the presence of perfused $\alpha$-agonists, the converse situation did not apply i.e. perfused acyl carnitine did not potentiate the responses to bolus injections of phenylephrine. Moreover, a concentration of 10µM palmitoyl carnitine actually depressed the maximal response to the agonist. Several possibilities exist to explain these findings.

This anomaly may have occurred simply as a result of the mode of application of drug, in that bolus injections may provide a high, localised concentration of drug transiently, whereas perfusions may achieve a more diffuse delivery of drug, attaining a steadier equilibrium between free drug in the perfusion medium and that interacting with the tissue, perhaps altering the response in several ways. Firstly, as long chain acyl carnitines are amphiphiles and thus can insert into biological membranes affecting fluidity *(Adams et al., 1979)*, when delivered as perfusions they may be more likely to exert such non-specific effects. Secondly, palmitoyl carnitine may induce a tachyphylaxis of the mesenteric vessels when in prolonged contact with the tissue thereby depressing its own effects. In fact, the response to bolus injections of the drug was reduced with time in the mesenteric vascular bed even after a period of 30 minutes washout, and furthermore, this effect may also offer an explanation for the lack of action of palmitoyl carnitine on the quiescient aorta, where the drug was applied directly into the organ bath, rather than this being attributable to any vascular selectivity of the compound.

Alternatively, it may be that palmitoyl carnitine is affecting some aspect of the tonic phase of contraction induced by prolonged stimulation of $\alpha$-adrenoceptors. When administered as bolus injections into the perfusion medium, phenylephrine elicits a transient contraction of the mesenteric vasculature i.e. a phasic response, however, perfusions of the agonist result in sustained contraction. There are likely to

be many differences in the mechanisms underlying these phases of vascular contraction and therefore a selective interaction of palmitoyl carnitine with some aspect of the tonic phase of mesenteric resistance vessel contraction may also account for the apparent discrepancies observed in its action in this preparation.

Palmitoyl carnitine attenuated the actions of both phenylephrine and acetylcholine when perfused at 10µM; at this concentration there was a reduction of response to higher doses of the agonists and significant depression of the maximum, possibly indicating non-specific effects of the amphiphile. Indeed, concentrations in excess of 1µM produced a marked foaming of the organ bath in isolated aorta experiments, suggesting a capacity of the drug to act as a detergent under these circumstances. It is worth noting that many of the existing studies involving palmitoyl carnitine and related compounds have been carried out at concentrations equal to or greater than 10µM, and therefore caution must be exercised in the interpretation of such data, albeit that equivalent concentrations of acyl carnitines have been reported to occur in the ischaemic myocardium *(Liedtke et al., 1978).*

In conclusion, palmitoyl carnitine exerts both vasoconstrictor and dilator effects exhibiting more profound activity in perfused resistance vessels. Its constrictor activity is greatly potentiated in the presence of $\alpha$-agonists, however, this does not appear likely to be the result of either direct $\alpha$-adrenoceptor stimulation or VOC activation.

# CHAPTER 5.

# PHARMACOLOGY OF LONG CHAIN ACYL CARNITINE ESTERS.

## DISCOVERY OF PALMITOYL CARNITINE ETHYL ESTER.

During the course of investigation into the vascular effects of palmitoyl carnitine it was decided, for convenience, to make up large batches of drug to be stored in solution in heat-sealed ampoules at 0°C. The solvent chosen was chloroform on the assumption of it being inert. However, it readily became apparent that prior storage of palmitoyl carnitine in this manner altered the pharmacological profile of the drug. Before commencing an experiment a fresh ampoule containing palmitoyl carnitine solution was thawed, the chloroform removed by bubbling with nitrogen and the drug redissolved in distilled water. Instead of the familiar vasoconstrictor response to bolus injections of palmitoyl carnitine observed in the isolated Langendorff perfused rat heart (fig.28), dose-related reductions in perfusion pressure were now observed with the drug that had undergone prior storage in chloroform (fig.28).

It was suggested that it was the 2% ethanol, included in standard laboratory chloroform as a stabiliser to prevent phosgene formation, that was reacting with the palmitoyl carnitine to form the ethyl ester of the compound (fig.29). This 'chloroform-produced' derivative of palmitoyl carnitine was designated PCE. The identity of the ethyl ester of palmitoyl carnitine was confirmed by chemical and spectroscopic analysis, and unambiguous synthesis of the compound. This novel synthetic ester of palmitoyl carnitine was code-named P1E. Following this serendipitous discovery some basic variations in structure were investigated, altering the ester function and fatty acyl chain length, and these compounds are listed in table 1.

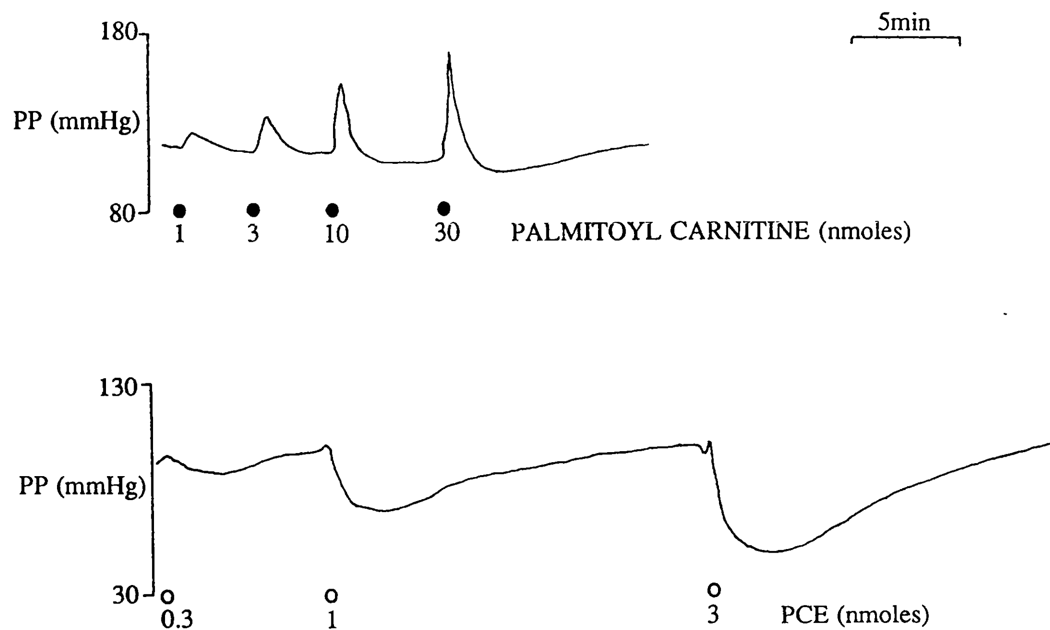



Figure 28. Typical traces illustrating the effects of palmitoyl carnitine (1-30nmoles) (*upper*) and the 'chloroform-produced' ethyl ester of palmitoyl carnitine, PCE (0.3-3nmoles) (*lower*) on perfusion pressure (PP) in the isolated Langendorff perfused rat heart.

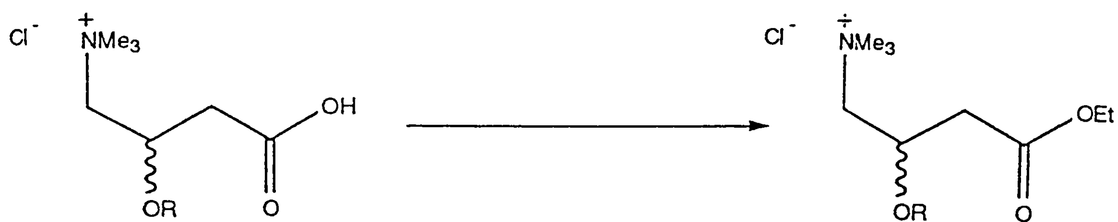
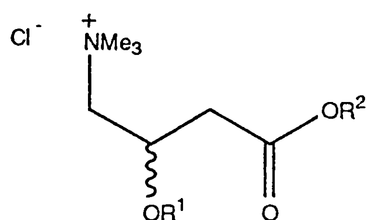



Figure 29. Conversion of palmitoyl carnitine to its ethyl ester when stored in chloroform (2% ethanol) in heat-sealed ampoules at $0^{0}$C. ($R = CO(CH_2)_{14}CH_3$).

Table 1. Guide to the structure and coding of synthetic compounds.




| *Code* | $R^1$ | $R^2$ |
|---|---|---|
| P1M | $CO(CH_2)_{14}CH_3$ | $CH_3$ |
| P1E | $CO(CH_2)_{14}CH_3$ | $CH_2CH_3$ |
| $PIP^i$ | $CO(CH_2)_{14}CH_3$ | $(CH_3)_2CH$ |
| S1E | $CO(CH_2)_{16}CH_3$ | $CH_2CH_3$ |
| $1P^i$ | H | $(CH_3)_2CH$ |
| $Pr1P^i$ | $COCH_2CH_3$ | $(CH_3)_2CH$ |

## 1)HEART.

### a) CONTROL RESPONSES.

Bolus injections of P1E, the ethyl ester of palmitoyl carnitine, caused dose-related vasodilations in the isolated Langendorff perfused rat heart that were both rapid in onset and sustained at higher doses (fig.30). A maximal response was produced by 10nmoles of P1E; higher doses produced a smaller fall in perfusion pressure preceded in several cases by a transient vasoconstriction possibly reflecting a non-specific effect of the drug. Small rises in resting tension were observed on addition of bolus injections of P1E and at no time was any effect on heart rate recorded.

The vasodilator activities of the synthetic ester P1E and the 'chloroform-produced' equivalent PCE are compared (fig.31a) providing pharmacological confirmation of previous chemical and spectroscopic analyses suggesting that palmitoyl carnitine was being esterified to P1E while undergoing storage in chloroform in heat-sealed ampoules. Figure 31b shows the decrease in tension observed with P1E and PCE when applied to the isolated rat heart; again both compounds exhibited a similar profile. Having established the identity of the novel vasodilator compound, structural variations of the molecule were investigated for pharmacological activity, alterations of chemical groups $R_1$ and $R_2$ being made (see table 1). Figure 32 shows the effect of increasing the fatty acid chain length by 2 carbon atoms, from palmitoyl to stearoyl, on vasodilator activity; both the maximal dilation elicited and the rate at which the decrease in perfusion pressure occurred were smaller with S1E than with P1E (fig.33). Complete removal of the fatty acid moiety ($1P^i$), the isopropyl ester of palmitoyl carnitine, or reduction of chain length to isobutyryl ($P^i1P^i$), the isobutyryl ester of isobutyryl carnitine, abolished any effect on coronary vascular tone (1-300nmoles, *n*=3) (data not shown).

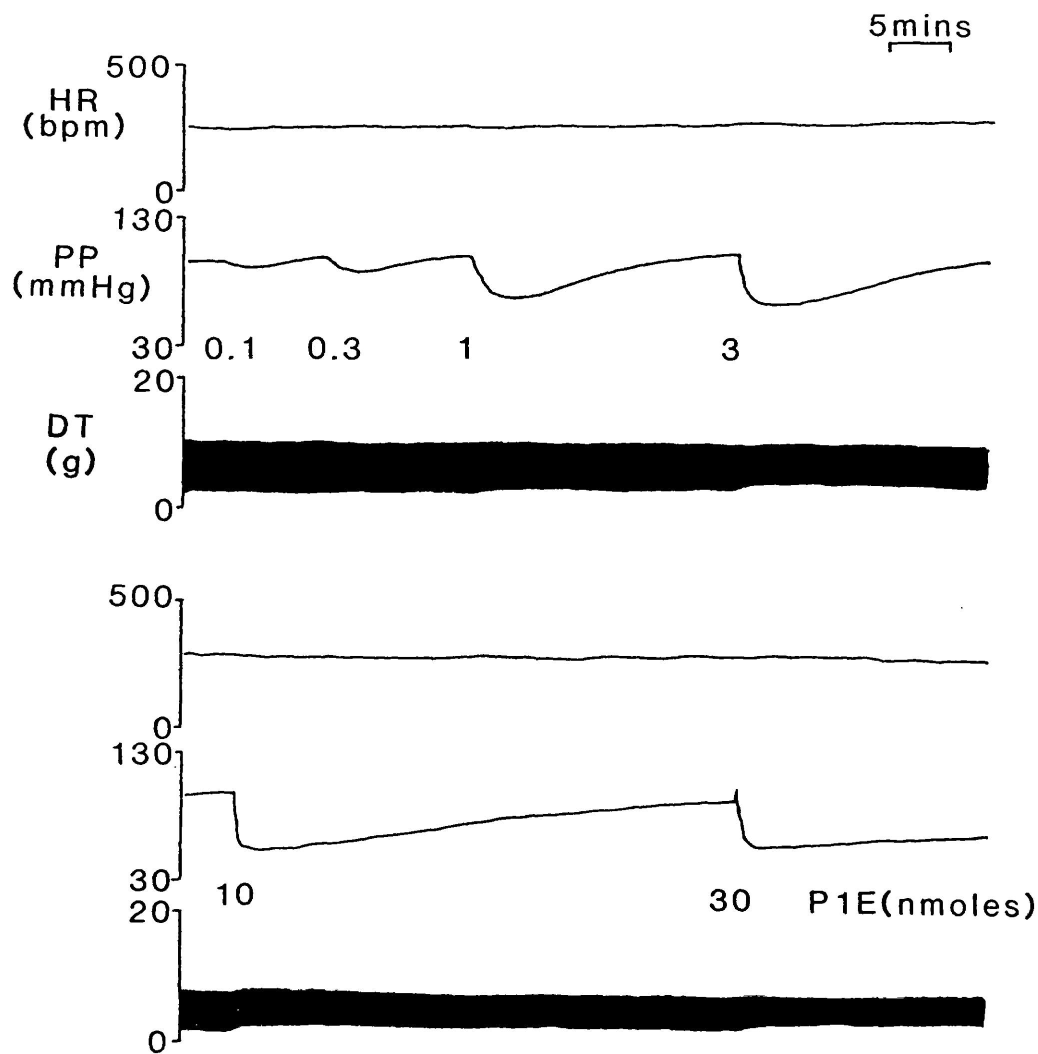



Figure 30. Effects of P1E (0.1-30nmoles) on heart rate (HR), perfusion pressure (PP) and developed tension (DT) in the isolated Langendorff perfused heart of the rat.

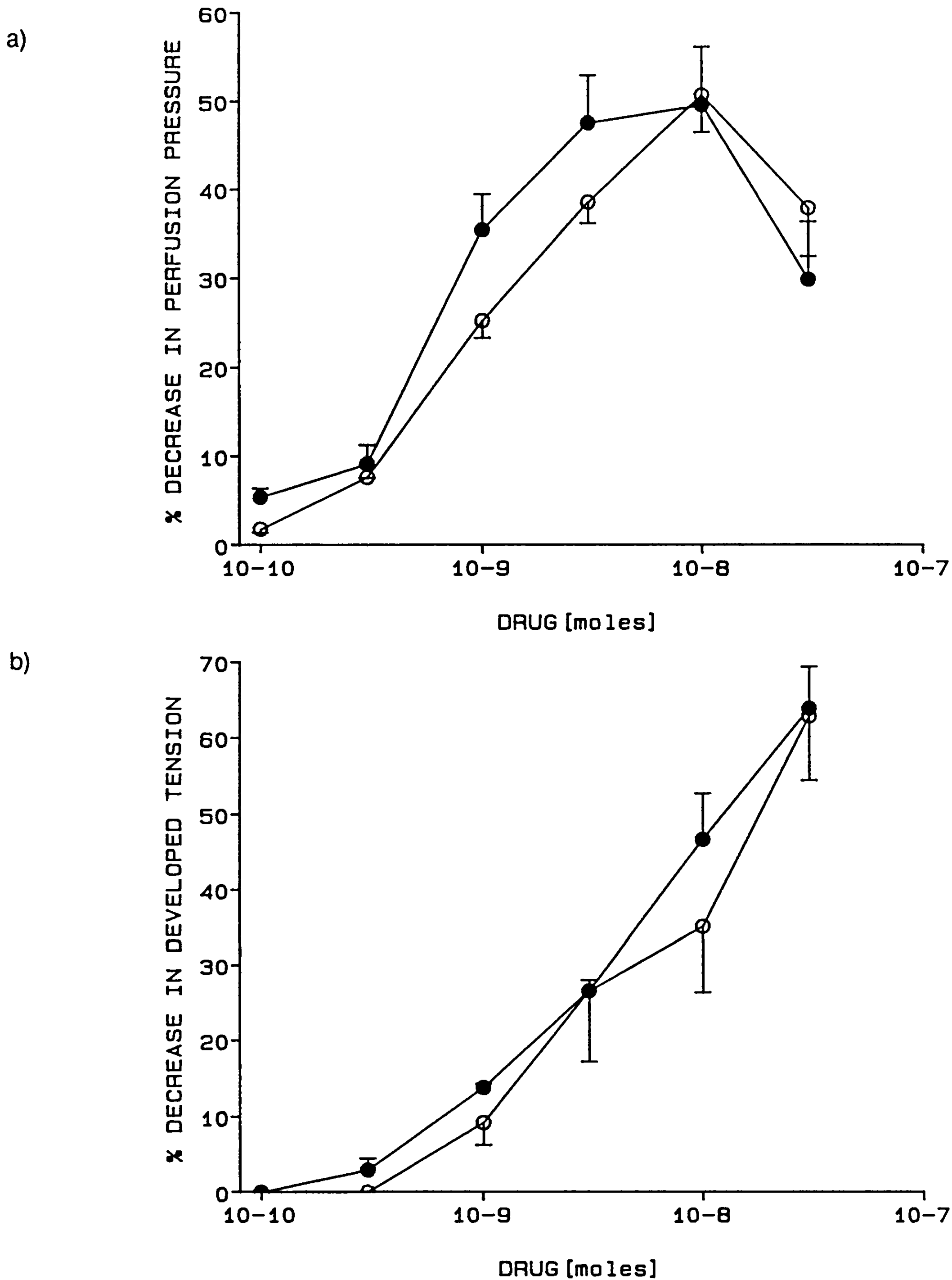



Figure 31. Comparison of the effects of the 'chloroform-produced' ethyl ester of palmitoyl carnitine (PCE, o) (100%=102.5±5.7mmHg, $n$=4) and the synthetic ester (P1E, •) (100%=95.8±3.5mmHg, $n$=4) on a) perfusion pressure and b) developed tension in the isolated perfused rat heart, expressed as % decrease from basal values. Vertical bars represent s.e.mean (*p<0.05).

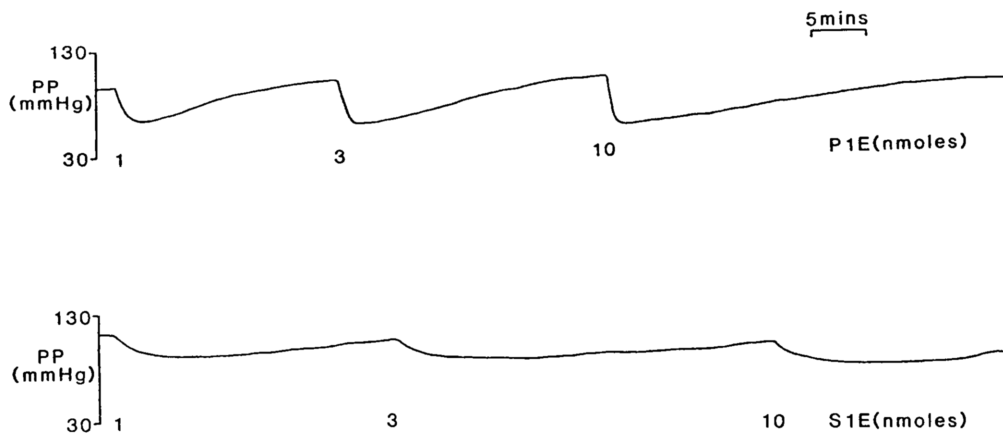



Figure 32. Effects of P1E (1-10nmoles) and S1E (1-10nmoles) on perfusion pressure (PP) in the isolated Langendorff perfused heart of the rat.

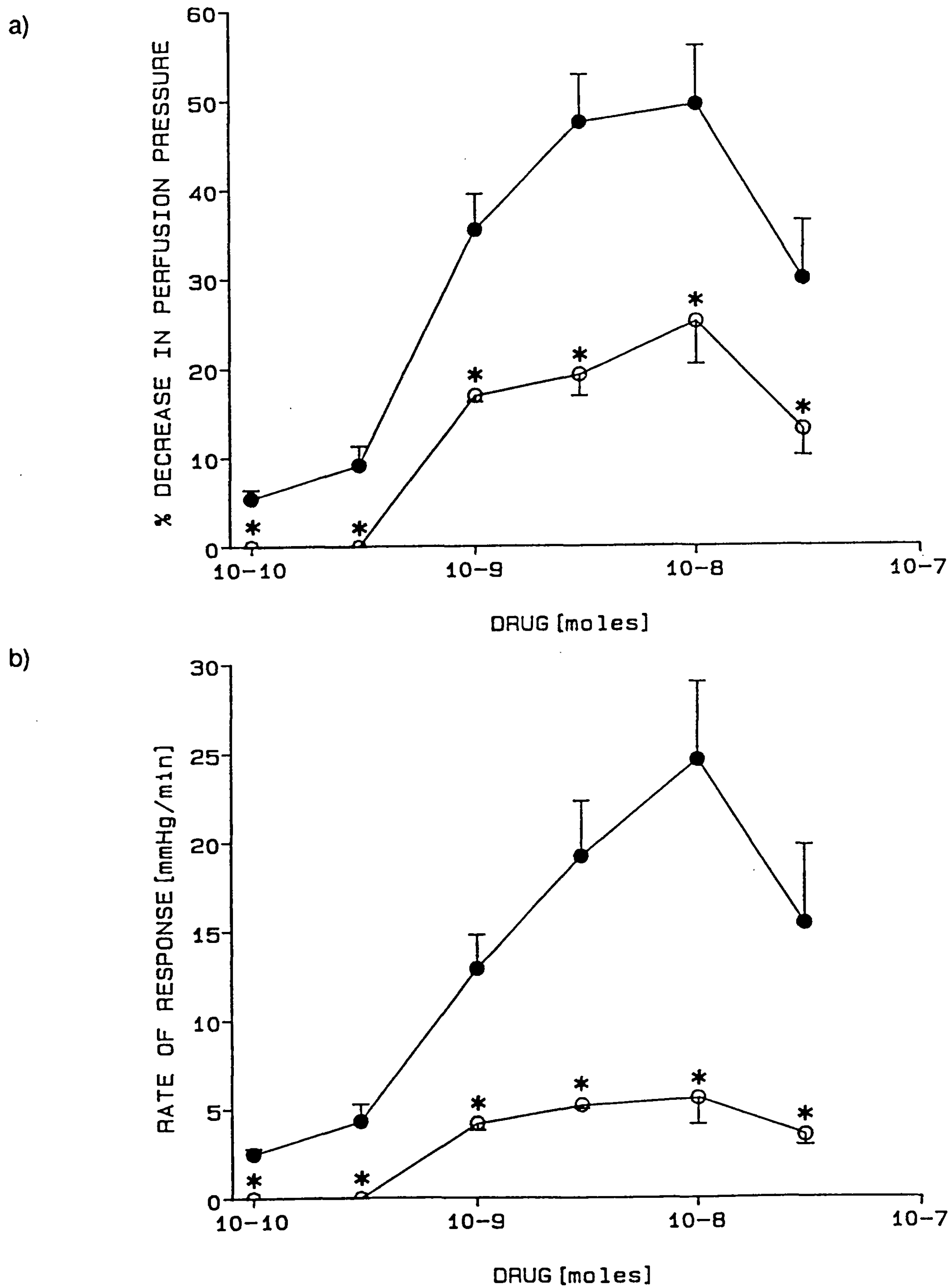



Figure 33. Effects of P1E (●), [100%=95.8±3.5mmHg, $n$=4] and S1E (o), [100%= 86.8±9.0mmHg, $n$=4] on perfusion pressure in the rat isolated Langendorff heart, expressed as a) % decrease from basal values and b) rate of response. Vertical bars represent s.e.mean (*p<0.05).

Substitution of the ethyl ester group with methyl (P1M) or isopropyl ($P1P^i$) groups did not alter the vasodilator activity of the compounds as markedly as altering the fatty acid chain, although a significant reduction in the response elicited by P1M compared to P1E or $P1P^i$ was observed with certain doses (0.1-3nmoles) (fig.34). A typical trace of the response of the isolated rat heart to $P1P^i$ is shown in figure 35, and in view of its potent vasodilator activity this compound was selected for use in most of the succeeding experiments investigating possible modes of action of the ester derivatives.

A comparison of the synthetic carnitine esters, P1E and $P1P^i$, with known vasodilators amyl nitrate, verapamil, cromakalim and iloprost is shown in figure 36. Vasodilations of similar magnitude to those elicited by the known agents were produced by the novel compounds at comparable doses, however, the duration of response was greatest with the carnitine esters.

In order to ascertain the exact concentration range over which $P1P^i$ was exerting its vasodilator effects on the coronary circulation, a cumulative concentration-response curve to the carnitine ester was performed (fig.37). Increasing concentrations of $P1P^i$ ($10^{-9}$-$10^{-6}$M) were perfused through the heart with a contact time of 10 minutes per concentration. Constant perfusion of $P1P^i$ decreased coronary perfusion pressure in a concentration-related manner and this effect was maximal at 0.1µM. ($EC_{50}$=16nM, *n*=4). At these concentrations there was little effect on developed tension and no effect on heart rate. However, a supramaximal concentration of $P1P^i$ (10µM) elicited a rise in perfusion pressure (53.5±1.5mmHg) and inhibition of developed tension within 16.0±0.3 minutes (data not shown). It is likely that these effects are non-specific, being due to the amphiphilic nature of the compound.

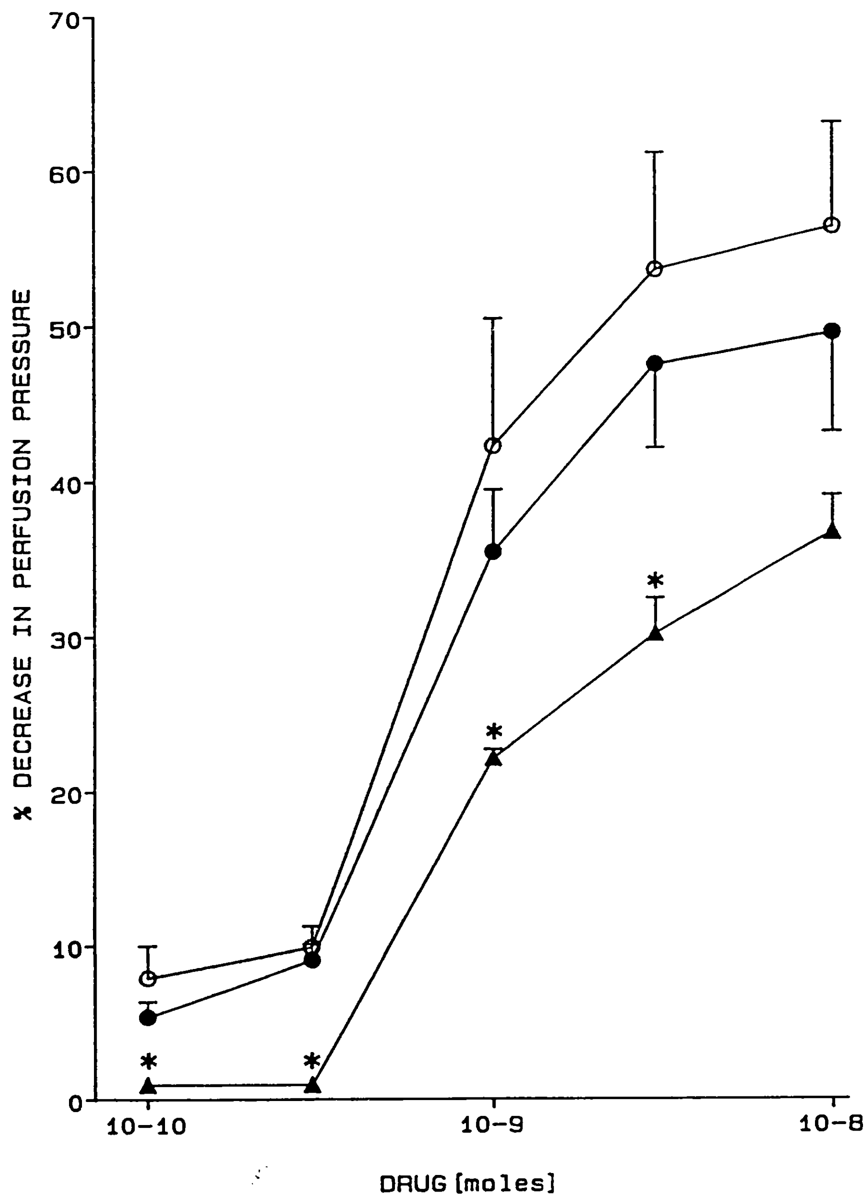



Figure 34. Effects of P1M (▲), [100%=92.5±6.7mmHg, *n*=4], P1E (●), [100%= 95.8±3.5mmHg, *n*=4] and P1P$^{i}$ (o), [100%=104.0±5.0mmHg, *n*=4] on perfusion pressure in the isolated Langendorff perfused heart of the rat, expressed as % decrease from basal values. Vertical bars represent s.e.mean (*p<0.05 compared with P1P$^{i}$ and P1E).

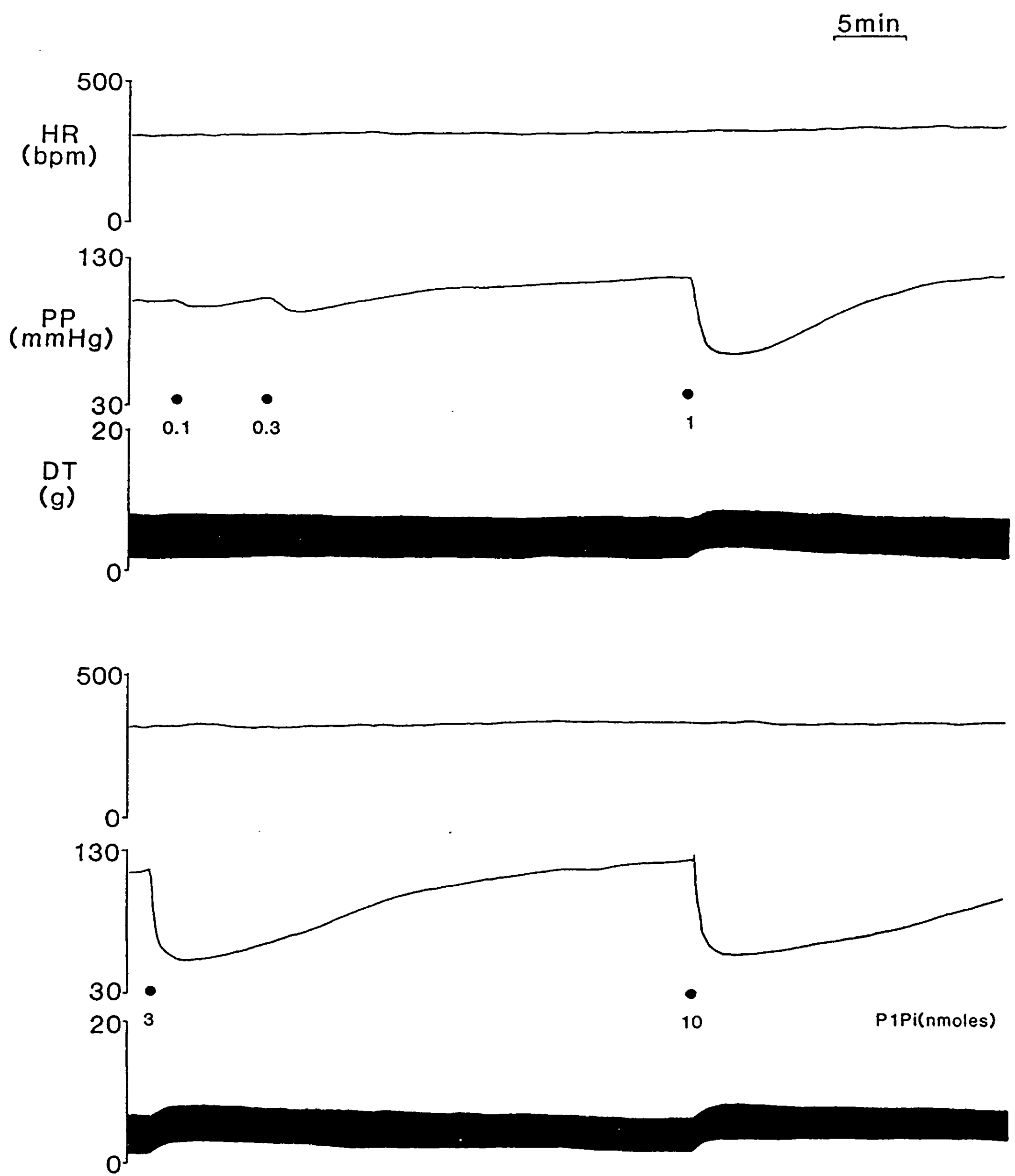



Figure 35. Typical trace showing the effects of P1P$^{i}$ (0.1-10nmoles) on heart rat (HR), perfusion pressure (PP) and developed tension (DT) in the isolated Langendorff perfused rat heart.

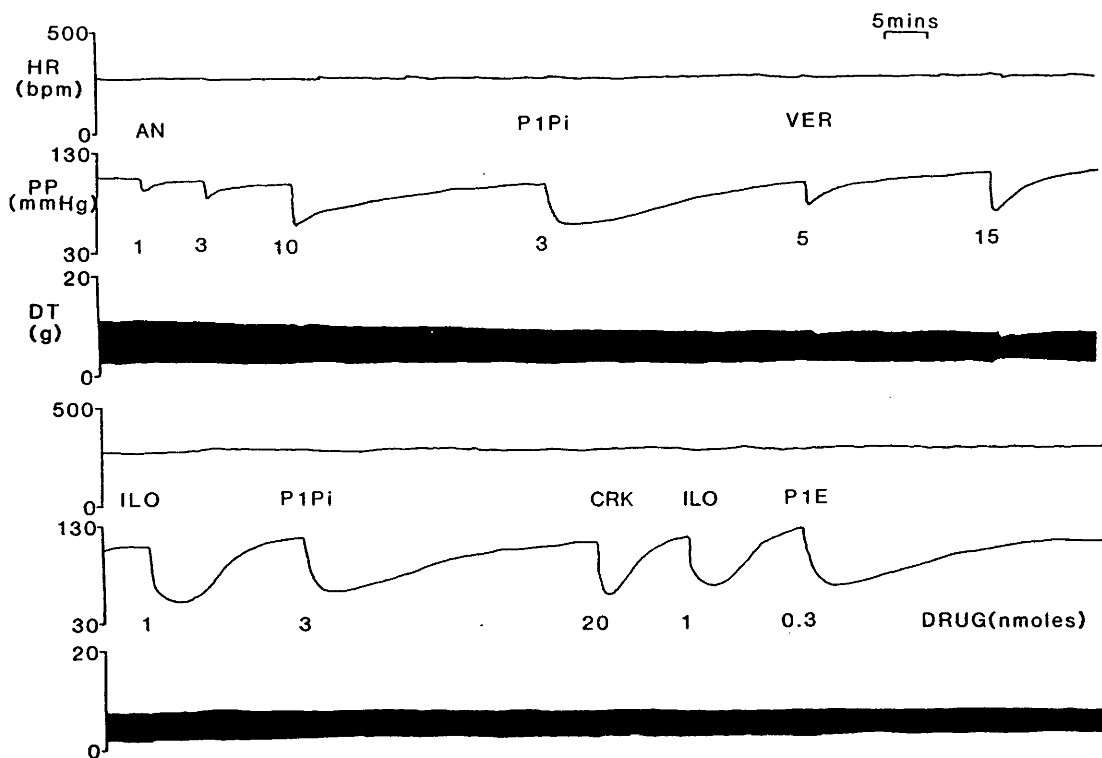



Figure 36. Comparison of the effects of P1E and P1P$^{i}$ with amyl nitrate (AN), verapamil (VER), iloprost (ILO) and cromakalim (CRK) on heart rate (HR), perfusion pressure (PP) and developed tension (DT) in the isolated Langendorff perfused rat heart.

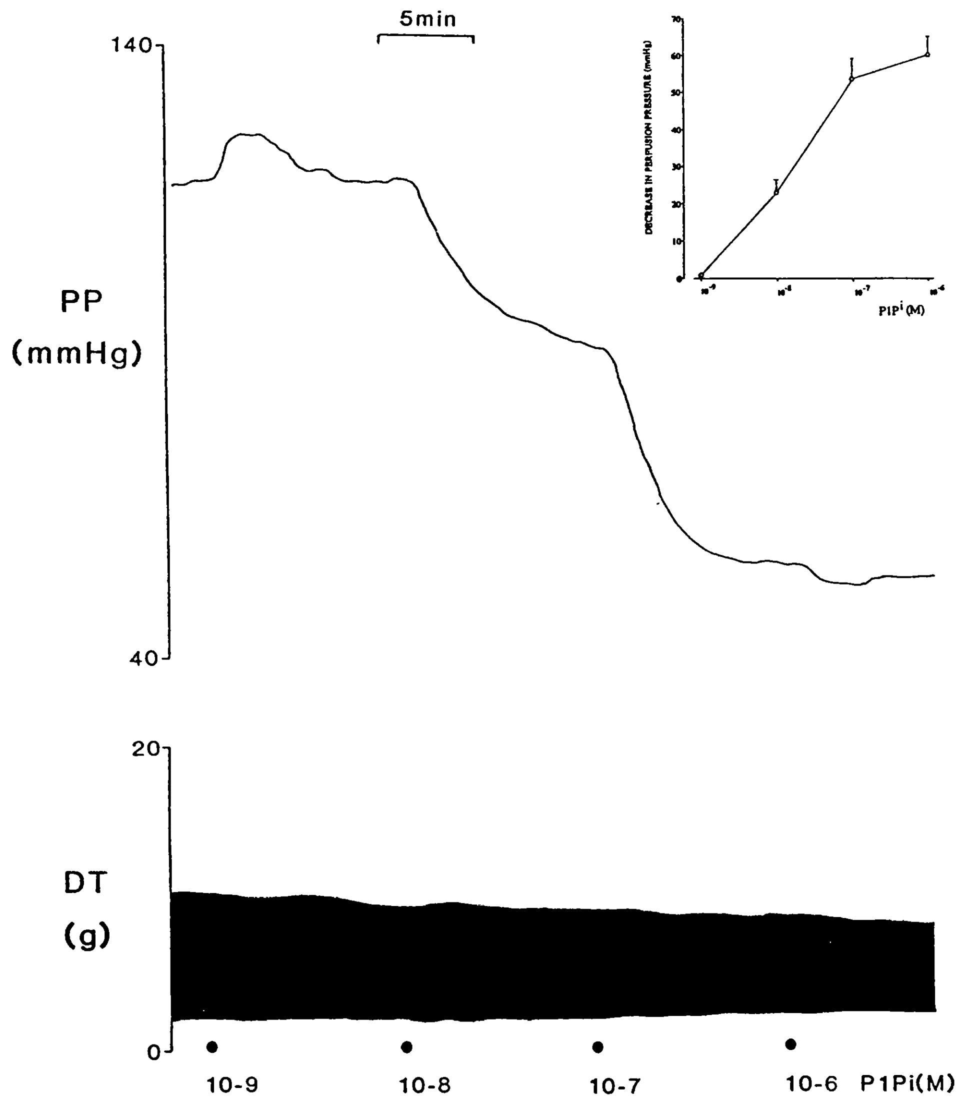



Figure 37. Typical trace showing the effects of cumulative concentrations of P1P$^i$ ($10^{-9}$-$10^{-6}$M) on perfusion pressure (PP) and developed tension (DT) in the isolated Langendorff perfused rat heart. Also shown are the mean falls in perfusion pressure at each concentration of P1P$^i$ ($n$=4) (*inset*). Vertical bars represent s.e.mean.

b) EFFECT OF AGENTS ON ACTION OF NOVEL COMPOUNDS.

In order to investigate the mechanism of action of the novel vasodilator carnitine esters in the Langendorff perfused heart the effect of certain compounds on responses to P1P$^{i}$ and derivatives was examined. The decrease in perfusion pressure elicited by a standard dose of PCE (10nmoles) was dependent on the basal tone of the preparation i.e. the greater the resting perfusion pressure the larger the vasodilator response (fig.38). This presented a problem when assessing the effect of certain agents on the action of the novel compounds. For example indomethacin, a cyclo-oxygenase inhibitor, decreased perfusion pressure when perfused through the isolated rat heart, and therefore direct comparison of responses to the carnitine esters in the presence and absence of the drug proved difficult to interpret.

The following protocol was adopted. Initially a control response to a standard dose of PCE (10nmoles) was obtained and, following recovery from the vasodilation, the heart was perfused with indomethacin and the same dose of PCE was added in the presence of this drug. The mean results of six such experiments suggest a reduction in the action of PCE in the presence of indomethacin; a control mean fall of 45.0±7.6mmHg compared with 26.2±8.2mmHg fall in hearts exposed to indomethacin ( 28μM ), however, a similar problem regarding lower basal perfusion pressures in the presence of enzyme inhibitor precluded firm conclusions about the potential invovement of cyclo-oxygenase products in the action of PCE being made. Figure 39 shows a trace obtained from an experiment in which the fall in perfusion pressure due to indomethacin ( 28μM ) was not sustained; in this instance the response to PCE was not attenuated. Subsequent experiments using P1P$^{i}$ employed a similar protocol comparing the effects of a standard dose of drug both in the presence and absence of a pharmacological agent. Table 2 summarises the effects of various agents administered in the perfusion medium on the vasodilator response to a standard dose of P1P$^{i}$ (1nmole).

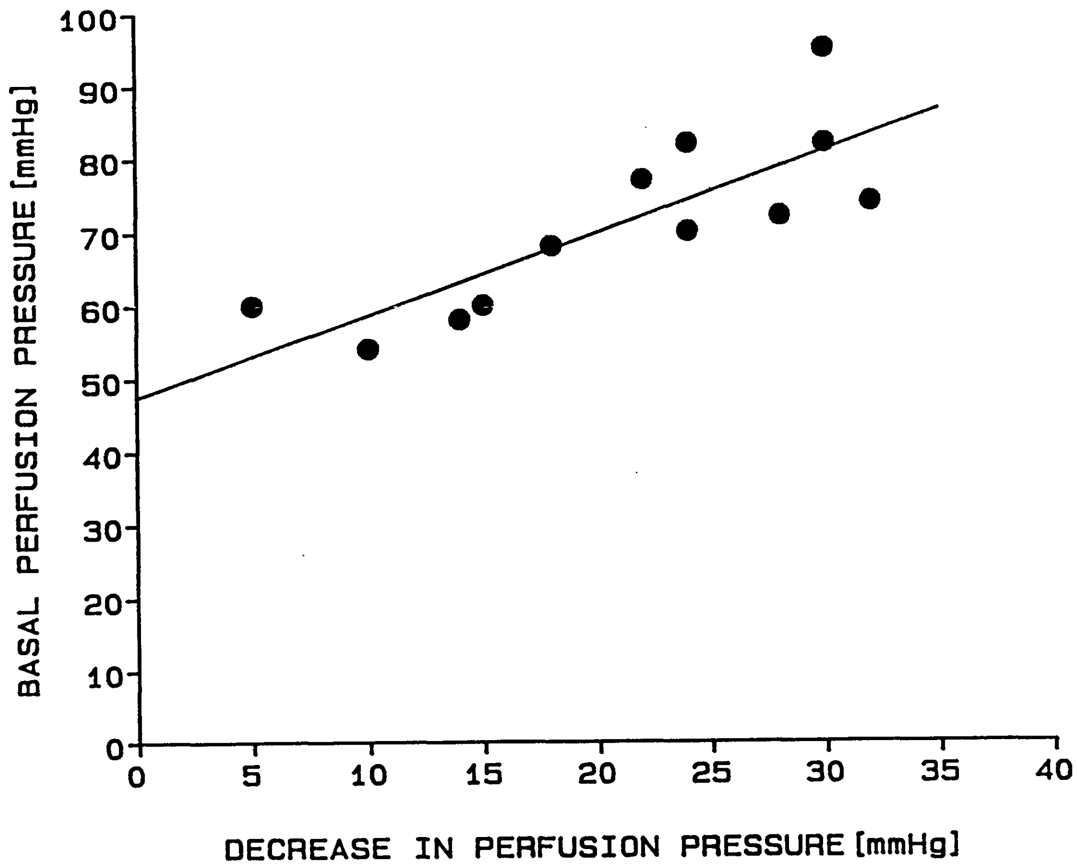



Figure 38. Correlation between basal perfusion pressure and the decrease in perfusion pressure elicited by a standard dose (10nmoles) of the 'chloroform-produced' ethyl ester of palmitoyl carnitine (PCE) in the isolated Langendorff perfused heart of the rat [$r$=0.81, $p<0.05$ ($n$=12)].

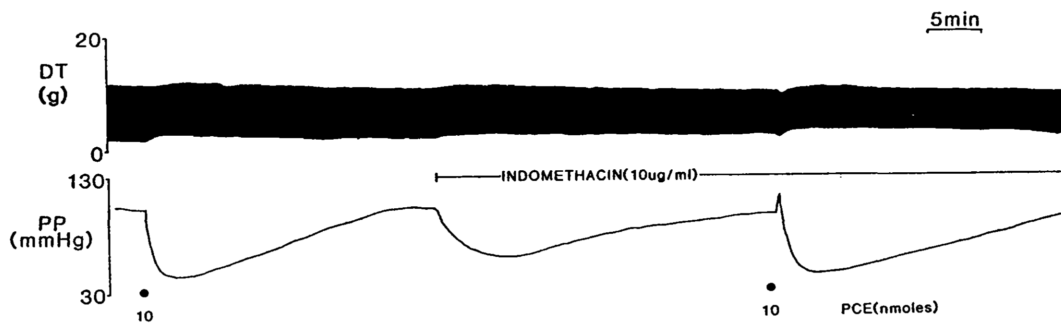



Figure 39. Trace showing the effects of indomethacin (28µM) on the action of a standard dose of PCE (10nmoles) on perfusion pressure (PP) and developed tension (DT) in the isolated Langendorff perfused rat heart.

Table 2. The effects of various pharmacological agents on the basal perfusion pressure and the vasodilator action of P1P$^{i}$ (1nmole) in the isolated Langendorff perfused heart of the rat. (*p<0.05, *n*=4).

| | BASAL PERFUSION PRESSURE (mmHg) | | VASODILATION (mmHg) | |
|---|---|---|---|---|
| DRUG | CONTROL | ±DRUG | CONTROL | ±DRUG |
| Flurbiprofen (10μM) | 84.5±2.2 | 85.8±4.2 | 30.8±1.0 | 28.5±4.0 |
| BW755C (10μM) | 81.8±7.6 | 71.5±10.0 | 25.0±6.0 | 17.2±5.1 |
| Methylene blue (10μM) | 79.8±6.0 | 66.2±12.3 | 24.8±3.7 | 11.0±6.9 |
| L-N$^{G}$-nitro arginine (100μM) | 91.5±5.3 | 110.0±7.1 | 30.8±1.2 | 32.8±2.5 |
| Glibenclamide (10μM) | 76.2±6.6 | 79.2±9.7 | 27.2±4.4 | 28.0±8.3 |
| Atropine (50nM) | 93.7±5.8 | 95.3±2.6 | 32.3±4.8 | 30.3±1.9 |
| D-Arg bradykinin (1μM) | 93.5±7.9 | 86.0±4.0 | 33.0±6.0 | 30.5±6.0 |
| Palmitoyl carnitine (1μM) | 76.0±3.0 | 73.0±3.0 | 26.0±3.5 | 19.6±3.0 |
| Palmitoyl carnitine (5μM) | 78.0±2.1 | 93.0±5.7 | 28.2±1.7 | 0 * |

1) Cyclo-oxygenase/lipoxygenase products.

Two inhibitors of the enzyme cyclo-oxygenase, indomethacin and flurbiprofen, were employed to assess whether the vasodilator action of P1P$^i$ was mediated via production of prostanoids e.g. prostacyclin, an endogenous smooth muscle relaxant. As mentioned previously, indomethacin (10µg/ml) exerted an effect on coronary vascular tone therefore making a direct comparison of P1P$^i$ responses in the presence and absence of drug difficult, however, flurbiprofen (10µM) did not alter basal tone of the preparation. In the presence of this inhibitor the vasodilator action of P1P$^i$ was not attenuated (fig.40)(table 2).

The effect of the dual cyclo-oxygenase/lipoxygenase inhibitor BW755C (10µg/ml) on the response to P1Pi was also examined. The fall in perfusion pressure elicited by the isopropyl ester was less, though not significantly, when BW755C was present, however, the basal coronary tone was also reduced by the inhibitor (table 2).

2) Endothelium-derived relaxant factor(s).

To investigate whether P1P$^i$ was exerting its vasodilator action via a release of endothelium-derived relaxant factor (EDRF), an inhibitor of the action of EDRF, methylene blue, was used to try to block the responses to the carnitine ester. The vasodilator response to P1P$^i$ was smaller in the presence of methylene blue (10µM), however, this is unlikely to be a specific inhibitory effect of the drug because the mean perfusion pressure was also lower in the presence of this agent (table 2). In one of four experiments the perfusion pressure was not reduced by methylene blue but slightly elevated, and in this situation the response to P1P$^i$ was not attenuated (fig.41).

In addition, the influence of an enzyme, superoxide dismutase (SOD), which prevents the degradation of EDRF by free radicals thereby potentiating its effects, on a submaximal dose of P1P$^i$ (0.3nmoles) was investigated. SOD, at concentrations

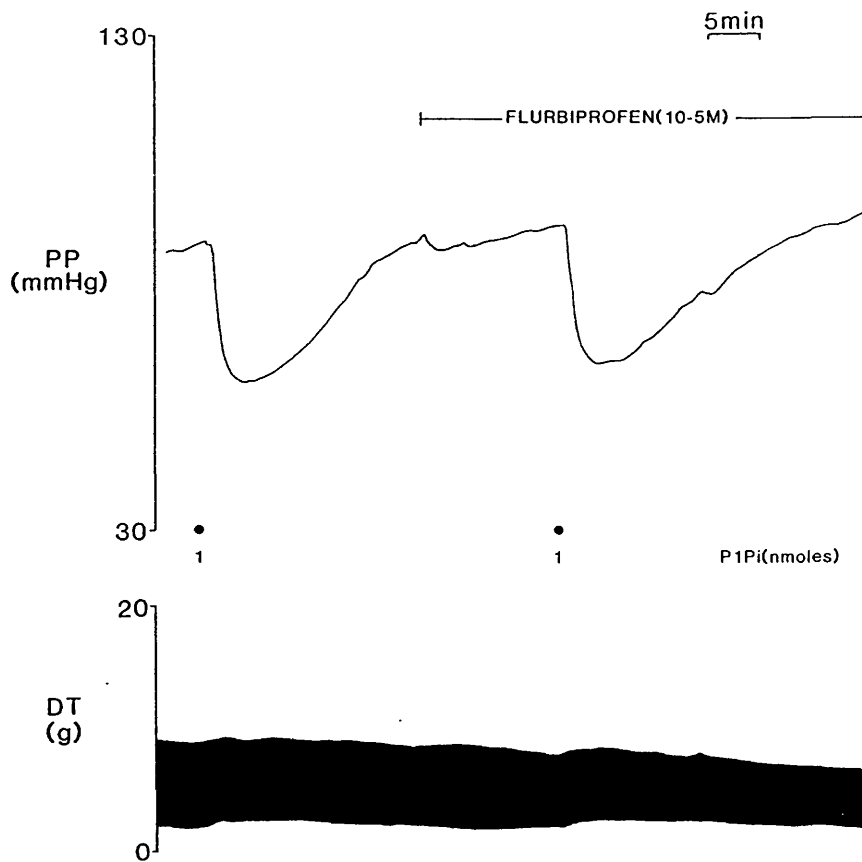



Figure 40. Typical trace showing the effects of flurbiprofen (10μM) on the action of a single dose of P1P$^{i}$ (1nmole) on perfusion pressure (PP) and developed tension (DT) in the isolated Langendorff perfused rat heart.

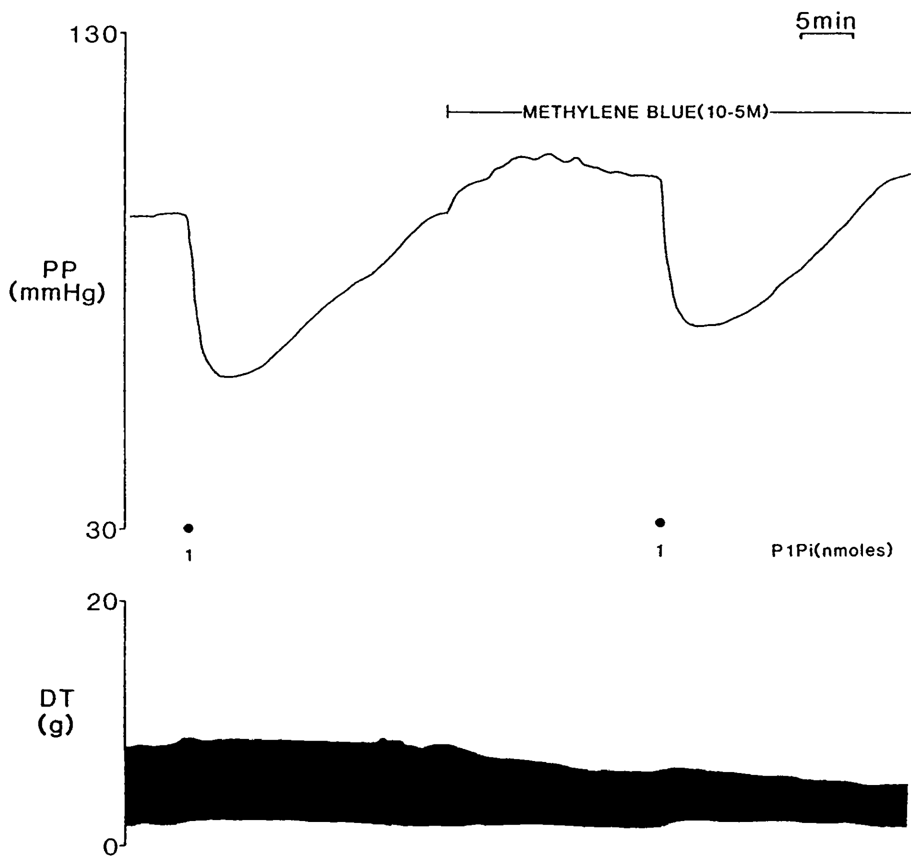



Figure 41. Trace showing the effects of methylene blue (10µM) on the action of a single dose of P1Pi (1nmole) on perfusion pressure (PP) and developed tension (DT) in the isolated Langendorff perfused rat heart.

of 20 & 50 units/ml, produced small, though not significant elevations of resting perfusion pressure, however, at these concentrations did not potentiate the vasodilator action of P1P$^{i}$ on the coronary vascular bed. In contrast, the vasodilator response to P1P$^{i}$ was unexpectedly attenuated with both concentrations of SOD used; the decrease in perfusion pressure elicited by P1P$^{i}$ was reduced from 13.9±2.0mmHg by 4.7±2.1 and 6.3±2.2mmHg in the presence of 20U/ml and 50U/ml, respectively (*n*=7).

Finally, the effect of the recently described inhibitor of endothelium-dependent vasodilation, L-N$^{G}$-nitro arginine (L-NOARG) *(Moore et al.,1990)* was investigated on the response to P1P$^{i}$. Figure 42 shows a typical trace obtained from one such experiment. Inclusion of L-NOARG (100µM) in the perfusion fluid increased the resting tone by 19.5±7.1mmHg, but did not alter the magnitude of vasodilation elicited by P1P$^{i}$ (1nmole).

3) Potassium channels.

Glibenclamide, a blocker of ATP-sensitive potassium channels *(Quast et al.,1988)*, was employed in a similar manner to above to assess any possible involvement of such channels in the vascular relaxant action of P1P$^{i}$. This drug did not affect resting perfusion pressure in the hearts tested, and therefore accurate comparison of the response to P1P$^{i}$, in the presence and absence of glibenclamide, was possible. The vasodilator action of P1P$^{i}$ was not attenuated by glibenclamide (10µM) (table 2), a concentration of drug that reduced the response to a submaximal dose of BRL38227 (30nmoles) by over 90% (*n*=4, data not shown).

4) Cholinergic action.

Atropine (5x10$^{-9}$M) did not reduce the magnitude of response to P1P$^{i}$ (table 2). At this concentration atropine abolished the vasoconstrictor effects of acetylcholine (1-10nmoles) and also the ability of higher doses to transiently cause the heart to cease beating (*n*=3, data not shown).

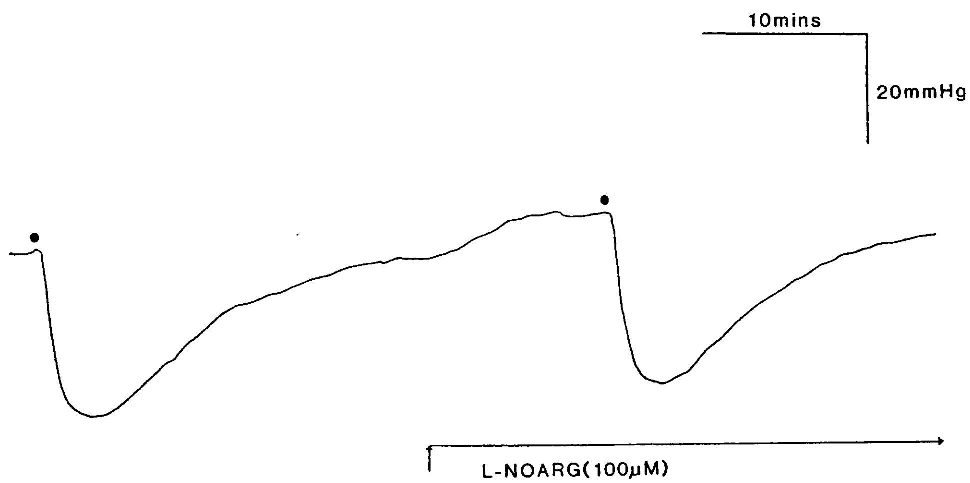



Figure 42. Typical trace showing the effects of L-$N^G$-nitro arginine (L-NOARG) (100μM) on the action of a single dose of $P1P^i$ (1nmole) on perfusion pressure in the isolated Langendorff perfused rat heart.

5) Bradykinin.

The possibility that P1P$^i$ was acting via stimulation of bradykinin receptors was also investigated using the selective bradykinin $B_2$-receptor antagonist D-Arg$^0$[Hyp$^3$,Thi$^{5,8}$,D-Phe$^7$]-bradykinin *(Regoli et al.,1990)*, however, the vasodilator action of P1P$^i$ was not significantly altered in the presence of this drug at a concentration of 100nM (table 2).

6) Palmitoyl carnitine.

In view of the similarity in structure and opposite vascular effects of palmitoyl carnitine and P1P$^i$, any possible antagonism of the vasodilator action of P1P$^i$ by the parent compound was investigated. Figure 43 shows typical traces obtained from experiments in which responses to P1P$^i$ were assessed in the presence of either 1µM or 5µM palmitoyl carnitine. The vasodilation to P1P$^i$ was reduced, though not significantly, with 1µM palmitoyl carnitine and totally abolished at the higher concentration (table 2), however, at 5µM there was also an inability of the coronary vasculature to respond to sodium nitroprusside (*n*=3, data not shown). In addition, there was an irreversible depression of developed tension in the heart associated with the higher dose of the acyl carnitine; control tension was 5.5±1.0g compared with 0.4±0.1g after 10 minutes perfusion with the drug (*n*=4).

7) Adenosine.

The influence of an enzyme responsible for the breakdown of adenosine, adenosine deaminase, on the response to PCE was examined to assess whether PCE was exerting its vasodilator action via release of this endogenous metabolite. In the perfused rat heart, bolus injections of adenosine (1-30nmoles) caused small, transient relaxations of the coronary vasculature in contrast to the larger, more sustained vasodilation elicited by PCE (10nmoles) (fig.44). Adenosine deaminase (1unit/ml) abolished the response to a submaximal dose of adenosine (3nmoles), an effect that persisted even after washout. However, the vasodilator action of PCE was

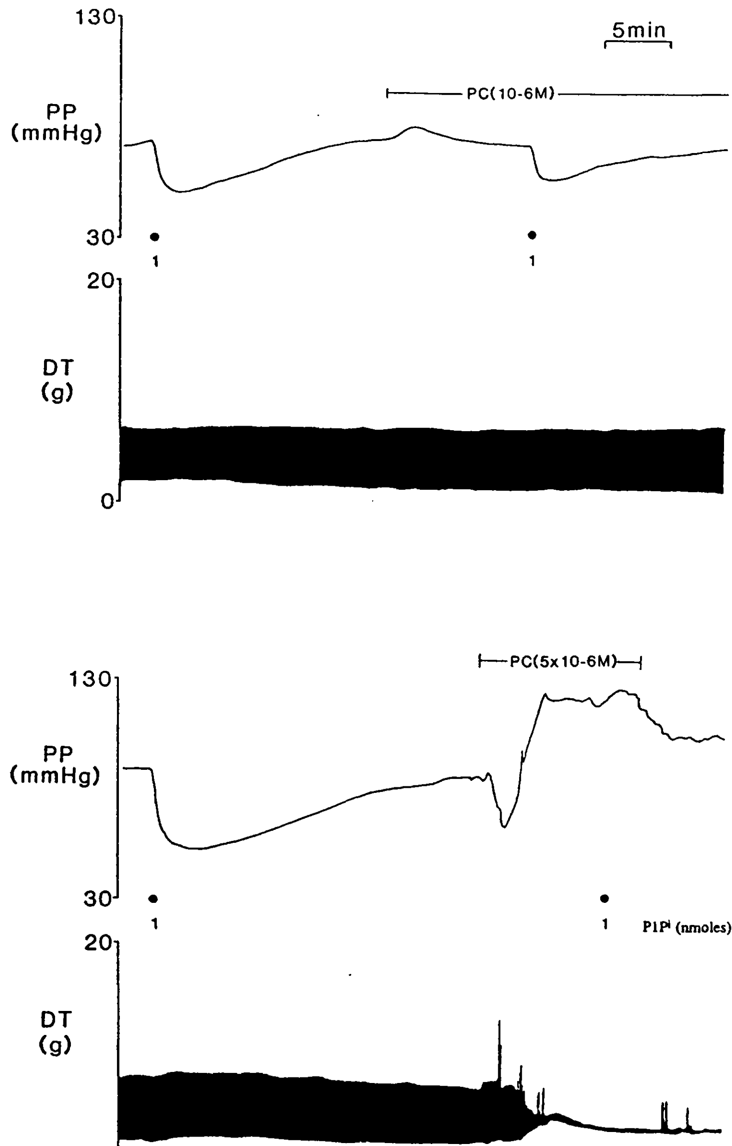



Figure 43. Typical traces showing the effects of 1µM (*upper trace*) and 5µM (*lower trace*) palmitoyl carnitine (PC) on the action of a single dose of P1Pi (1nmole) on perfusion pressure (PP) and developed tension (DT) in the isolated Langendorff perfused rat heart.

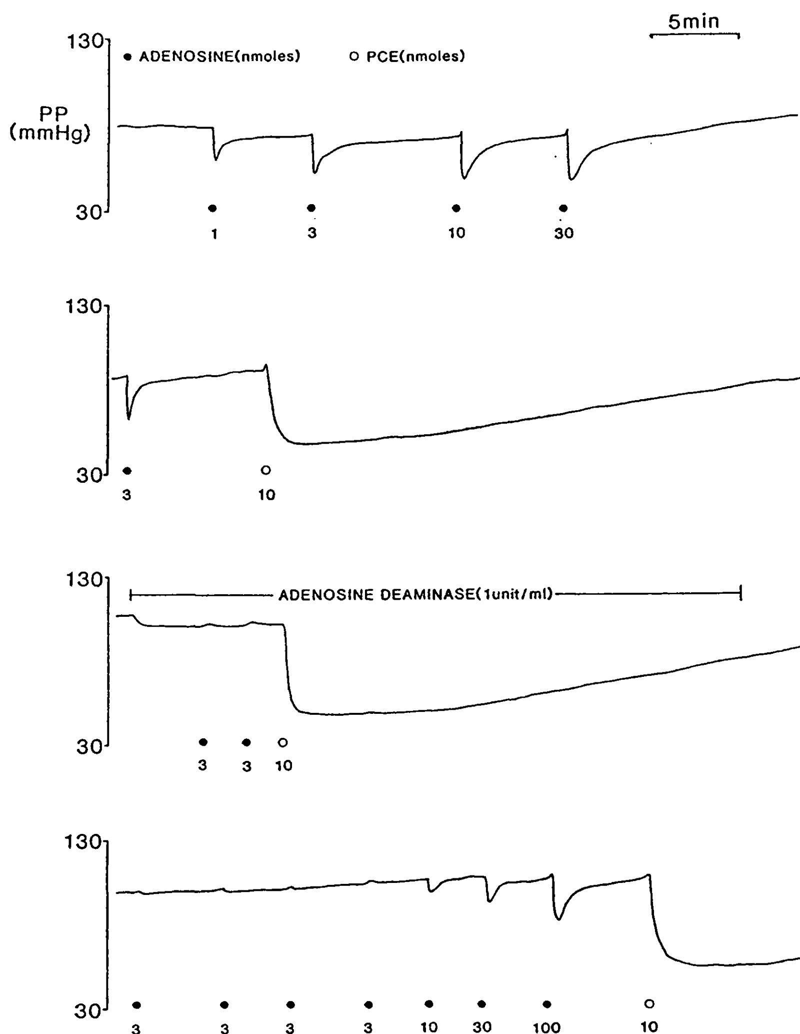



Figure 44. Typical trace showing the effects of adenosine deaminase (1unit/ml) on the action of adenosine (•, 1-100nmoles) and PCE (o,10nmoles) on perfusion pressure (PP) in the isolated Langendorff perfused rat heart.

not affected by this enzyme; control decrease in perfusion pressure produced by PCE (10nmoles) was 36.0±5.4mmHg compared with 35.0±7.8mmHg in the presence of adenosine deaminase ($n$=4).

8) Phospholipase $A_2$.

Mepacrine ($10^{-7}$M), which inhibits phospholipase $A_2$ ($PLA_2$) an enzyme responsible for membrane phospholipid degradation, reduced the vasodilation produced by the methyl ester of palmitoyl carnitine, P1M (0.3nmoles), though not significantly; control decrease in perfusion pressure was 14.5±4.6mmHg compared with 5.8±2.0mmHg in the presence of mepacrine ($n$=4). However, at this concentration of mepacrine, the response to P1M did not recover to control values following a period of washout (7.8±2.0mmHg). It was not feasible to examine higher concentrations of mepacrine on the response to P1M, because micromolar concentrations produced severe, irreversible depression of developed tension and contracture.

c) EFFECT OF PCE ON $^{86}$RUBIDIUM EFFLUX.

To further investigate the mode of action of the palmitoyl carnitine derivatives the effect of PCE on efflux of radiolabelled rubidium, a marker of potassium movement, from preloaded rat hearts, was examined. Figure 45 shows the efflux of $^{86}$Rb from rat hearts, expressed as efflux rate constant (erc/min) with time, elicited by single bolus doses of PCE (1-100nmoles). There was no rise in $^{86}$Rb efflux induced by a bolus injection of 1nmole of PCE, a dose which produced a submaximal relaxation of the coronary vasculature, however, at higher doses (10 & 100 nmoles) the efflux was transiently increased attaining a maximum within a few minutes of drug administration. Further addition of PCE (10nmoles) to the perfused heart, at a point when the coronary bed appeared maximally dilated, produced neither a decrease in perfusion pressure nor an increase in $^{86}$Rb efflux from the tissue (fig.45, *bottom trace*). The relationship between $^{86}$Rb efflux and vasodilation due to PCE is shown in figure 46.

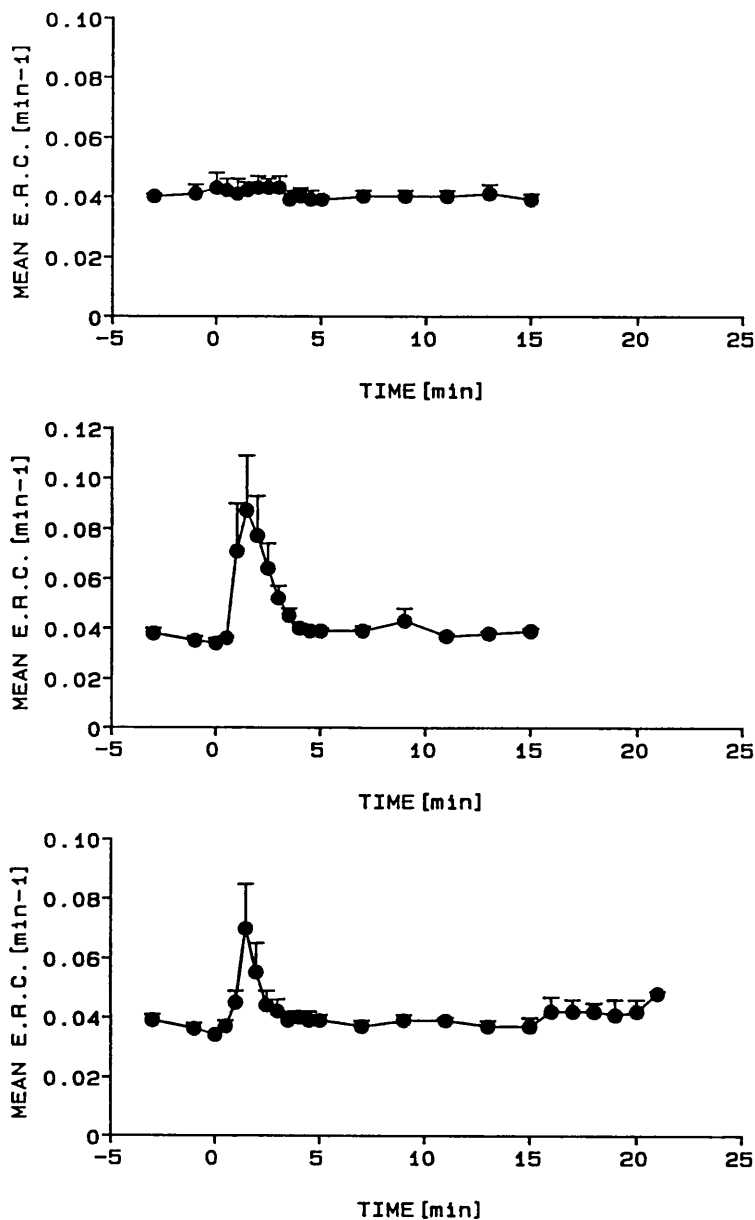



Figure 45. Effects of the 'chloroform-derived' ethyl ester of palmitoyl carnitine (PCE) (1-100nmoles) on $^{86}$rubidium efflux from isolated Langendorff perfused rat heart, expressed as mean efflux rate coefficient (E.R.C.). Doses of PCE 1nmole (*top, n*=4), 10nmoles (*middle, n*=4) and 100nmoles (*bottom, n*=4) were administered at time=0. Additionally, a single dose of PCE (10nmoles) was added at time=15 minutes (*bottom*) following the initial dose of PCE. Vertical bars represent s.e.mean.

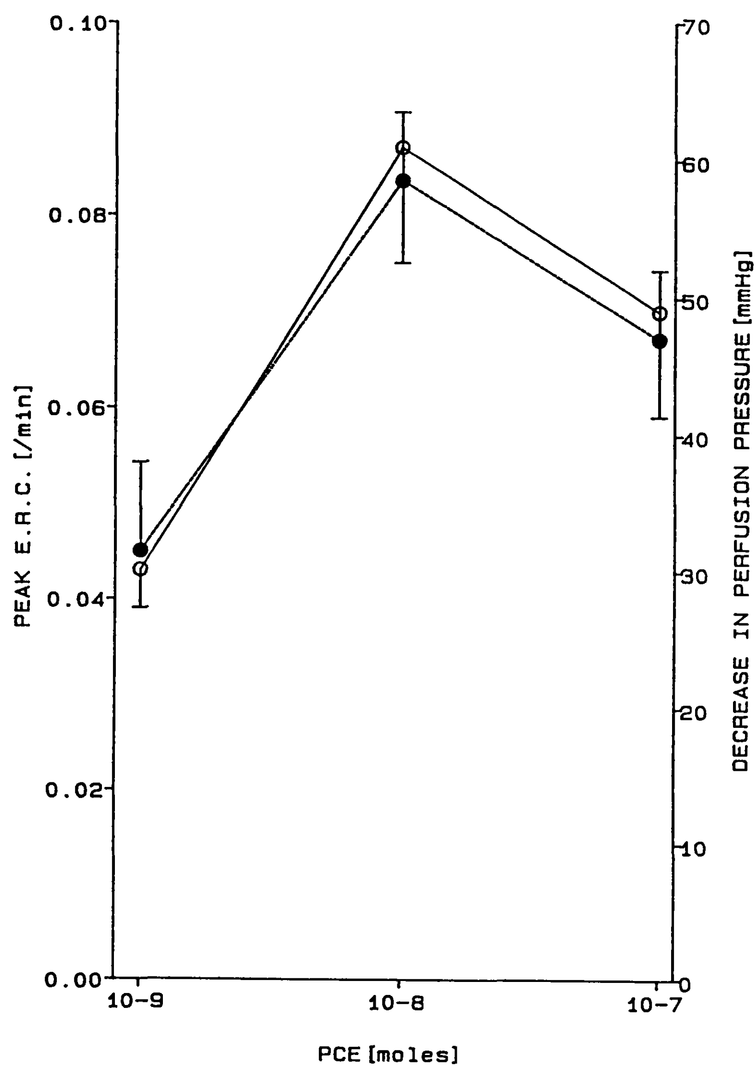



Figure 46. Comparison of the effects of PCE (1-100nmoles) on perfusion pressure (●) in and peak $^{86}$rubidium efflux (o) from preloaded isolated Langendorff perfused rat hearts ($n$=4).

Similar experiments involving the synthetic ethyl ester (P1E), however, have failed to provide confirmation of the results obtained with PCE.

d) EFFECT OF EXTRACELLULAR CALCIUM CONCENTRATION.

The effect of changing the calcium concentration of the perfusion solution on the action of PCE was assessed in the rat heart. Calcium concentration was increased cumulatively from 1.2mM to 4.8mM and then back to 1.2mM for a further control response. The data obtained suggest an inverse relationship between extracellular calcium concentration and magnitude of vasodilation elicited by a standard dose of PCE (1nmole) (fig.47); the higher the calcium concentration the smaller the vascular response.

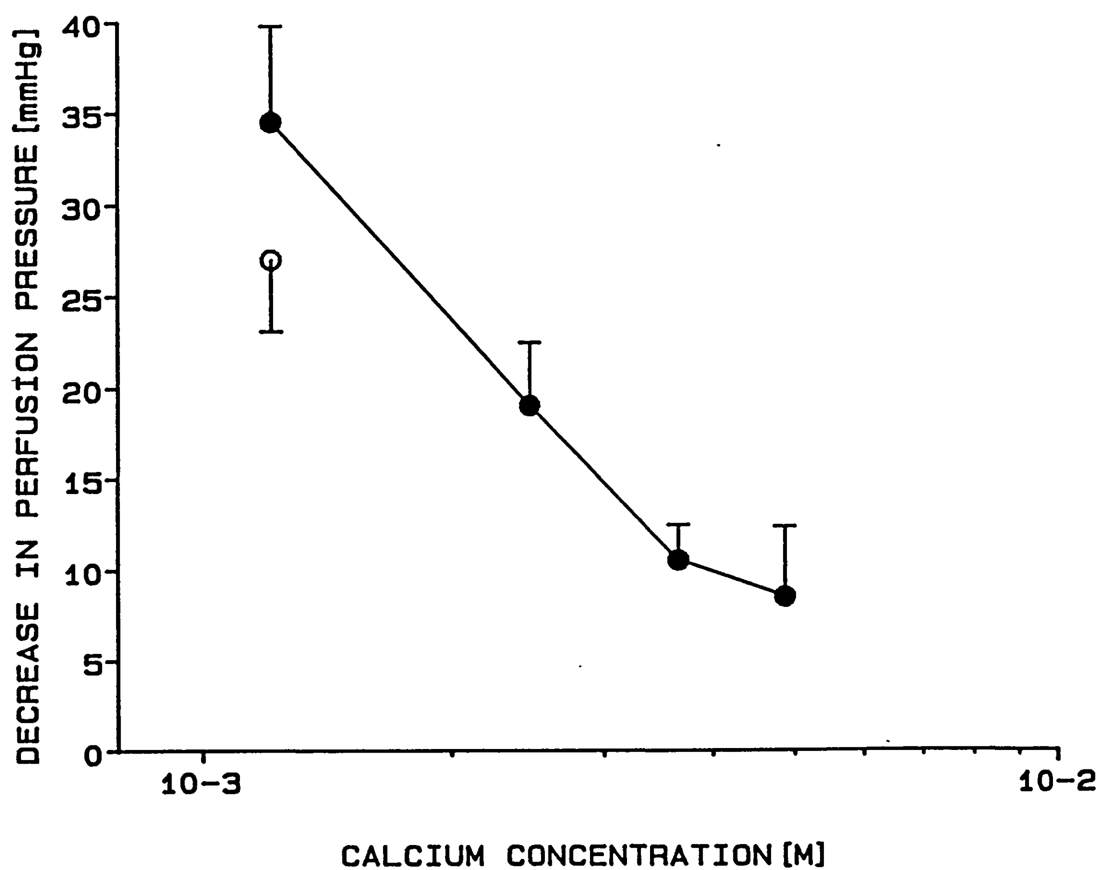



Figure 47. Effects of cumulatively increasing extracellular calcium concentration (1.2-4.8mM) on the vasodilator action of a single dose of PCE (10nmoles (•)) in the isolated Langendorff perfused rat heart. The response to a further dose of PCE (10nmoles) following recovery at 1.2mM [$Ca^{2+}$] is also shown (o). Vertical bars represent s.e.mean (*n*=4).

## 2) MESENTERIC VASCULAR BED.

a) PRECONTRACTED PREPARATIONS.

In order to examine the effects of the novel carnitine ester $P1P^i$ on the mesenteric vascular bed, the basal tone of the preparation first had to be raised artificially to allow expression of any vasodilator activity of the drug. This was achieved using three constrictor agents, phenylephrine, endothelin and potassium, included in the perfusion medium at a constant concentration.

1) Endothelin.

Figure 48a shows a typical trace of the effect of bolus injections of $P1P^i$ on the isolated perfused mesenteric vascular bed of the rat, precontracted with endothelin (1nM). The peptide, endothelin-1, produced a gradual increase in the basal tone of the preparations, reaching a plateau of 90.6±8.0mmHg, and subsequent addition of the carnitine ester produced gradual decreases in perfusion pressure reaching a maximum at about 30nmoles (fig.48b). Associated with higher doses of the drug (>10nmoles) was a vasoconstrictor action which preceded the fall in vascular tone, whilst at supramaximal doses $P1P^i$ elicited solely a pressor response.

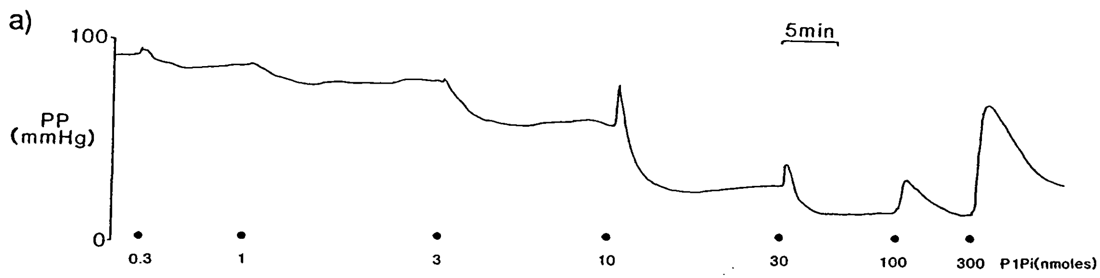
2) Phenylephrine.

In those vascular beds that were precontracted with phenylephrine (10µM), the profile of activity of $P1P^i$ was similar (fig.49). Basal tone was raised to 144.0±12.8mmHg on addition of phenylephrine, and under these conditions the isopropyl ester of palmitoyl carnitine produced a relaxant action on the mesenteric vasculature with associated constrictor effects apparent at higher doses (>10nmoles).

3) Potassium.

Resting tone in the preparations was increased to a level of 127.0±13.6mmHg by inclusion of high potassium (110mM) in the perfusion medium ($Na^+$ reduced to

a)




b)

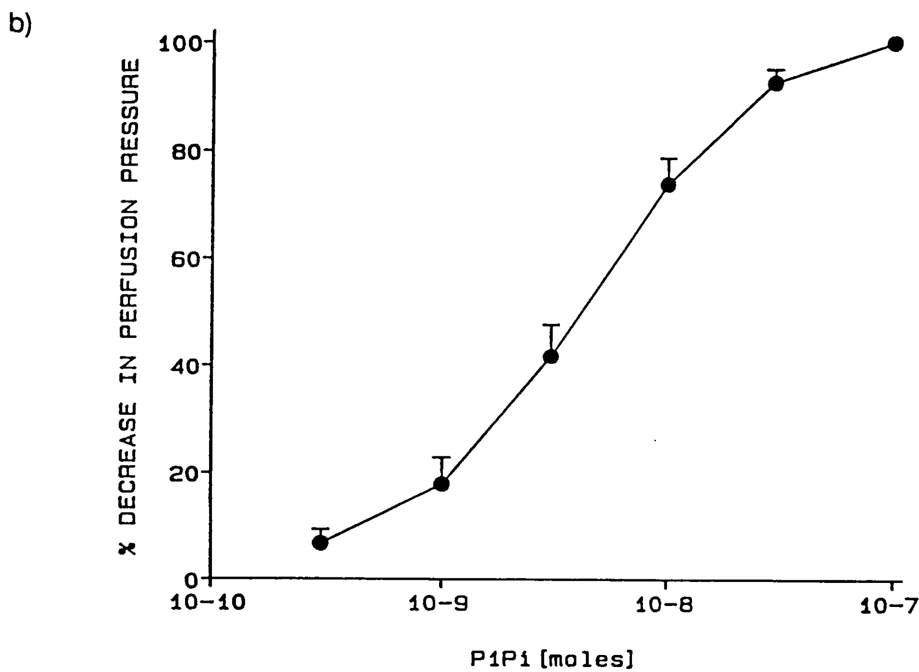



Figure 48. a) Typical trace showing the effects of P1P$^i$ (0.3-300nmoles) on perfusion pressure (PP) in the isolated perfused mesenteric vascular bed of the rat precontracted with endothelin (1nM). b) Vasodilator action of P1P$^i$ in rat mesenteric vascular beds precontracted with endothelin (1nM), expressed as % cumulative decrease in perfusion pressure (100%=61.6±5.5mmHg, *n*=9).

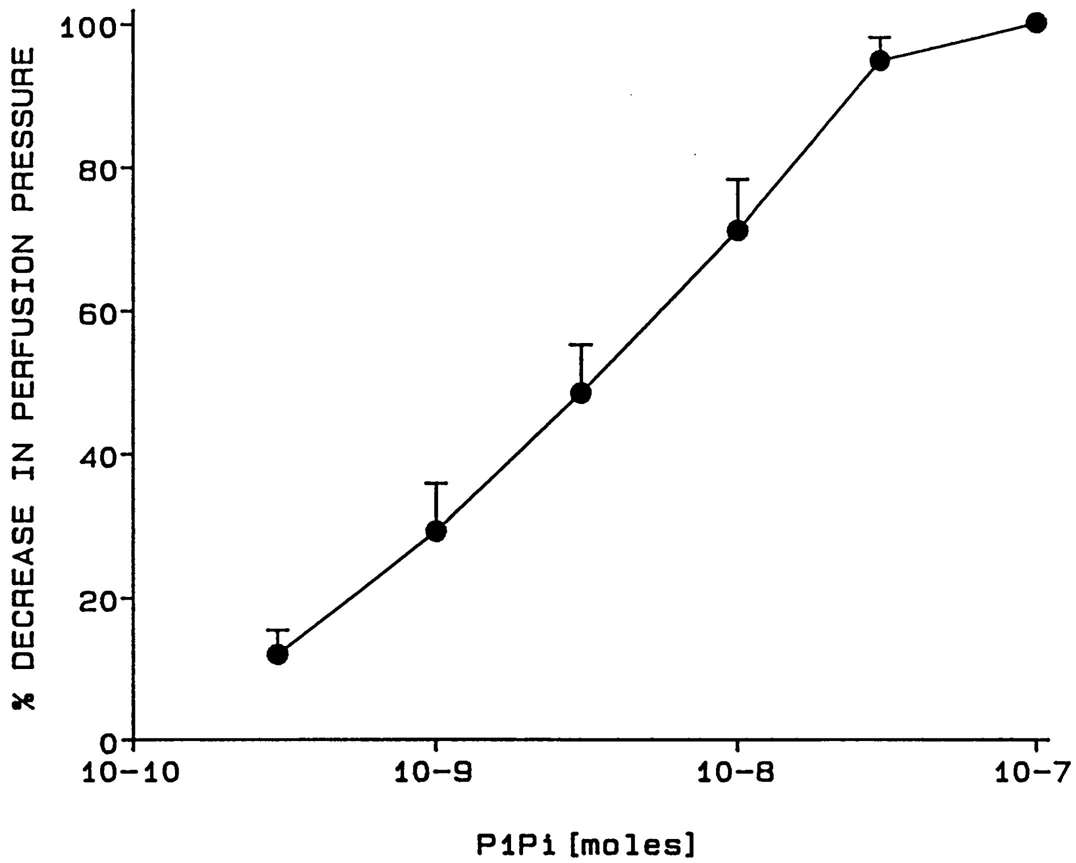



Figure 49. Effects of P1P[i] (0.3-100nmoles) on perfusion pressure in isolated perfused mesenteric vascular beds of the rat precontracted with phenylephrine (10μM), expressed as % cumulative decrease in perfusion pressure (100%= 120.5±38.9mmHg, $n$=5).

maintain osmolarity), and in these precontracted beds P1P[i] induced a dose-related fall in perfusion pressure (fig.50). At higher doses of the ester (>10nmoles) the response was clearly biphasic; initially there was a rapid drop in perfusion pressure followed by a slower, more sustained relaxation of the mesenteric vessels. However, in the presence of high potassium there was not the same degree of vasoconstriction associated with higher doses of P1P[i], in fact only one dose (300nmoles) exhibited this action (fig.50).

In a separate group of experiments the mesenteric vessels were allowed to recover from responses to P1Pi before addition of the succeeding dose of drug (fig.51), and under these conditions the biphasic vasodilator response was clearly seen. Figure 52 illustrates the relationship between the 'fast' and 'slow' components of the P1P[i] response; both components are dose-related and appear to mirror each other.

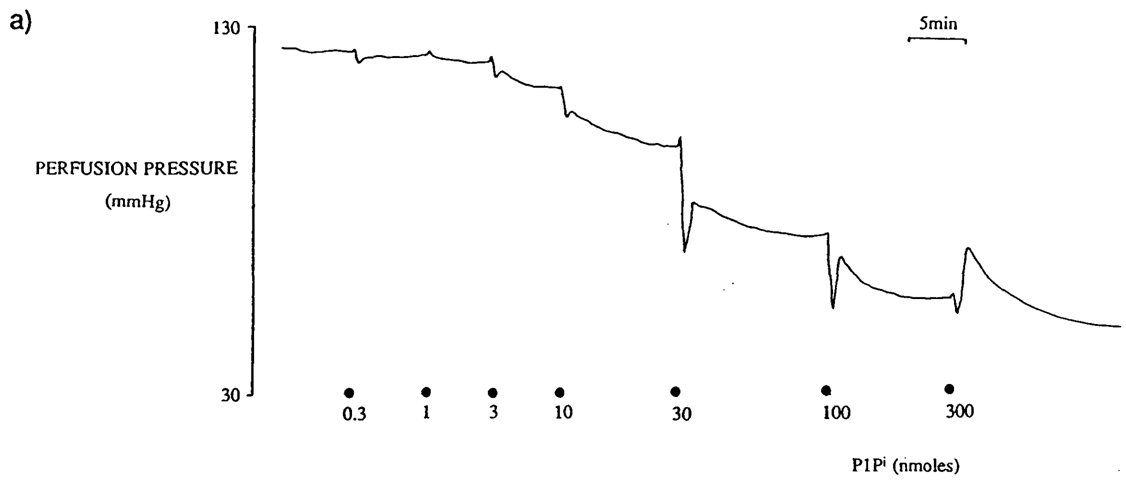
In addition, the effects of the calcium channel blocker verapamil were investigated in potassium depolarised mesenteric vascular beds, and the relaxant action compared to that of P1P[i] (fig.53). Both agents elicited dose-related vasodilation, however, verapamil appeared the more potent drug.

b) EFFECT OF P1Pi ON CALCIUM-INDUCED CONTRACTION.

Having established that the synthetic carnitine ester exerted a relaxant action on precontracted mesenteric vascular beds, displaying a more rapid effect in potassium-depolarised tissues, the possibility that P1P[i] was acting via blockade of voltage-operated calcium channels (VOCs) was investigated.

Cumulative concentration-response curves were constructed to calcium (0.1-10mM) in potassium-depolarised (110mM) mesenteric vascular beds, and in control preparations the tissue sensitivity did not alter over the experimental period (fig.54). The effect of P1P[i] on calcium-induced contraction was investigated by constructing a control concentration-response curve to calcium chloride and,

a)




b)

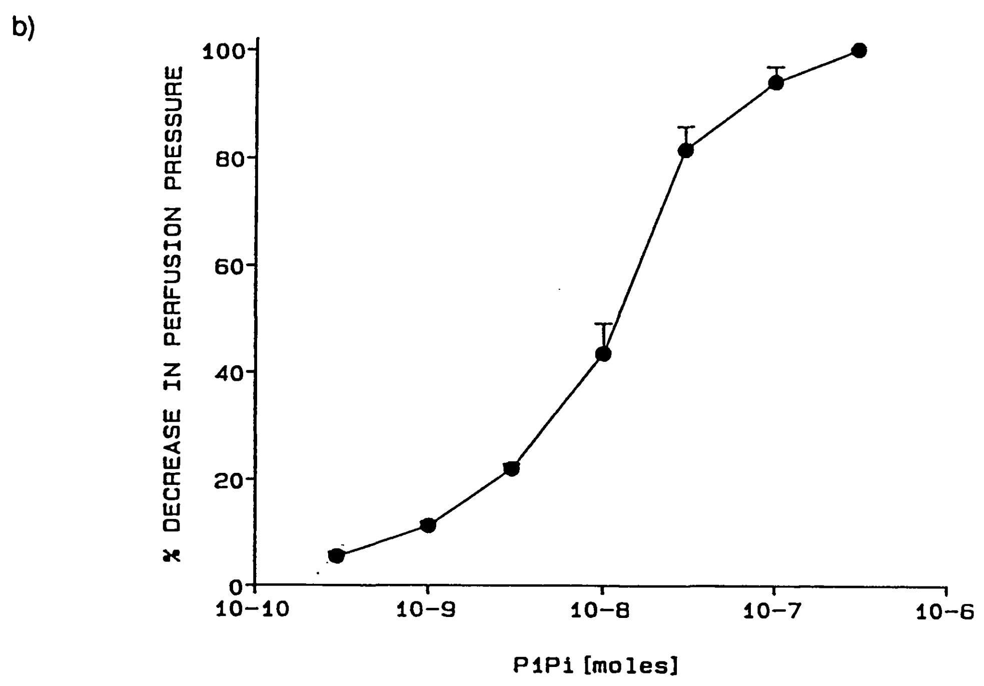



Figure 50. a) Typical trace showing the effects of $P1P^i$ (0.3-300nmoles) on perfusion pressure in the isolated perfused mesenteric vascular bed of the rat precontracted with high potassium (110mM). b) Vasodilator action of $P1P^i$ in rat mesenteric vascular beds precontracted with high potassium (110mM), expressed as % cumulative decrease in perfusion pressure (100%=99.0±16.2mmHg, *n*=4).

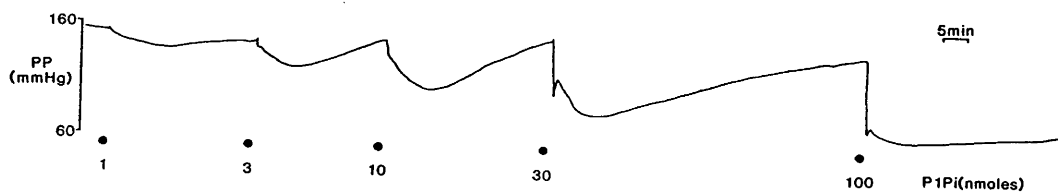



Figure 51. Typical trace showing the effects of P1P$^{i}$ (1-100nmoles) on perfusion pressure (PP) in the isolated perfused mesenteric vascular bed of the rat precontracted with high potassium (110mM).

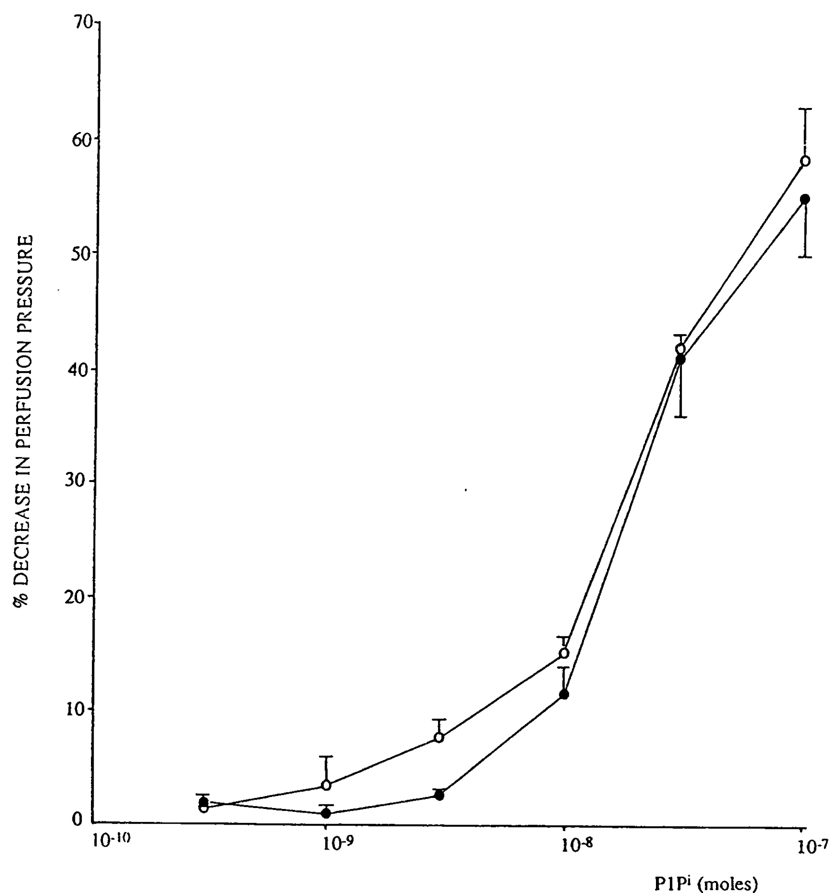



Figure 52. Comparison of the 'fast' (●) and 'slow' (o) components of the P1Pi-induced vasodilation of isolated perfused rat mesenteric vascular beds precontracted with high potassium (110mM), expressed as % decrease in perfusion pressure from basal values (100%=96.0±21.3mmHg, *n*=4).

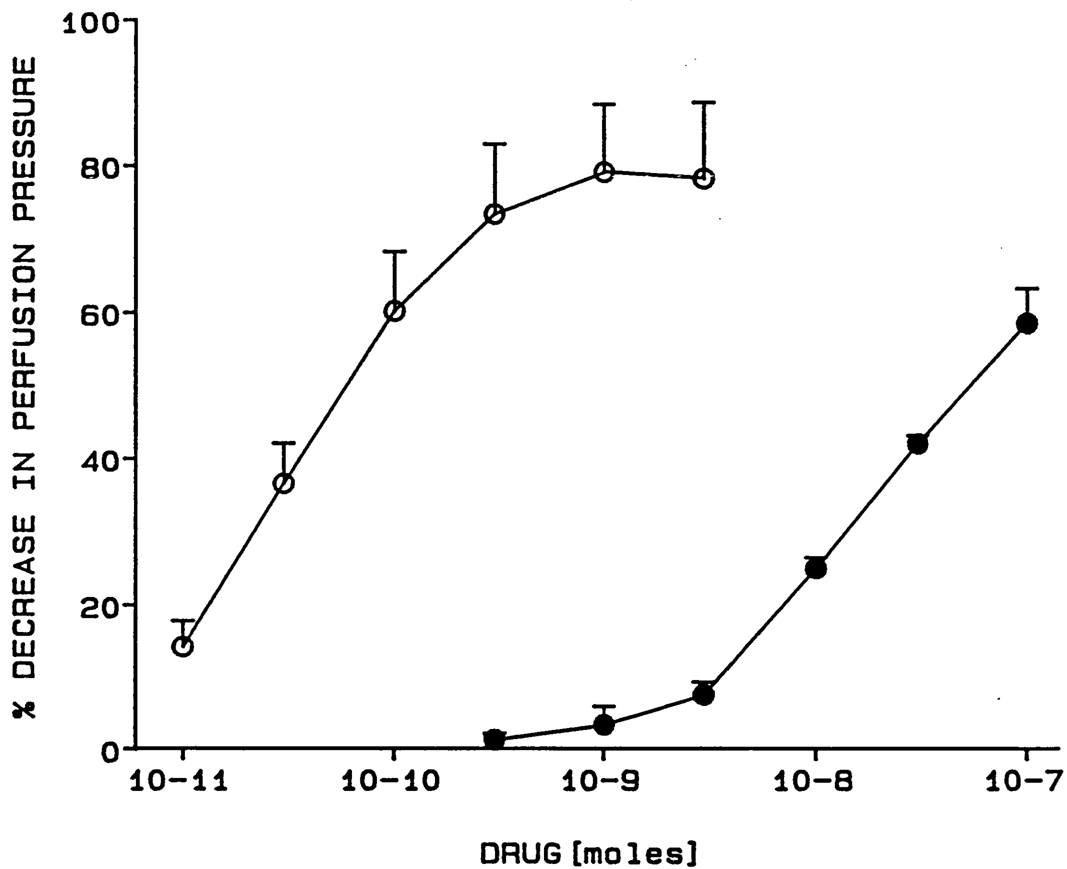



Figure 53. Comparison of the vasodilator effects of verapamil (o, $n$=6) and P1P$^{i}$ (•, $n$=4) in the isolated perfused mesenteric vascular bed of the rat precontracted with high potassium (110mM), expressed as % decrease from basal values.

following washout of the calcium, perfusing the vascular bed with $P1P^i$ (0.1-1.0μM) and repeating the cumulative addition of calcium in the presence of the ester (fig.55a). $P1P^i$ significantly attenuated the contraction induced by calcium in a concentration-dependent manner (fig.55b); at all concentrations of calcium the contractile response of the vascular bed was reduced by $P1P^i$.

### 3) GUINEA-PIG TAENIA-COLI.

#### a) EFFECT OF $P1P^i$ ON CALCIUM-INDUCED CONTRACTION.

A similar protocol was adopted to assess whether $P1P^i$ exerts an antagonistic effect on contractile responses to calcium in the guinea-pig taenia coli preparation depolarised with high potassium (110mM). Figure 56 shows the effect of a single concentration of $P1P^i$ (1μM) on the response to calcium in this preparation. There was a change in sensitivity of the taenia coli with time, however, the response to calcium was not significantly altered by the isopropyl ester of palmitoyl carnitine.

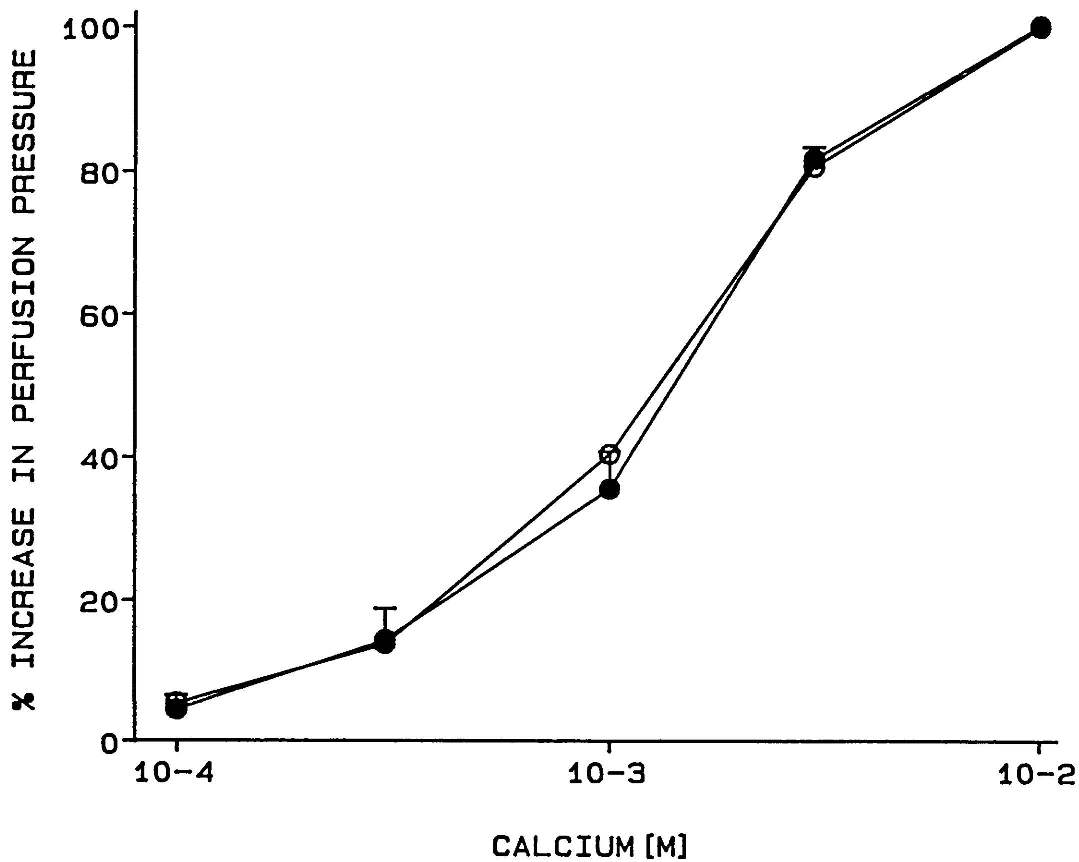
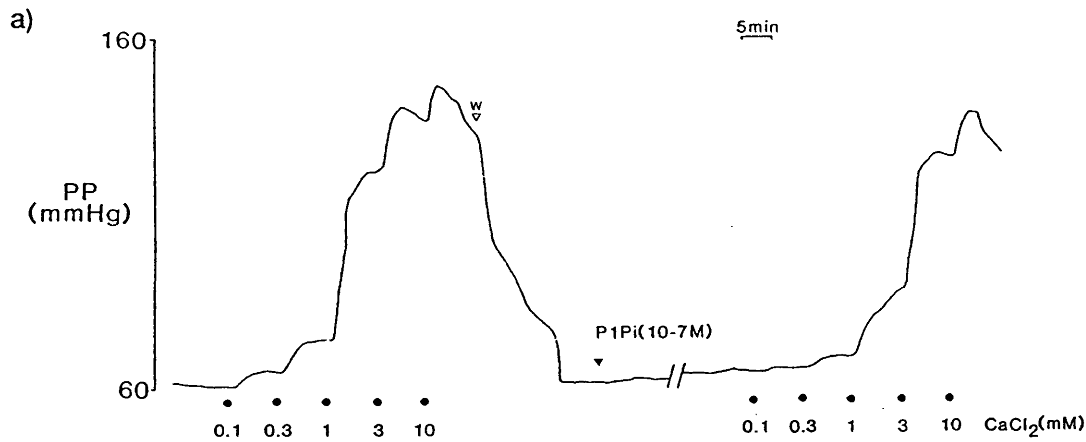



Figure 54. Effects of repeated administration of calcium ($10^{-4}$-$10^{-2}$M) to rat isolated perfused mesenteric vascular beds depolarised with high potassium (110mM). Data expressed as % cumulative increase in perfusion pressure for initial control concentration-response (•) and for 2nd concentration-response (o) performed 40 minutes later (*n*=4).



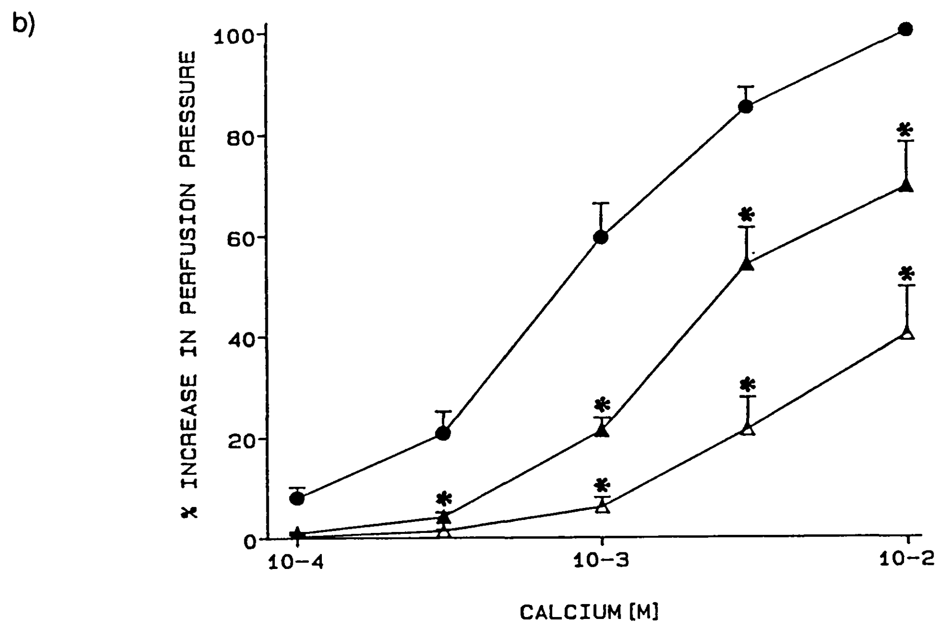




Figure 55. a) Typical trace showing the effects of P1P[i] (0.1μM) on the increase in perfusion pressure elicited by cumulative concentrations of calcium chloride (0.1-10mM) in the mesenteric vascular bed of the rat depolarised with high potassium (110mM). b) Effects of 0.1μM (▲, *n*=4) and 1.0μM (△, *n*=4) P1P[i] on the constrictor response to calcium in potassium-depolarised mesenteric vascular beds, expressed as % increase in perfusion pressure of maximal response of control curve (●, 100%=88.5±7.5mmHg, *n*=8). Vertical bars represent s.e.mean (*p<0.05).

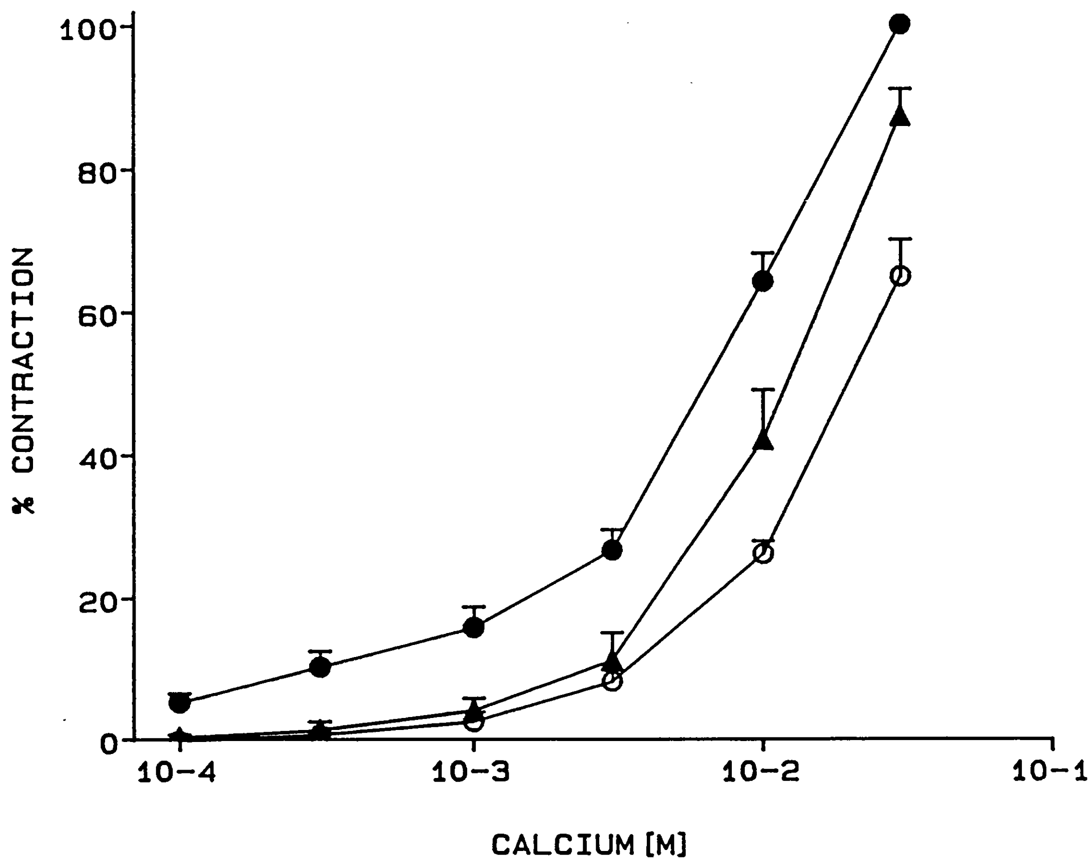



Figure 56. Comparison of the effects of 1μM P1P[i] (▲,*n*=4) on the contractile response to calcium in guinea-pig taenia coli depolarised with high potassium (110mM) with that of a time-matched control (o, *n*=4), expressed as % increase of the maximal contraction of the initial control concentration-response (●, 100%= 5.0±0.45g, *n*=8).

## 4) AORTA.

### a) PRECONTRACTED TISSUES.

Having examined the effects of P1P$^i$ on fine resistance vessels i.e. the coronary and mesenteric vascular beds, the action of P1P$^i$ on the isolated thoracic aorta of the rat, prepared as rings, was investigated. In order to assess the vascular relaxant effect of the ester, the tissues were first precontracted using either phenylephrine, endothelin or high potassium.

#### 1) Phenylephrine.

Aortic rings were precontracted with phenylephrine ($10^{-7}$M) and the integrity of the endothelium assessed by addition of acetylcholine (1µM) to the organ bath; tissues that showed less than 80% relaxation were assumed to possess a damaged endothelial cell layer and were discarded. In addition, in some experiments the endothelium was mechanically removed before examining the effects of P1Pi on the aorta.

Phenylephrine ($10^{-7}$M) produced a rise in tension of 0.46±0.07g and 0.69±0.03g in aortic rings with and without an intact endothelial cell layer, respectively. Figure 57 shows typical traces obtained when P1P$^i$ (0.4-12.8µM) was added cumulatively to such precontracted preparations; no appreciable relaxant action of P1P$^i$ was observed in either rubbed or unrubbed tissues (fig.58). Additionally, in some preparations the tissue was washed following application of P1P$^i$, recontracted to phenylephrine ($10^{-7}$M), and relaxed by addition of papaverine (1-30µM) to confirm that mechanical damage of the aorta had not occurred during the course of the experiment (*n*=3, data not shown).

The effect of altering the potassium concentration of the bathing solution, from 5.9mM to 3.2mM, on the response of phenylephrine precontracted aortae to P1P$^i$ was also investigated to examine whether the modified Krebs solution used in experiments on perfused rat hearts was allowing manifestation of latent vasodilator

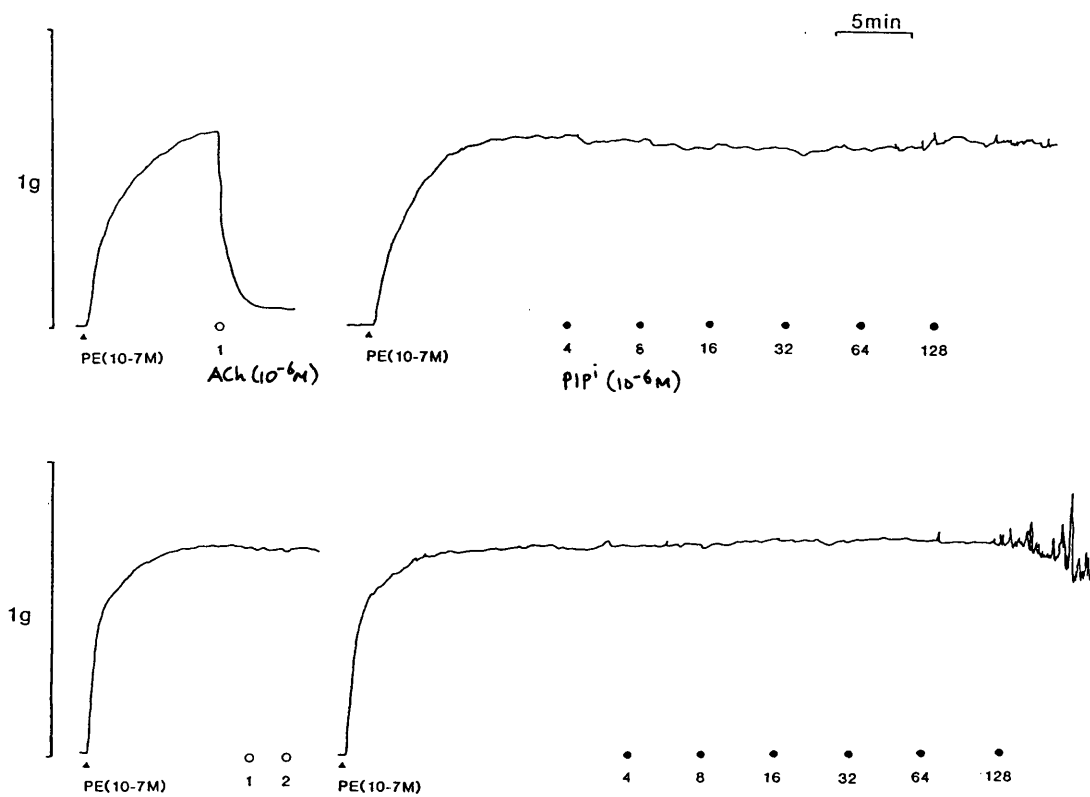



Figure 57. Effects of acetylcholine (1-2μM) and P1P$^{i}$ (0.4-12.8μM) added cumulatively on tension(g) in rat thoracic aortic rings with the endothelium either intact (*upper trace*) or artificially removed (*lower trace*) precontracted with phenylephrine (0.1μM).

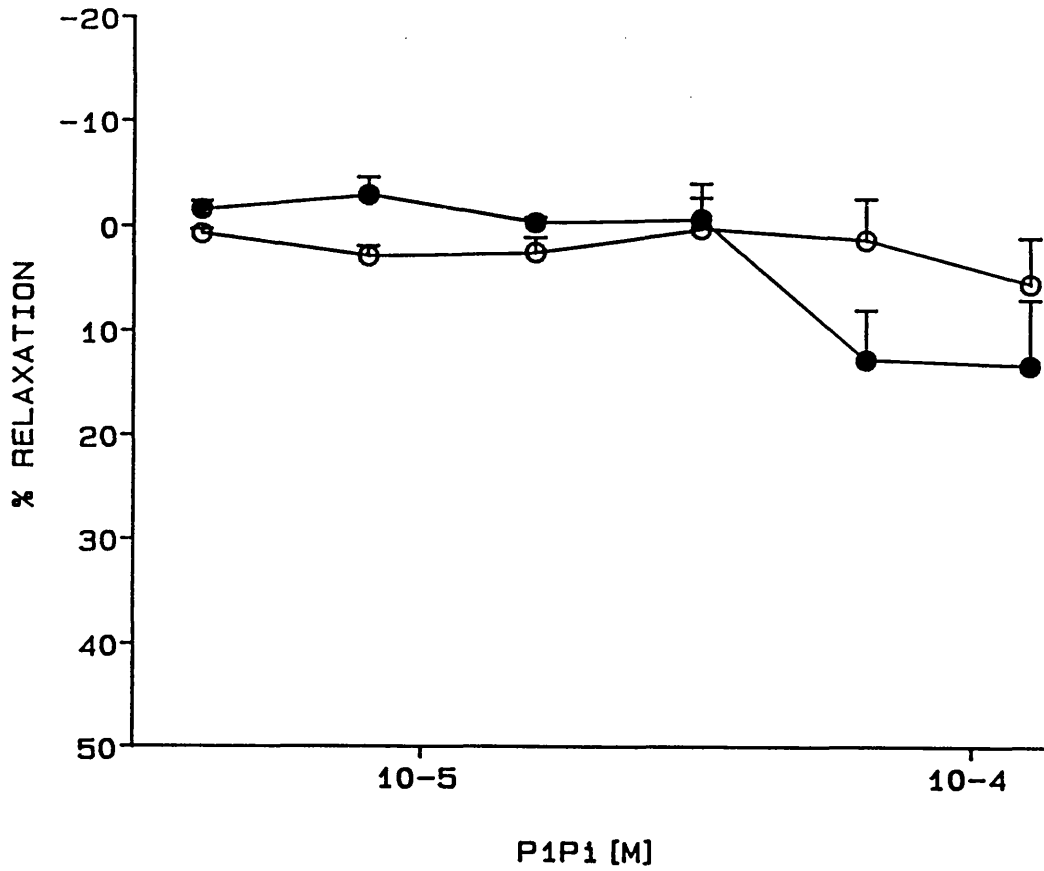



Figure 58. Effects of P1P[i] (0.4-12.8μM) on phenylephrine-precontracted rat thoracic aortic rings with endothelium intact (•) or artificially removed (o), expressed as % relaxation of induced tone (*n*=12).

activity. Similar results to those obtained previously with P1P[i] using 5.9mM [$K^+$] were found with 3.2mM [$K^+$] in the modified Krebs-Henseleit solution i.e. no relaxation was produced by the ester (*n*=4, data not shown).

2) Potassium.

Potassium chloride was also used to precontract aortic rings, and concentrations of 80mM and 110mM elicited rises in vascular tone of 0.81±0.08g and 0.86±0.10g, respectively. A typical trace illustrating the response to P1P[i] (0.5-64μM) in the presence of the higher potassium concentration is shown in figure 59. P1P[i] caused gradual relaxation of potassium-precontracted aortae, exhibiting slightly more effect at 110mM than 80mM (~50% peak relaxation compared with ~30%, respectively) (fig.60).

3) Endothelin.

Endothelin-1 (1nM) induced a gradual rise in tension, reaching a plateau level (0.46±0.02g) about 20 minutes after inclusion in the organ bath (fig.59). P1P[i] (4-64μM) caused a relaxation of endothelin precontracted preparations reaching ~60% of the induced tone at the highest concentration used (fig.60).

b) EFFECT ON AGONIST RESPONSE.

The effect of the synthetic carnitine ester P1P[i] on the response of the rat aorta to two agents, phenylephrine and acetylcholine, was also investigated.

1) Phenylephrine.

Aortic rings were contracted with phenylephrine ($10^{-7}$M) and, following a plateau response to the drug, acetylcholine (1μM) was introduced into the organ bath to assess endothelial integrity in a similar manner to that previously described; again, less than 80% relaxation of the tissue was taken to be indicative of endothelial cell damage and these preparations discarded. The aortae were then

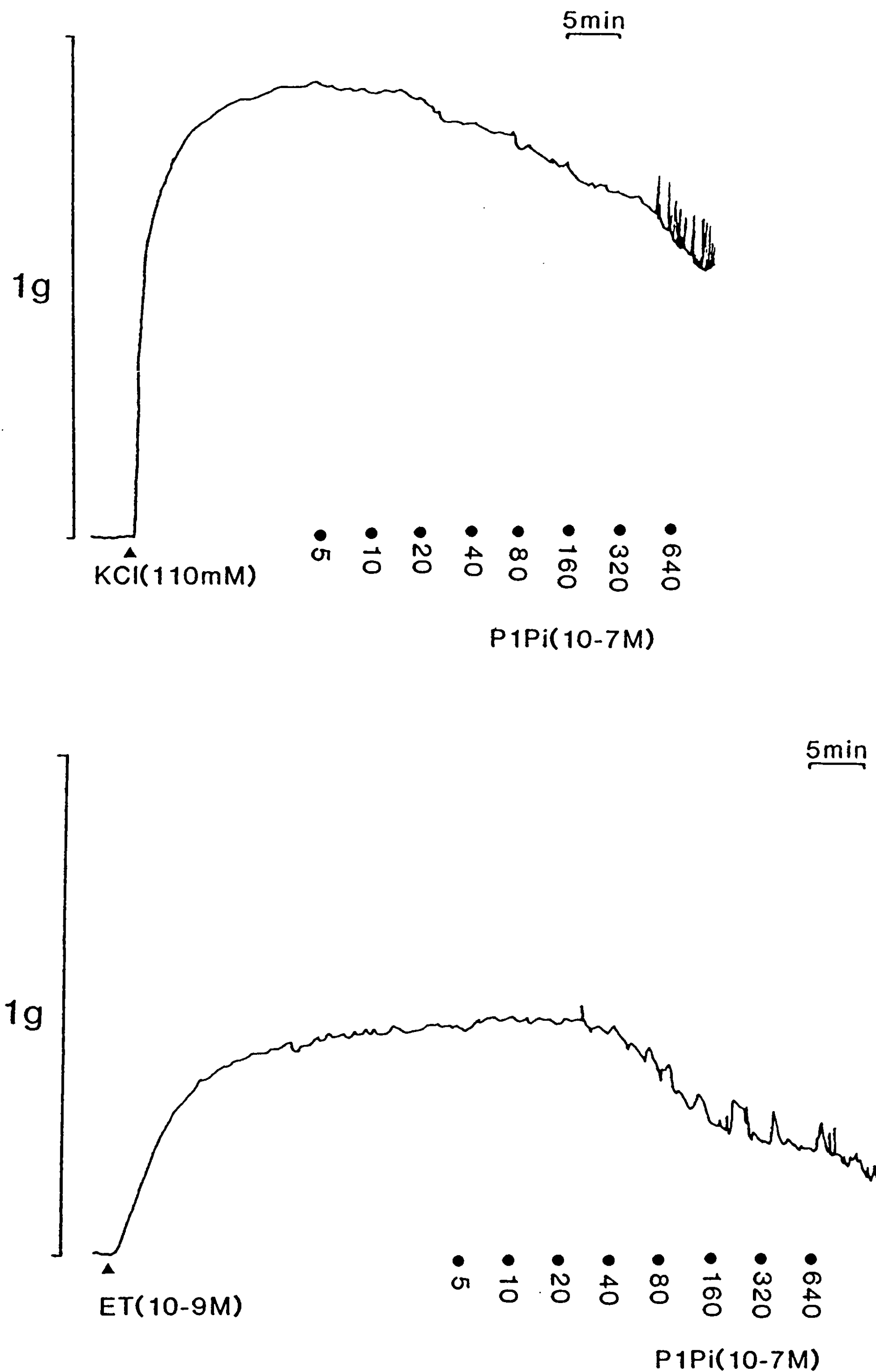



Figure 59. Typical traces showing the effects of P1P$^{i}$ (0.5-64μM) added cumulatively to the organ bath on tension in rat aortic rings precontracted with either high potassium (110mM) (*upper trace*) or with endothelin (1nM) (*lower trace*).

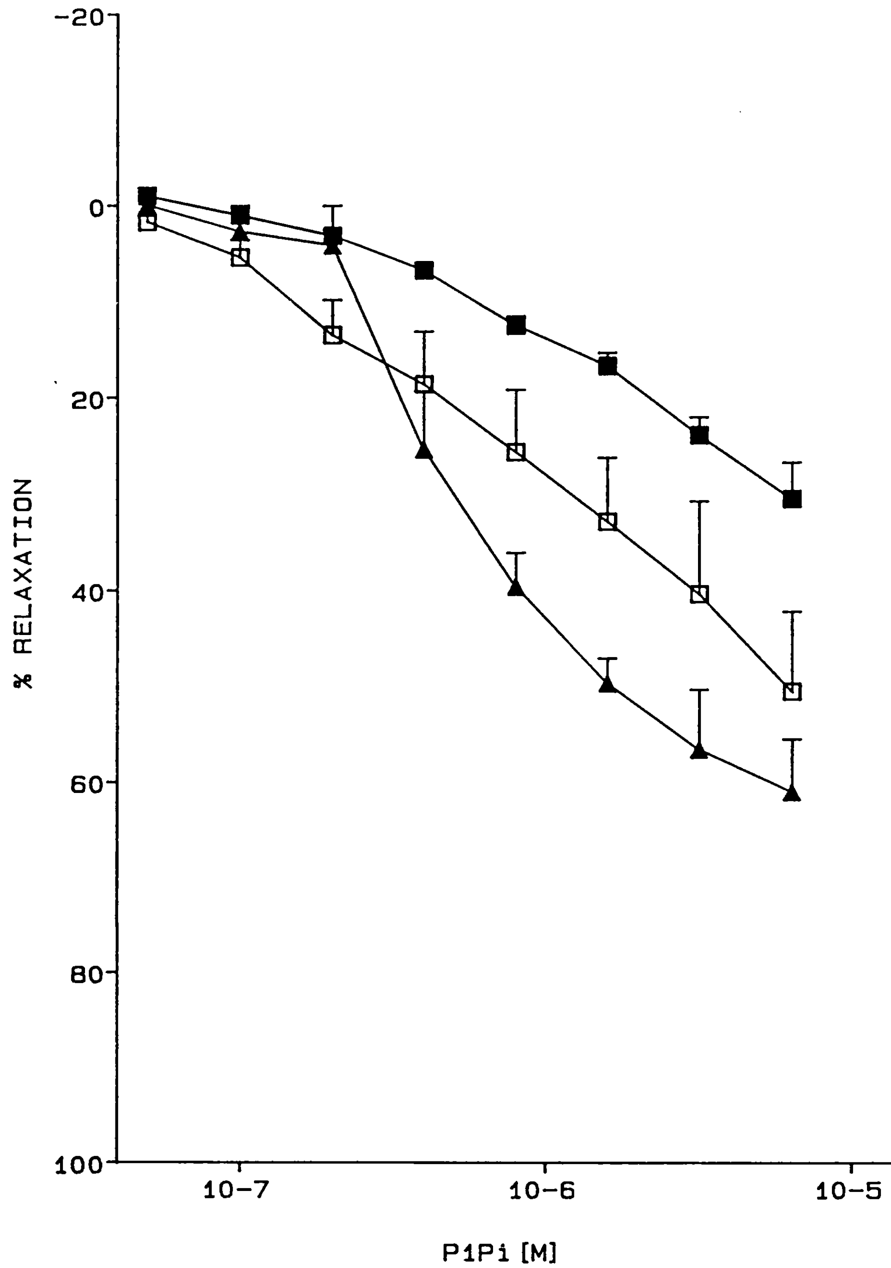



Figure 60. Relaxant effects of P1P^i (0.5-64.0μM) on rat aortic rings precontracted with either 80mM (■, *n*=4) or 110mM (□, *n*=4) potassium chloride, or with 1nM endothelin (▲, *n*=3), expressed as % decrease of induced tone.

washed and, after a recovery period, cumulative concentration-response curves to phenylephrine were constructed. Figure 61 shows that sensitivity of the preparations did not vary with time.

The effect of P1P$^i$ on the response to phenylephrine was assessed adopting the same protocol as above except that, 15 minutes prior to the second concentration-response curve to the $\alpha$-adrenoceptor agonist, the tissue was incubated with a fixed concentration of P1P$^i$ and then responses to phenylephrine repeated in the presence of the synthetic ester. P1P$^i$ (0.1-1μM) had no effect on the contractile effect of phenylephrine, however, a higher concentration of 10μM produced a significant attenuation of the response (fig.62); there was a non-parallel shift in the concentration-response curve with a depressed maximal contraction to phenylephrine.

2) Acetylcholine.

The effect of P1P$^i$ on acetylcholine-induced relaxations of aortic rings, precontracted with phenylephrine ($10^{-7}$M), was investigated. Tissues were precontracted, endothelial integrity assessed as before, washed and allowed to recover. Preparations were recontracted with phenylephrine ($10^{-7}$M) and cumulative concentration response curves to acetylcholine constructed; initially a control curve was obtained and then further curves in the presence of increasing concentrations of P1P$^i$ (0.1-10μM). Concentrations of P1P$^i$ up to 1μM did not affect the relaxant action of acetylcholine, however, at 10μM the drug significantly attenuated this response (fig.63). The response to acetylcholine in the absence of P1P$^i$ did not appreciably change throughout the experimental period (data not included).

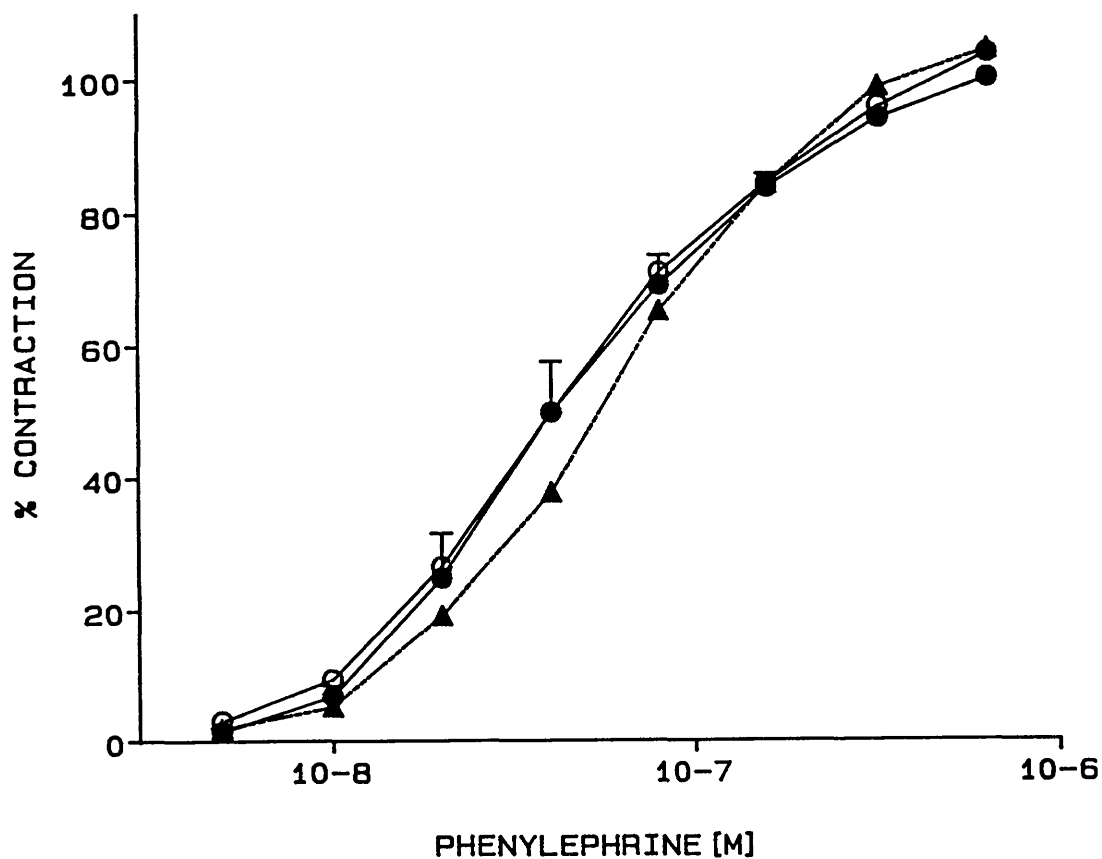



Figure 61. Effects of repeated addition of cumulative concentrations of phenylephrine (5-640nM) on tension in rat thoracic aortic rings, expressed as % contraction of the maximal control response. Control (•), 2nd (o) and 3rd (▲) concentration-response curves were performed allowing 30 minute recovery periods between each curve (100%=0.85±0.08g, *n*=6).

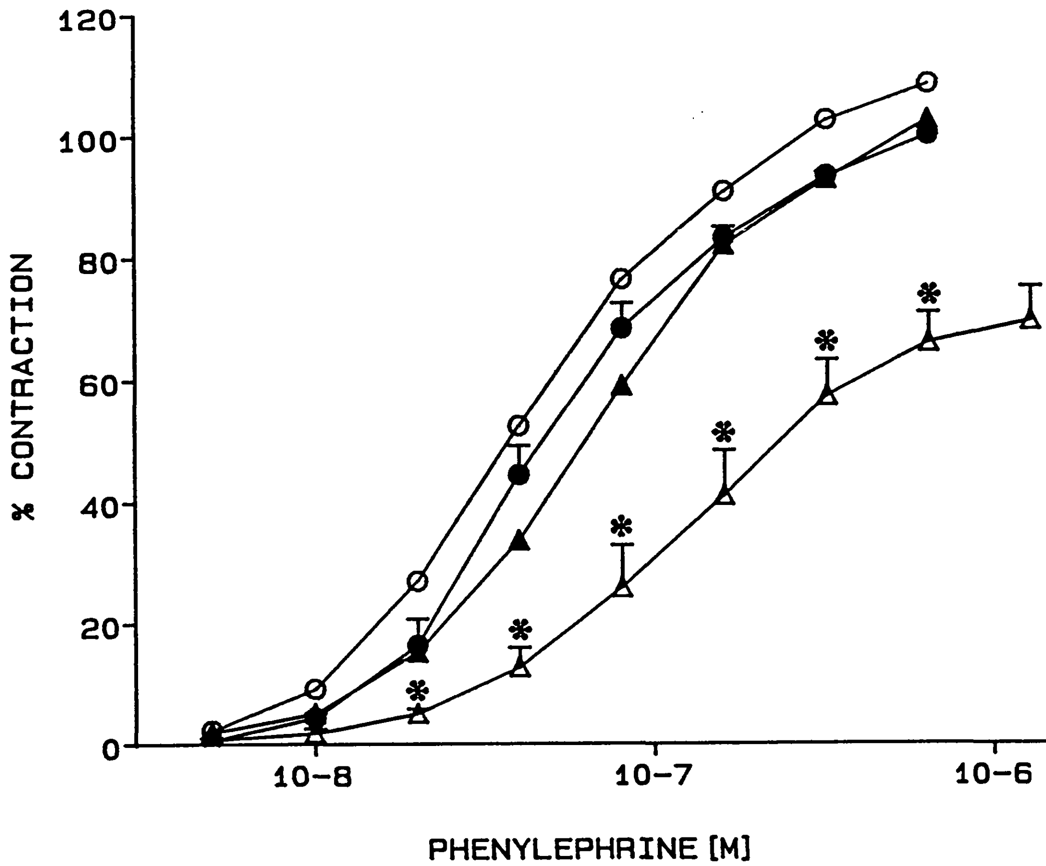



Figure 62. Effects of 0.1μM (o), 1.0μM (▲) and 10μM (Δ) P1P[i] on the contractile response of rat thoracic aortic rings to cumulative concentrations of phenylephrine, expressed as % contraction of the maximal response of the initial control curve (●, 100%=0.76±0.1g, *n*=4). Vertical bars represent s.e.mean (*p<0.05).

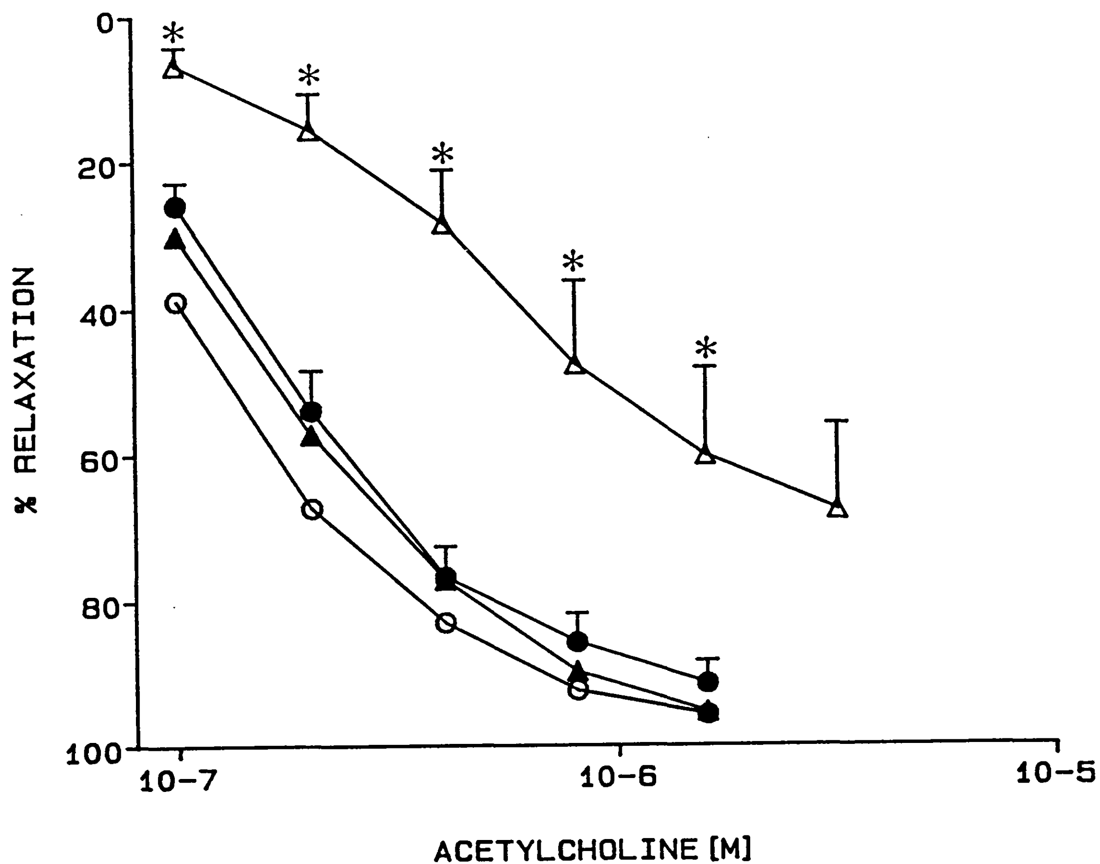



Figure 63. Effects of P1P[i] on the relaxation of rat thoracic aortic rings, precontracted with phenylephrine (0.1μM), elicited by cumulative concentrations of acetylcholine. Data expressed as % relaxation of induced tension in the presence of 0.1μM (o, 100%=0.67±0.06g), 1.0μM (▲, 100% = 0.55±0.08g) and 10μM (△, 100% =0.43±0.08g) P1P[i] compared with the initial control response (●, 100%= 0.73±0.06g). Vertical bars represent s.e.mean (*$p<0.05$, $n$=6).

## 5) ELECTROPHYSIOLOGY.

### a) PAPILLARY MUSCLE.

The effect of palmitoyl carnitine and its isopropyl ester, $P1P^i$, on the cardiac action potential of guinea-pig papillary muscle was investigated. Initially control values of action potential parameters were obtained in the absence of drug (table 3) and then, following perfusion of the muscle with a fixed concentration of either palmitoyl carnitine or $P1P^i$ for 20 minutes, further impalements were carried out in the presence of drug.

Table 3. The Cardiac Action Potential of the Guinea-pig Papillary Muscle (*n*=40, 8-12 impalements per papillary muscle).

| Parameter. | Control value. |
|---|---|
| Resting membrane potential (RMP) | 80.0±0.7mV |
| Upstroke velocity (dV/dt) | 215.9±8.7Vs$^{-1}$ |
| Amplitude | 103.0±1.3mV |
| T20 | 77.3±2.8ms |
| T50 | 187.9±2.2ms |
| T90 | 223.9±2.1ms |

(T values represent the time taken for % repolarisation of the action potential e.g. 20% (T20))

Palmitoyl carnitine (10-100µM) produced a concentration-related depression of the cardiac action potential (fig.64); the resting membrane potential became more

positive, amplitude decreased in magnitude, and the time taken for the action potential to repolarise was reduced. This effect is also illustrated in figure 65 which shows a series of action potentials recorded from a single cell, constantly impaled for a period of 20 minutes, in the presence of 100μM palmitoyl carnitine.

In contrast to palmitoyl carnitine, however, P1P$^i$ (100μM) was without effect on the action potential (fig.66). In view of its lack of action on cardiac muscle, the effects of P1P$^i$ on other types of muscle, including both vascular and nonvascular preparations, were examined.

b) RABBIT EAR ARTERY.

Resting membrane potential was recorded from ear artery cells for a control period, the artery then exposed to P1P$^i$ (1μM) for 20 minutes, and further impalements obtained in the presence of drug. In these experiments P1P$^i$ had no significant effect on the membrane potential (fig.67), however, the potassium channel activator, cromakalim (1μM), caused a large hyperpolarisation of the artery when tested using a similar protocol (fig.67); cromakalim altered resting potential from -55mV to -80mV.

In order to ascertain whether the isopropyl ester was exerting its action only in partially depolarised blood vessels, the effects of P1P$^i$ (1-10μM) was assessed on membrane potential in ear arteries already exposed to phenylephrine (10μM). Control impalements were carried out as described earlier, the muscle then exposed to phenylephrine for 10 minutes and further recordings made. Following this, increasing concentrations of P1P$^i$ (1-10μM) were incorporated into the bathing medium containing phenylephrine, and after an equilibrium period of 10 minutes additional impalements of drug treated cells were carried out. Figure 68 illustrates the depolarising action of the $\alpha_1$-adrenoceptor agonist phenylephrine, which altered membrane potential to a new resting level of approximately -33mV. P1P$^i$ (1-10μM) was without significant effect on the membrane potential in the presence of phenylephrine (fig.68).

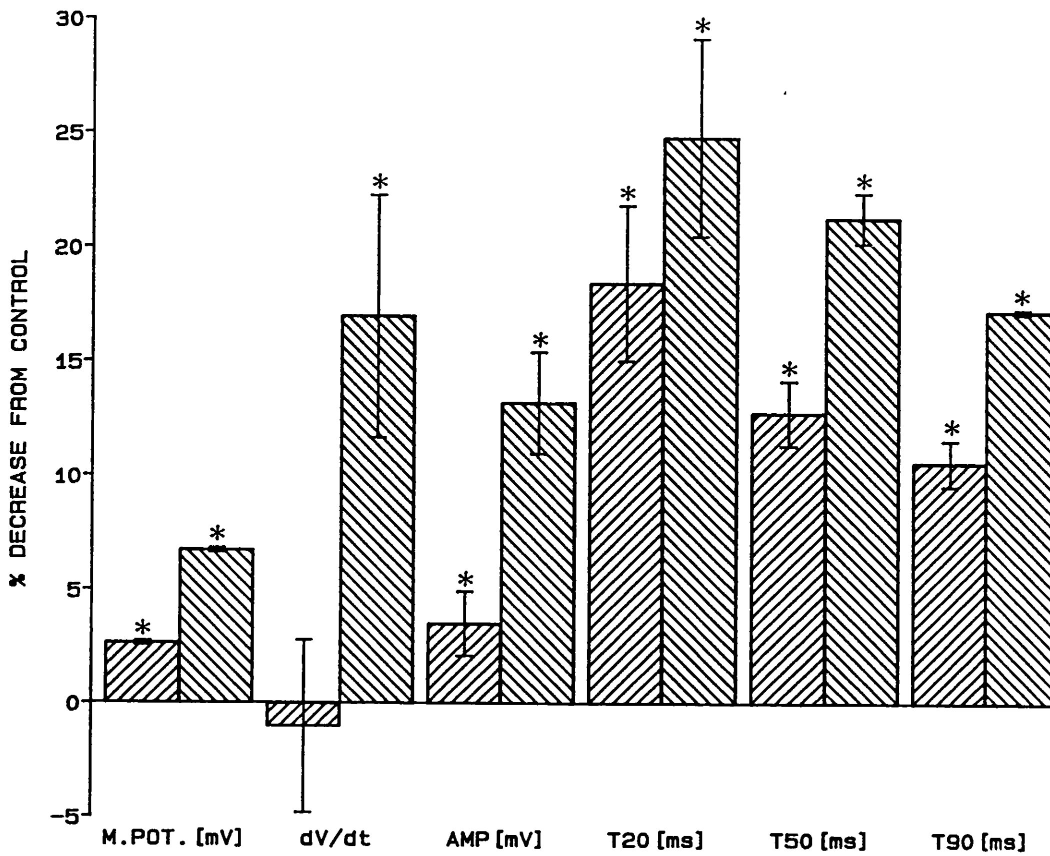



Figure 64. Effects of 10μM (▨) and 100μM (▧) palmitoyl carnitine on the cardiac action potential of guinea-pig papillary muscle, expressed as % decrease from control values. Parameters measured are membrane potential (M.POT.), upstroke velocity (dV/dt), amplitude (AMP), and the time taken for 20%, 50% and 90% repolarisation of the action potential (T20, T50 & T90, respectively). Vertical bars represent s.e.mean (*$p<0.05$, $n$=40).

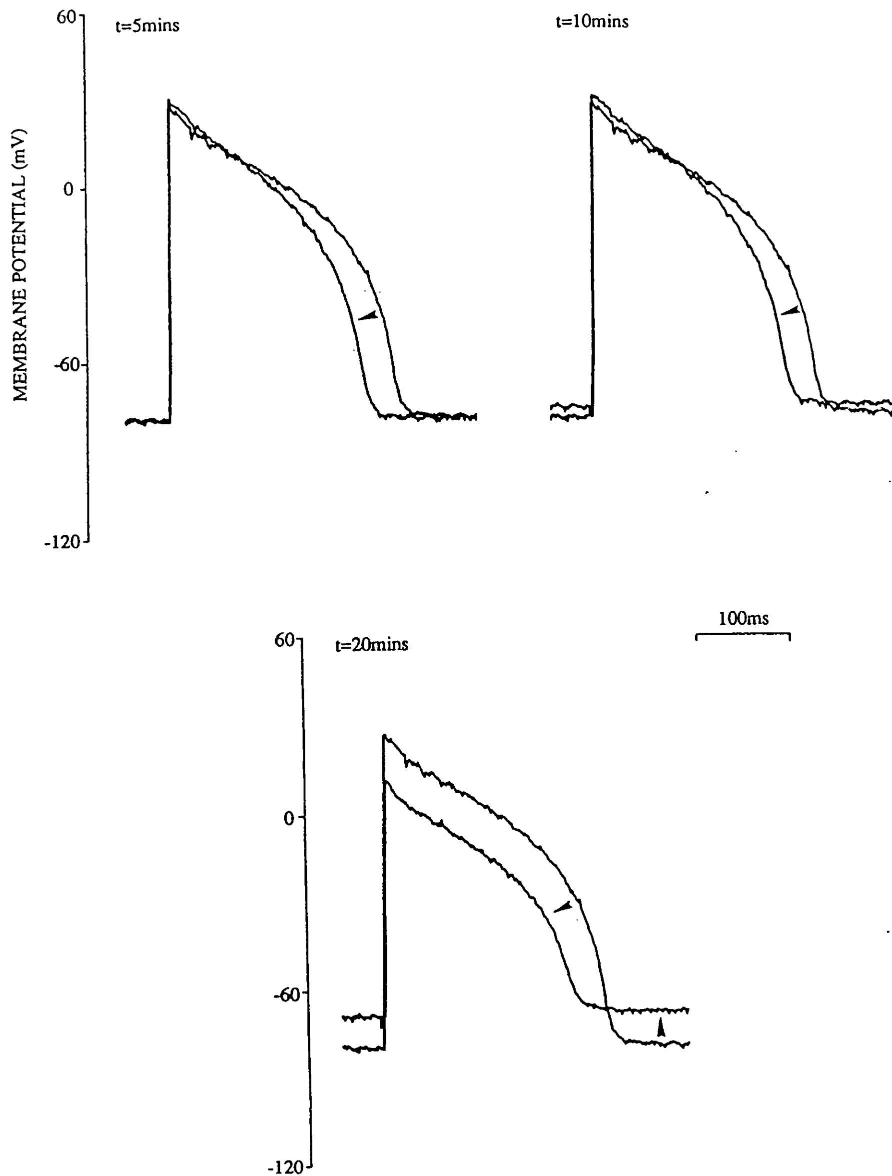



Figure 65. Effects of palmitoyl carnitine (100μM) on the cardiac action potential measured from a single cell of guinea-pig papillary muscle constantly impaled for a period of 20 minutes. Action potentials recorded at 5, 10 and 20 minutes (*indicated by arrows*) are superimposed over a control potential recorded before addition of palmitoyl carnitine.

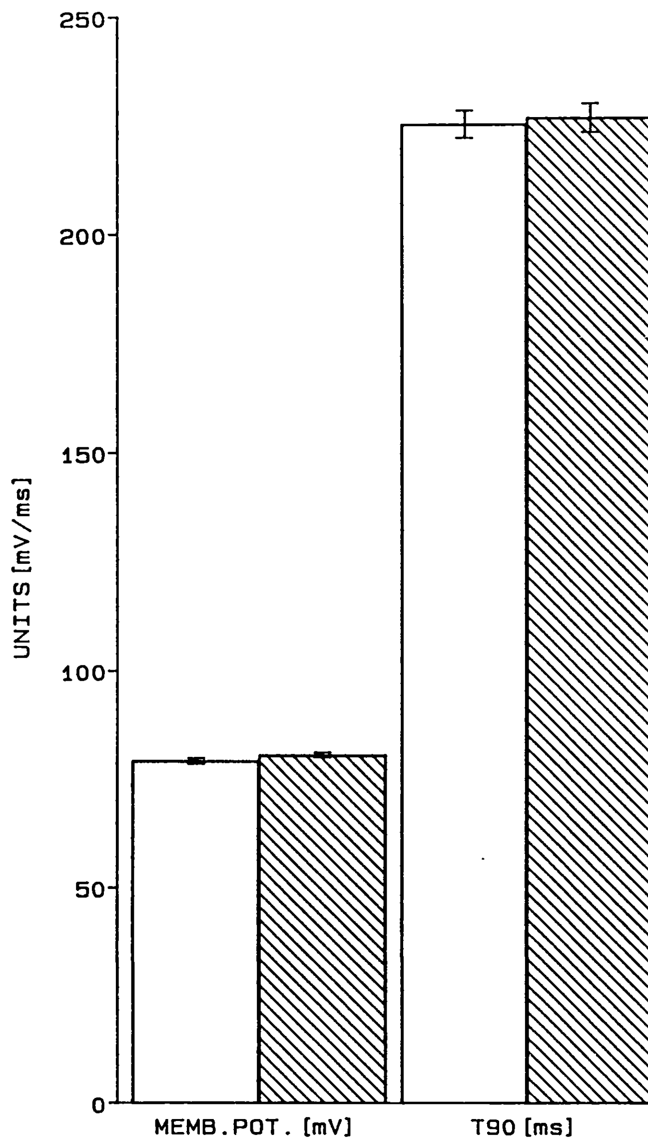



Figure 66. Effects of P1P[i] on the cardiac action potential from guinea-pig papillary muscle, expressed as resting membrane potential (MEMB.POT.) and time taken for 90% repolarisation (T90) in cells exposed to 100μM P1P[i] (*hatched bars)* for 20 minutes compared with control values (*empty bars*). Vertical bars represent s.e.mean (*n*=30).

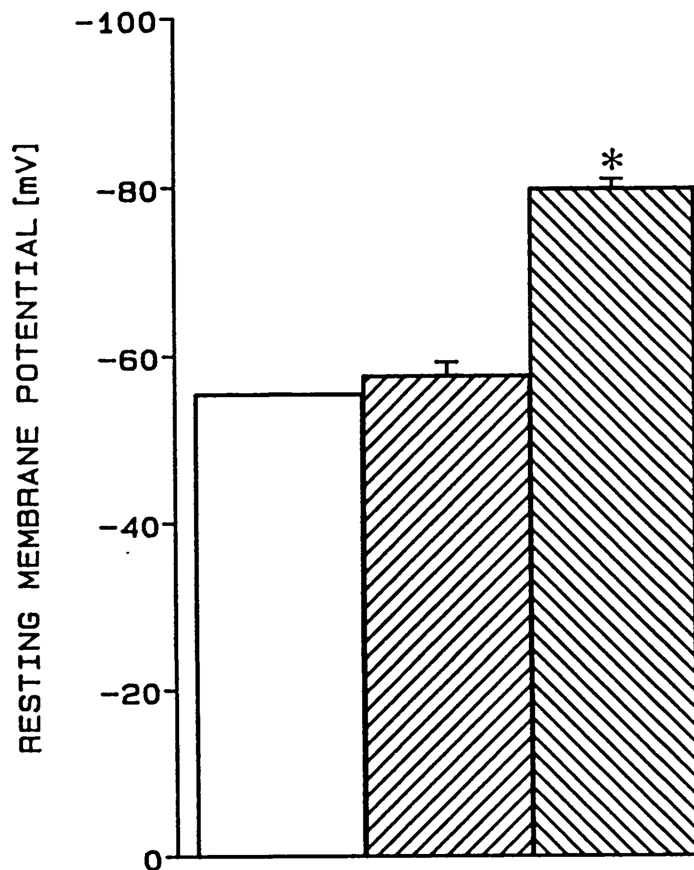



Figure 67. Effects of P1P[i] (1µM, ▨ ) and cromakalim (1µM, ▧ ) on resting membrane potential recorded from single cells of the rabbit ear artery, compared with control values ( □ , *n*=15, 3 preparations). Vertical bars represent s.e.mean (*$p<0.05$).

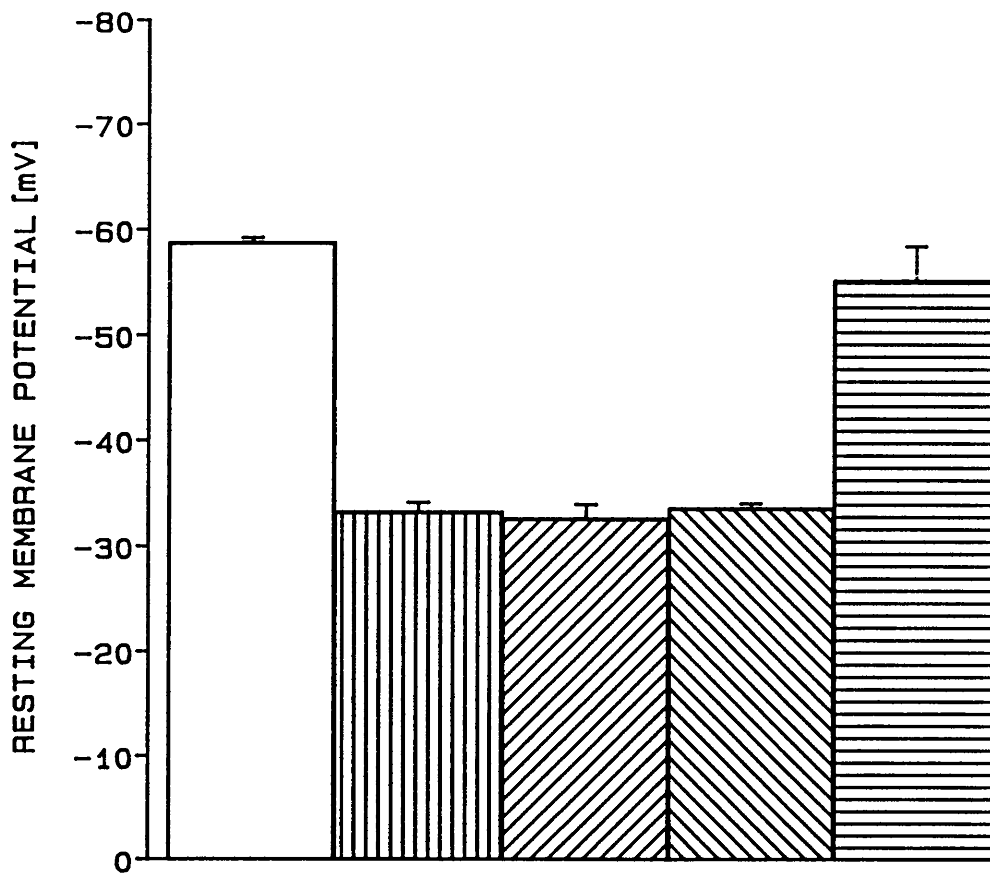
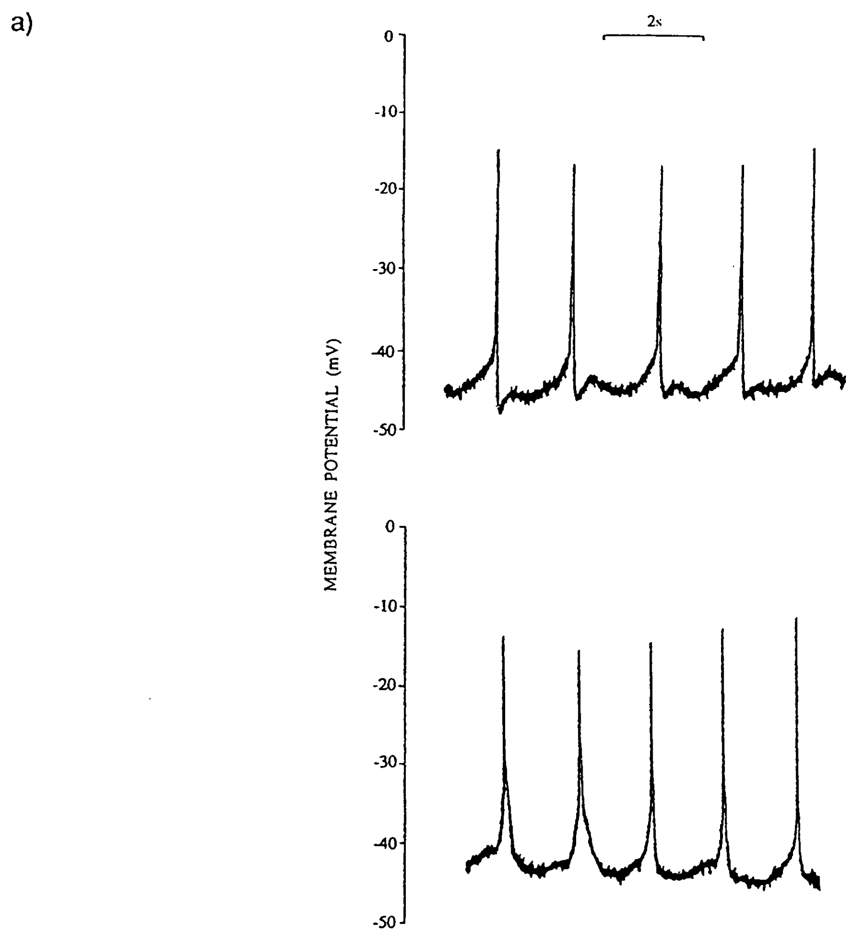



Figure 68. Effects of 1µM ( ▨ ) and 10µM ( ▧ ) P1P[i] on resting membrane potential recorded from cells of rabbit ear artery depolarised with phenylephrine (10µM, ▥ ). Also shown are initial control values ( □ ) and recovery values following a 30 minute washout period ( ▤ ). Vertical bars represent s.e.mean (*n*=15, 3 preparations).

c) GUINEA-PIG TAENIA COLI.

A similar protocol was adopted for assessment of any action of $P1P^i$ on resting membrane potential in the taenia coli preparation of the guinea-pig. Control impalements were carried out following an equilibration period, $P1P^i$ (1µM) included in the bathing solution and the tissue exposed to drug for 20 minutes before further impalements were made. A typical trace of the electrical activity recorded from the smooth muscle cells is shown in figure 69a, and it can be seen that $P1P^i$ (1µM) did not affect the 'spiking' of these cells, nor did it affect resting membrane potential (fig.69b).

a)




b)

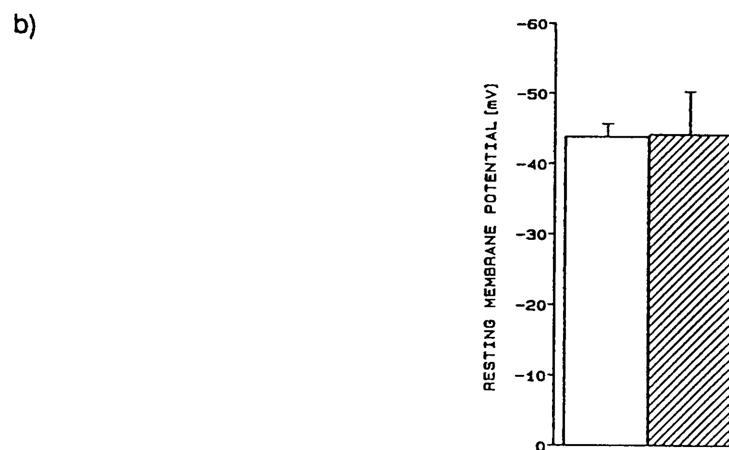



Figure 69. a) Electrical recordings from cells of the guinea-pig taenia coli in the presence of P1P[i] (1μM, *lower trace*) with control impalements (*upper trace*). b) Effects of P1P[i] (1μM, *hatched bar*) on resting membrane potential in guinea-pig taenia coli cells, compared with control pre-drug values (*empty bar*). Vertical bars represent s.e.mean (*n*=12, 3 preparations).

## 6) INFLUENCE OF THE ENDOTHELIUM.

### a) CULTURED ENDOTHELIAL CELLS.

Preliminary studies were carried out at the William Harvey Institute (London) investigating the effects of $P1P^i$ on cultured bovine aortic endothelial cells. The cells were packed into a column perfused with a physiological buffer, and the perfusate dripped onto a bioassay cascade comprising rabbit aorta and mesenteric artery, denuded of endothelium and precontracted with U46619 (30nM).

Figure 70 illustrates the response of the isolated blood vessels in such an experiment. Bradykinin (1ng) required injection through the column in order to produce a relaxation of the bioassay tissues, and this effect diminished down the cascade. In contrast, glyceryl trinitrate was able to exert its relaxant action directly on the smooth muscle without prior contact with the endothelial cells. $P1P^i$ (100nmoles) was without effect when applied directly over the tissues, however, produced a large, sustained relaxation of the vascular preparations when administered through the endothelial cell column. The action of $P1P^i$ diminished down the bioassay cascade, suggesting that this compound was exerting its action via release of some relaxant factor from the endothelial cells that possessed a short duration of action.

This experiment has been repeated, however, further experiments are obviously required involving smaller doses, different esters and palmitoyl carnitine itself, before firm conclusions can be drawn.

### b) EFFECT OF ENDOTHELIAL DAMAGE ON THE VASODILATOR ACTION OF $P1P^i$.

Experiments were performed using the mesenteric vascular bed of the rat to assess the endothelial dependency of the vascular relaxant action of $P1P^i$ (fig.71). Mesenteric vascular beds were precontracted with phenylephrine (10μM) and, following a plateau response to the drug, dose-responses were carried out either to

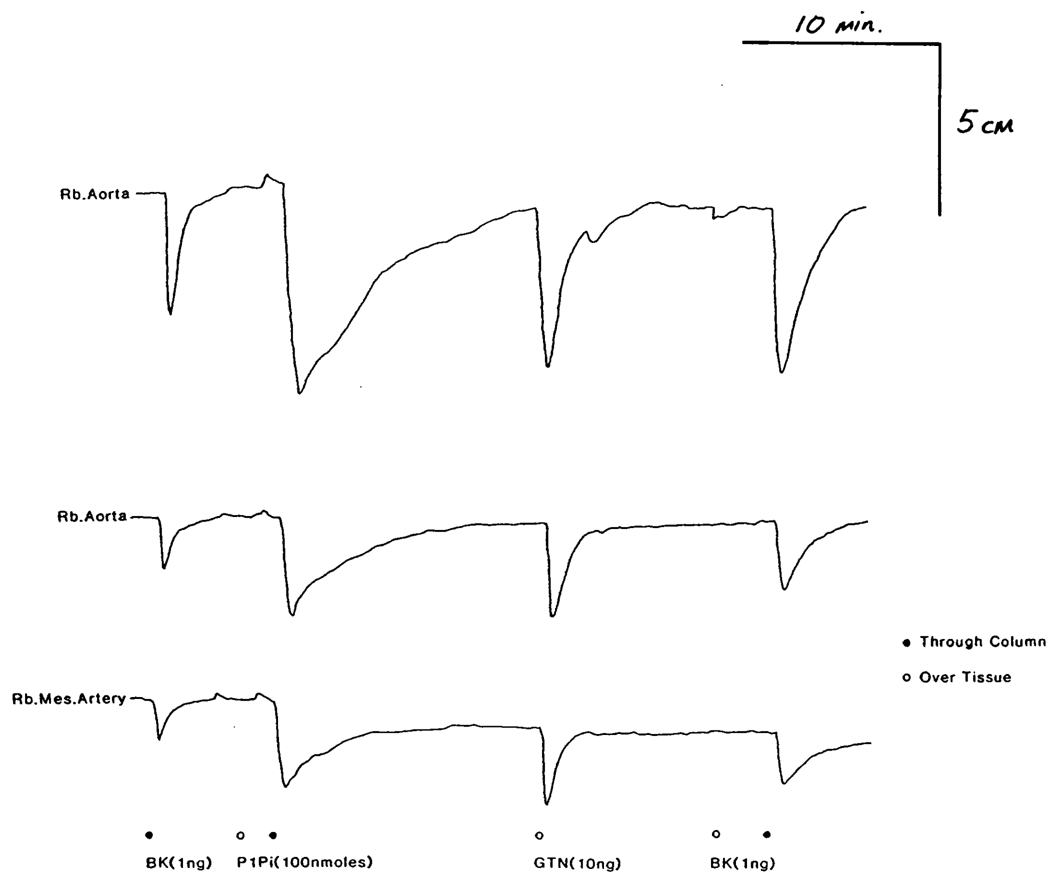



Figure 70. Typical trace showing the effects of P1P$^{i}$ on EDRF release from cultured endothelial cells. P1P$^{i}$ (100nmoles) was administered either directly over bioassay tissues (o), comprising rabbit aorta and mesenteric artery, or via a column containing bovine aortic endothelial cells (•). Also shown are the effects of bradykinin (BK)(1ng) and glyceryl trinitrate (GTN)(10ng).

acetylcholine (0.1-10pmoles) or to P1P$^i$ (1-30nmoles). The tissues were washed and 8mls of air injected through the bed following recovery. Phenylephrine was again administered to precontract the preparations and the dose-response to the vasodilators repeated in the air-damaged tissues.

Acetylcholine caused a dose-related transient vasodilation in phenylephrine precontracted preparations, however, this action was abolished in the air-damaged tissues (fig.71), indicating that the endothelial cell layer had been damaged by administration of air. P1P$^i$, however, elicited a slower, more sustained relaxation of the precontracted mesenteric bed, but this action was not attenuated following injection of air, in fact it was slightly potentiated (fig.72).

### 7) *IN VIVO* RESPONSES TO P1P$^i$.

Male Wistar rats were anaesthetised and cannulated for recording of blood pressure and heart rate. P1Pi was administered intravenously as bolus injections, via the right jugular vein, and a comparison made with the calcium antagonist verapamil (fig.73).

P1P$^i$ (0.3-1µmoles) induced small, transient falls in blood pressure with concomitant decreases in heart rate, whilst at the highest dose used (3µmoles) there was also an increase in heart rate following the initial drop. In contrast verapamil (50-500nmoles) caused a larger, more sustained hypotensive effect, but also exerted a negative chronotropic effect.

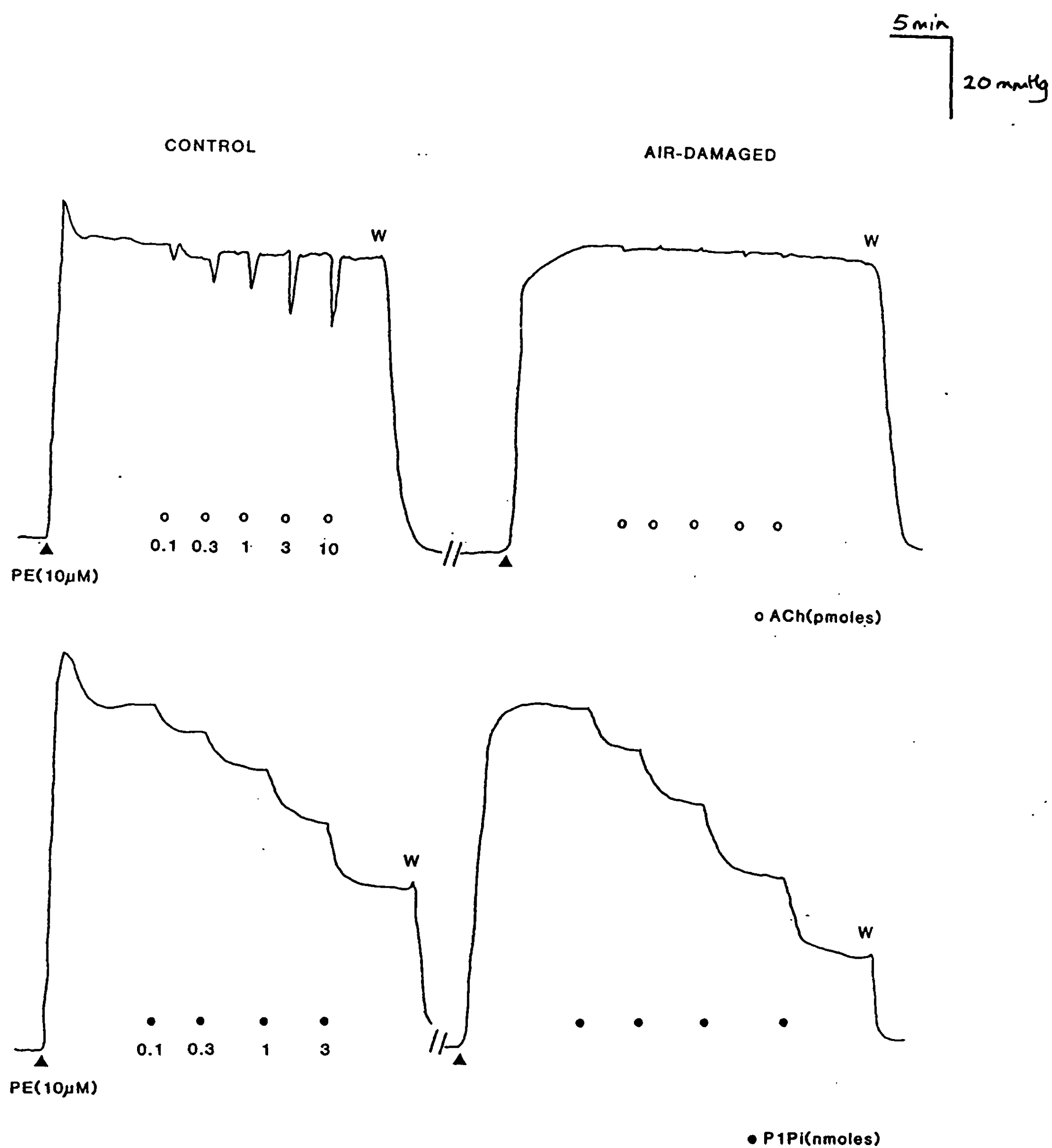



Figure 71. Typical traces showing the effects of damaging the endothelium of rat isolated mesenteric vascular beds by air injection on the vasodilator actions of acetylcholine (0.1-10pmoles, *upper trace*) and P1P$^i$ (0.1-3nmoles, *lower trace*) in phenylephrine-precontracted preparations.

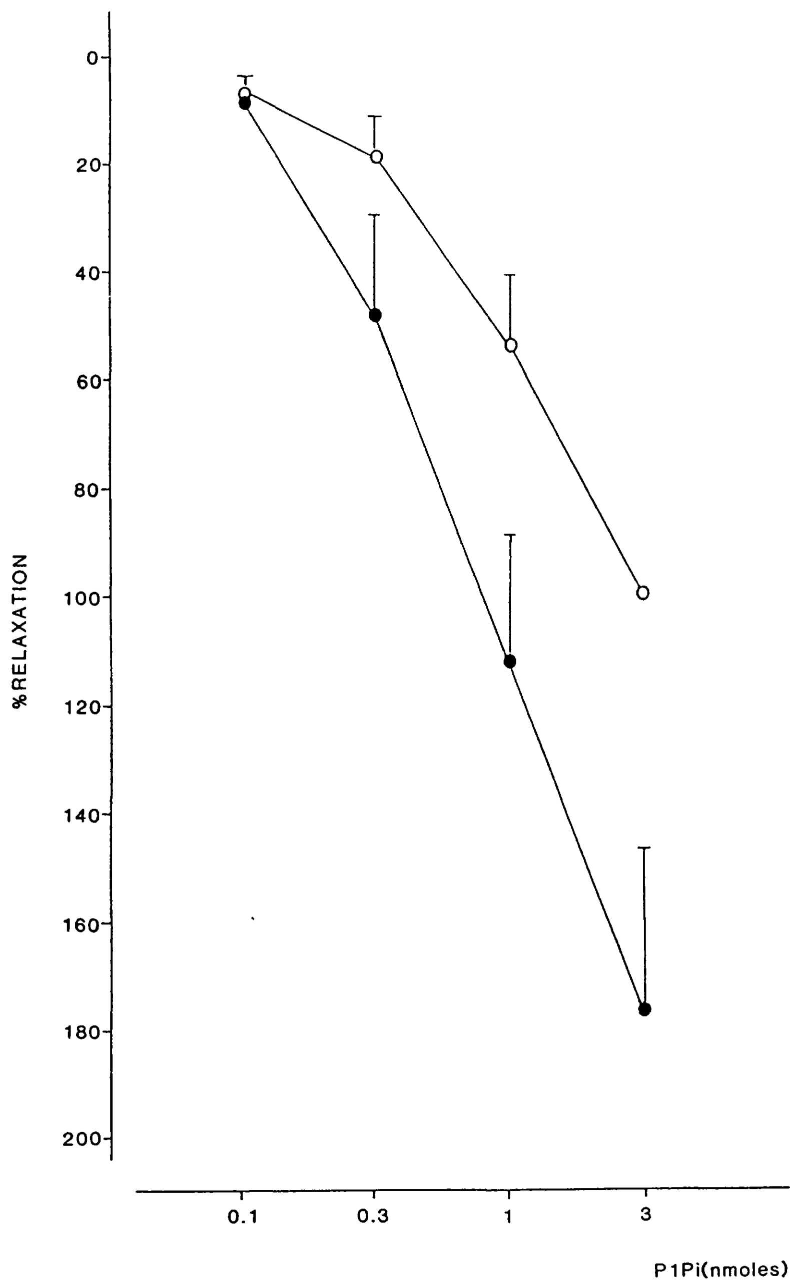



Figure 72. Effects of P1P$^{i}$ (0.1-3nmoles) on perfusion pressure in control (o) and air-damaged (●) isolated perfused rat mesenteric vascular beds precontracted with phenylephrine (10µM). Vertical bars represent s.e.mean ($n$=4).

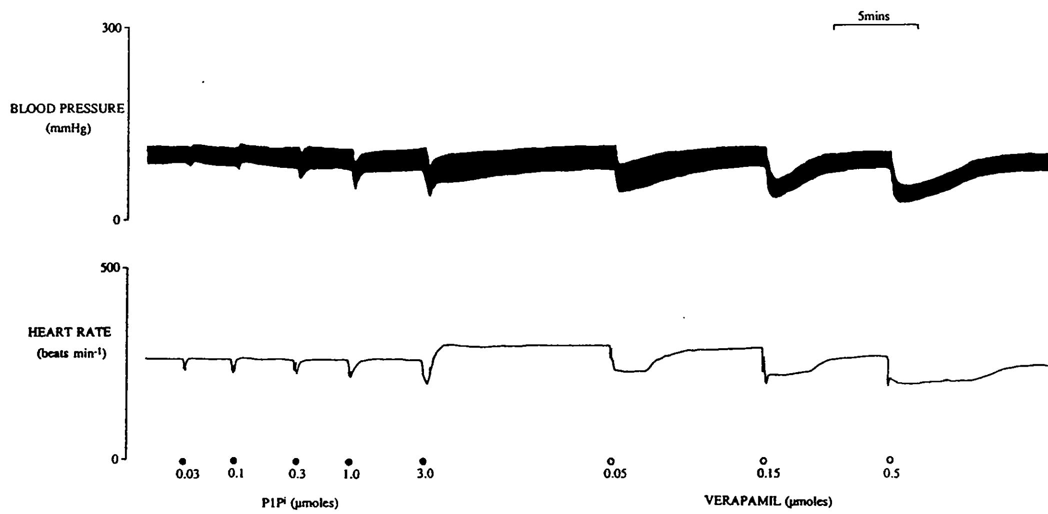



Figure 73. Typical trace showing the effects of P1P$^i$ (•, 0.3-1.0µmoles) and verapamil (o, 50-500nmoles) on blood pressure and heart rate in the anaesthetised rat ($n$=3).

# CHAPTER 6.

# DISCUSSION.

Esterification of the palmitoyl carnitine molecule produced compounds that exerted a profound, long-lasting vasodilator action in the perfused rat heart. Therefore, a small change in structure fundamentally alters the vascular activity of the molecule; P1E and related compounds exhibited essentially opposite effects on the coronary circulation to those of the parent drug. Why should such a small alteration produce a difference in activity, and how do the novel, synthetic esters of palmitoyl carnitine produce their effects?

Firstly, esterification of the parent acyl carnitine removes the negatively charged moiety of the compound i.e. the carbonyl group, leaving an overall positive charge on the molecule, conferred by the quaternary nitrogen group. In addition, the conformation of the molecule will be altered as a consequence. Under physiological conditions palmitoyl carnitine will be able to form an internal salt i.e. a Zwitterion formed by the interaction of the carbonyl and quaternary nitrogen groups, leaving the fatty acyl chain extended like a tail. However, the presence of an ester group in the molecule will prevent such an interaction and the conformation will be altered accordingly. Preliminary investigation of the structure-activity relationships of the molecule suggest that fatty acyl chain length is important with regard to the magnitude of vasodilation elicited in the coronary circulation. This may be indicative of a receptor mechanism underlying the effects of the esters, or may simply relate to the lipophilicity of the compound and hence the ability to intercalate into biological membranes. Alteration of the ester function of the molecule, however, did not produce such marked changes in activity, possibly indicating that the ability of the compounds to produce a vasodilator instead of a vasoconstrictor action resides in there not being a free carbonyl group present. The fact that there was a slight increase in the activity of the compound with increasing carbon length of ester group i.e. P1P$^i$ possessed the greatest coronary dilator action, may indicate some ste ric hindrance of the action of an esterase to breakdown the drug. Indeed, the duration of action of P1P$^i$ *in vivo* was transient. Further investigation into the structure-activity relationships of the molecule is obviously required to attempt to resolve the above problems.

The mode(s) of action of the novel esters of palmitoyl carnitine remains unclear at present in spite of the many routes of investigation carried out in the present study, including employment of specific antagonists of known vasodilators in isolated vascular preparations, electrophysiological studies and bioassay coupled to perfused endothelial cell cultures. However, certain modes of action appear unlikely from the data obtained and the possibilities narrowed for future investigation of $P1P^i$ and related compounds.

For example, the involvement of products of the cyclooxygenase pathway appears unlikely in the coronary relaxant action of $P1P^i$ as the vasodilator action of this compound in the heart was not affected by flurbiprofen. However, there was no concomitant, direct measurement of products released from the heart such as 6-keto-$PGF_1$ to conclusively prove this. Additionally, the specificity of the cyclooxygenase inhibitors used is open to question in view of their varying effects on perfusion pressure in the perfused heart. For example, indomethacin elicited a vasodilator response whereas flurbiprofen was without effect on coronary tone. The dual cyclooxygenase/lipoxygenase inhibitor BW755C also produced a fall in perfusion pressure, however, this effect may be due to an inhibition of leukotriene production, as these products of the lipoxygenase pathway are potent coronary vasoconstrictors *(Letts & Piper,1982; Roth & Lefer,1983).*

In a similar manner, certain agents that were employed to investigate a potential role of EDRF in the action of the carnitine esters also produced variable and apparently contradictory effects on coronary tone. For example, methylene blue, an inhibitor of the actions of EDRF *(Martin et al.,1985)*, caused a drop in perfusion pressure in three out of four hearts, whilst superoxide dismutase, which prevents EDRF breakdown *(Gryglewski et al.,1986)*, produced a slight elevation in basal pressure. The reason(s) for these apparent anomalies is not clear at present, however, there have been studies in which the specificity of action of these agents has been questioned. For example, SOD does not potentiate the vasodilator effect of acetylcholine in either the perfused kidney or mesenteric vascular bed of the rat

*(Bhardwaj & Moore,1988)* or its relaxant action in rabbit aorta *(Silin et al.,1985)* casting doubt on the integrity of this compound as a selective pharmacological tool for the study of EDRF in perfused organs. In the present study, SOD did not potentiate the vasodilator action of $P1P^i$ but actually attenuated it, however, for the reasons stated above firm conclusions cannot be drawn from these experiments about the involvement of EDRF in the action of $P1P^i$. Similarly, although the response to $P1P^i$ was significantly reduced in the presence of methylene blue, in three out of four hearts, the selectivity of this effect is questionable. For example methylene blue, in addition to its recognised ability to reversibly inhibit the effects of endothelium-dependent vasodilators *(Martin et al.,1985; Bhardwaj & Moore,1988)*, is also capable of blocking prostacyclin production via a mechanism independent of inhibition of soluble guanylate cyclase *(Martin et al.,1989)*. Therefore, the results obtained using these compounds in the present study to investigate a possible role for EDRF in the action of $P1P^i$ need to be treated cautiously.

Perhaps a better agent to employ in this context is the recently described inhibitor of EDRF synthesis, L-$N^G$-nitro arginine (L-NOARG), which attenuates endothelium-dependent vasodilatation in both rat mesenteric vascular bed and rabbit aorta without affecting responses to nitroprusside *(Moore et al.,1990)*, unlike methylene blue which inhibits the action of nitrovasodilators *(Martin et al.,1985)*. In accord with the study of Moore and co-workers (1990), who observed a constrictor action of L-NOARG, this compound produced a significant elevation of coronary tone, possibly via inhibition of basal EDRF production thereby indicating a role for EDRF in coronary vascular reactivity. L-NOARG did not, however, affect the vasodilator response to $P1P^i$, suggesting that this action is not mediated via EDRF production.

In contrast, supportive evidence for the involvement of EDRF in the action of the carnitine esters came from data obtained using the perfused endothelial cell column where $P1P^i$ caused release of some factor from the cultured cells that

produced relaxation of endothelial denuded bioassay tissues. However, the dose required to elicit a response was high i.e. 100nmoles, and the final concentration reaching the cells is unknown. Therefore, since the esters of palmitoyl carnitine are amphiphiles and therefore will influence membrane fluidity at sufficient concentration, it is possible that they will induce leakage of cellular contents, including EDRF, via a non-specific effect rather than an intrinsic action of the compounds. Further work is obviously required involving a greater dose range of synthetic ester and also control data using other amphiphilic compounds before firm conclusions can be drawn from these experiments. The bulk of evidence, however, from other experiments seems to suggest that $P1P^i$ and related compounds are not exerting their vasodilator action via the stimulation of EDRF production.

For example, in addition to the results obtained in the perfused heart discussed above, other experiments have shown that there was no appreciable difference between the action of $P1P^i$ in precontracted aortae possessing either an intact or mechanically damaged endothelium. Furthermore, $P1P^i$ was able to produce some relaxation of aortae precontracted with high potassium, under which conditions the relaxant effect of acetylcholine is inhibited *(Taylor et al.,1988)*. However, perhaps the most convincing data came from experiments carried out in the perfused mesenteric vascular bed where the response to the isopropyl ester of palmitoyl carnitine was not attenuated in air-damaged preparations, in contrast to the vasodilator responses to acetylcholine which were abolished in such tissues. However, histological evidence showing the condition of the endothelial cell layer would be beneficial before ruling out the influence of EDRF in the action of P1Pi.

$P1P^i$ does not appear to mediate its vasorelaxant action via the opening of potassium channels in a similar manner to cromakalim. Firstly, supramaximal concentrations of the ester did not affect resting membrane potential in cardiac, vascular or non-vascular smooth muscle. If the drug were acting via opening of potassium channels $P1P^i$ should have elicited a hyperpolarisation of the membrane; this clearly was not observed. However, this evidence alone is not sufficient to

disprove that P1P$^i$ has a site of action distinct from membrane potassium channels as the relaxant effect of the drug has not yet been demonstrated in the ear artery preparation and its lack of effect on membrane potential may merely reflect a selectivity of P1P$^i$. More convincing evidence, however, was obtained from experiments in which the coronary dilator action of P1P$^i$ was not attenuated by a concentration of glibenclamide that almost completely abolished responses to the potassium channel activator BRL38227. The possibility that P1P$^i$ and related esters can activate other potassium channels in the coronary circulation cannot be excluded at present, especially as PCE was shown to elicit release of radiolabelled rubidium from preloaded rat hearts, albeit at high doses. It has been demonstrated that both resting and stimulated rubidium efflux from rat aorta do not accurately represent potassium movement *(Smith et al.,1986)* and therefore higher doses of drug may be required to demonstrate such effects using rubidium as tracer. For example, cromakalim elicits relaxant effects on vascular smooth muscle at lower concentrations than are required to stimulate rubidium efflux *(Weir & Weston,1986)*. The results of experiments in the present study, however, using PCE to stimulate rubidium efflux are inconclusive, since the exact degree of esterification of palmitoyl carnitine in the 'chloroform-produced' PCE is unknown and thus residual effects of the parent drug may be present, and also the results were not reproducible using the synthetic compound P1P$^i$. Finally, the fact that P1P$^i$ itself can elicit vasodilations in mesenteric vascular beds precontracted with 110mM potassium would seem to suggest that it is acting via a mechanism other than activation of potassium channels.

Several other possibilities for the mechanism of action of P1P$^i$ seem unlikely from the series of experiments carried out in the heart using selective blocking agents. For example, the direct stimulation of either bradykinin $B_2$ or muscarinic receptors by P1P$^i$ does not appear to account for its coronary vasodilator action as responses to this drug were not affected by D-Arg$^0$[Hyp$^3$,Thi$^{5,8}$,D-Phe$^7$]-bradykinin or atropine, respectively. In addition, the potential involvement of phospholipase $A_2$ in the action of P1P$^i$ was difficult to assess in view of the detrimental effects of the

enzyme inhibitor mepacrine, which at micromolar concentrations produced irreversible damage of the heart. Finally, the effects of P1P$^i$ do not appear to be mediated via the release of endogenous adenosine in the heart, however, it is assumed in these experiments that adenosine deaminase has access to the smooth muscle i.e. the site at which adenosine is produced, and therefore would be able to inactivate any adenosine produced in response to P1P$^i$. A preferable future protocol would involve use of specific adenosine $A_2$ receptor blockers.

Palmitoyl carnitine has been shown to exert effects on calcium movement, such as release from intracellular stores *(Messineo et al.,1986)* and it has also been postulated to be an endogenous membrane channel opener *(Spedding & Mir,1987)*. The possibility arises, therefore, that the esters of palmitoyl carnitine may exert opposing effects on calcium movement within the cell. P1P$^i$ inhibited the vasoconstrictor responses to calcium in the mesenteric vascular bed of the rat depolarised by high potassium, indicating an ability of P1P$^i$ to either antagonise movement of calcium across the plasma membrane via VOCs or to directly affect some aspect of the pathway involved in maintainance of tonic contraction. This action of P1P$^i$ did not occur in the non-vascular smooth muscle investigated i.e. the taenia coli preparation, indicating selectivity in the action of the drug. The coronary dilator action of PCE was reduced by raising extracellular calcium, an effect consistent with the hypothesis that the esters of palmitoyl carnitine are producing their vasodilation via a calcium antagonistic effect , however, increased extracellular calcium would also be expected to reduce the effects of any vasodilator indirectly by increasing the free intracellular calcium available for contraction. A further possibility is that binding of P1P$^i$ is inhibited under such conditions since it has been shown that millimolar concentrations of calcium inhibit binding of certain calcium antagonists *(Garcia et al., 1986)*.

P1P$^i$ elicited full relaxation of mesenteric vascular beds precontracted with either phenylephrine, endothelin or potassium with similar potency, however, in the latter case the response to P1P$^i$ was biphasic, comprising an initial fast relaxation

followed by a slower, more prolonged component. This variation may occur as a consequence of the different electrical states of the smooth muscle membrane induced by the agonists used to precontract the tissue. For example, the membrane potential will have been depolarised by less than 7mV by endothelin *(Wallnofer et al.,1989)*, about 30mV by phenylephrine if comparable to noradrenaline *(Mulvany et al.,1982; Wallnofer et al.,1989)* and fully depolarised by 110mM potassium (calculated from the Nernst equation). Additionally, the mechanisms involved in maintaining contraction in mesenteric resistance vessels by these agents is different; high potassium-induced tone is sustained by extracellular calcium influx via VOCs *(Van Breemen & McNaughton,1970)*, whereas phenylephrine promotes an influx of calcium via predominantly potential-insensitive channels, and the action of endothelin remains unclear although certain evidence suggests that contraction is not exclusively sustained by calcium influx into the cell *(Sagiura et al.,1989)*. The fact that $P1P^i$ exhibited a similar potency in vasodilating mesenteric vascular beds precontracted with each of these agents may imply a site of action distal to the pathways whereby they raise intracellular calcium to a sufficient level for contraction; possibly a direct effect on contractile proteins involved in the maintainance of tonic contraction, e.g. interference with 'latch-state' *(Dillon et al.,1981)* or other cellular proteins interacting with cytoskeletal structures *(Rasmussen et al.,1987)*. Alternatively, the esters of palmitoyl carnitine may be exerting effects on calcium extrusion/sequestration mechanisms within the cell, however, at present it is only possible to speculate on the mechanism of action of $P1P^i$ and related compounds. Interestingly, the fact that there was a biphasic response to $P1P^i$ in high potassium-precontracted mesenteric vascular beds may suggest more than one mechanism underlying its effects, and therefore is an obvious area for further investigation. Finally, it is worth noting that $P1P^i$ was able to cause vasoconstriction in this preparation; it seems likely, however, that this is a non-specific amphiphilic effect since it occurred at supramaximal doses when complete relaxation of the precontracted tissue had been achieved.

The effects of $P1P^i$ in the rat aorta occured at much higher concentrations and were less profound than those in the heart; a concentration of 0.1µM $P1P^i$ caused almost maximal vasodilation in the perfused heart whilst producing no appreciable relaxation of precontracted aortae. It appears, therefore, that $P1P^i$ possesses a selectivity for fine resistance vessels. The degree of involvement of intracellular calcium release decreases and that of extracellular calcium increases when going from conduit to resistance vessels *(Cauvin et al.,1982)* in the maintainance of contraction, possibly indicating some interference of $P1P^i$ with calcium influx into the cell. Alternatively, differences may exist between the pathways or agents responsible for contraction within the vessels. So far, little investigation of the subcellular pathways and contractile apparatus has been carried out in resistance vessels, even though this type of blood vessel is more important in the physiological regulation of blood flow.

In conclusion, the experiments investigating the mode of action of $P1P^i$ and related compounds have produced much negative, and in certain cases inconclusive data i.e. where the selectivity of the agent employed is doubtful, but have narrowed the possibilities. The action of $P1P^i$ does not appear to be endothelium-dependent but an effect on the smooth muscle directly, possibly via more than one mechanism.

# CHAPTER 7.

# CONCLUSIONS.

The work presented here examin ing the cardiovascular effects of long chain acyl carnitines has shown that, far from being a class of compound exerting solely non-specific amphiphilic actions, these fatty acid derivatives are capable of exhibiting complex profiles of activity on vascular tissue. At present their mode(s) of action is unclear, however, the variation in response to palmitoyl carnitine between mesenteric vascular beds precontracted with either $\alpha$-adrenoceptor agonists or endothelin-1, causing a potentiated vasoconstriction or a vasodilation respectively, clearly warrants further investigation. The mechanism(s) involved are likely to be complicated since related compounds such as palmitoyl lysophosphatidylcholine are capable of producing biphasic responses in either situation.

Perhaps more interestingly, the current research has led to the discovery of a series of novel, vasodilator compounds whose action appears to be selective for resistance blood vessels, most notably those of the coronary circulation. Addition of the ester group to the acyl carnitine molecule changes its activity from that of a predominantly vasoconstrictor compound to a potent vasodilator, also losing the marked detrimental mechanical effects on the heart, within its effective concentration-range, associated with the parent drug. The underlying mechanism(s) of action of these vasodilator derivatives is currently unknown and, although the possibilities have been narrowed down by the present research, the scope for further investigation appears limitless.

Firstly, exploration of structure-activity relationships of the long chain acyl carnitine esters may yield important information about the chemical groups required for the molecule to produce vasodilator effects. Basic research in this area has already revealed that changing the ester function does not radically alter the capacity of the molecule to cause vasodilation, however, the question of whether the change in activity from palmitoyl carnitine to that of its ester derivative resides in their not being a negatively charged moiety i.e. carboxylic acid group, present should be addressed. In order to answer this the ester group could be replaced by another

function such as an amide or an alkyl group, thereby removing the negative charge, leaving the molecule with a net positive charge. Additionally, if these compounds still possess dilator activity it is possible that their *in vivo* action may be more prolonged since they will not be potential substrates for esterase enzymes in the body.

Alteration of the fatty acid chain length from palmitoyl to stearoyl causes a decrease in vasodilator activity. It would be of interest, therefore, to investigate a greater spectrum of fatty acyl chain lengths. For example, increasing saturated chain lengths from octanoyl ($C_8$) to icosanoyl ($C_{20}$), including some odd number carbon chain lengths and also some unsaturated fatty acids e.g. oleoyl. This will help to clarify whether a specific fatty acid chain is important, perhaps to interact selectively with a receptor site, or whether a lipophilic group is required to help the molecule insert into or pass across a lipid membrane.

Perhaps the most interesting aspect of the molecule to examine is that of the positively charged quaternary nitrogen group. It is possible that part or all of the vasodilator activity of the ester derivatives resides in a specific interaction of this charged group with a receptor site, such as a membrane protein or ion channel, an action that is prevented in palmitoyl carnitine by the formation of a Zwitterion. In order to investigate this the long chain acyl carnitine ester could be altered to the free base i.e. an uncharged molecule. Additionally, the methyl groups on the nitrogen atom could be replaced, partially or fully, by larger groups such as ethyl and isopropyl, or by smaller hydrogen atoms. Obviously, there are limitless alterations that can be made to the basic molecule that are not mentioned above, however, further investigation will hopefully lead to a greater understanding of the structural features required to produce vasodilation and possibly provide some clue to the site of action of these compounds.

Secondly, the acyl carnitine esters appear to be selective for resistance blood vessels such as the mesenteric and coronary vascular beds, whilst having little effect in conduit vessels such as the thoracic aorta. It would be interesting to further

examine the actions of these compounds on other vascular preparations e.g. tail artery, perfused kidney, perfused hind-limb, portal vein, and also some non-vascular smooth muscle. Additionally, the action of acyl carnitine esters in different species should be examined.

Finally, the ultimate goal of future research would be to elucidate the underlying mechanism(s) of action of long chain acyl carnitine esters. There is likely to be more than one mechanism involved, especially since in high potassium-precontracted mesenteric vascular beds, P1Pi elicited biphasic relaxations. This would therefore be one aspect of the acyl carnitine esters' vasodilator action to investigate further. In addition, another problem that should be resolved is that of the apparent ability of these compounds to produce a sizeable release of EDRF from cultured aortic endothelial cells yet not appear to mediate their vasodilator activity in isolated vascular preparations via such a release. As mentioned previously, further experimentation with cultured cells is required.

Other possible areas of investigation include the use of cultured cells or cellular fractions such as sarcoplasmic reticulum, to examine the effects of acyl carnitine esters on intracellular calcium movement, employing fluorescent probes or radiolabelled tracers, or work with enzymes such as protein kinase C, recently shown to be stimulated or inhibited by a number of lipid-containing compounds, the effect dependent on the charge of the molecule (*Epand & Lester, 1990*). Similarly, other potential targets for $P1P^i$ and related compounds are G-proteins, which are also modulated by cationic amphiphilic molecules, possibly via a receptor-independent mode of action (*Mousli et al.,1990*). Lastly, to further explore the relevance of the positive charge to the activity of the acyl carnitine esters, the effects of highly negatively-charged compounds such as heparin and dextran sulphate on the response to $P1P^i$ in the heart could be investigated, since they are able to abolish endothelium-dependent vascular relaxations to polycations (*Thomas et al.,1989b*).

In conclusion, acyl carnitine esters are a new and interesting series of vasodilator compounds whose mechanism of action is at present unknown. Their mode of action does not appear to conform to that of many currently investigated drugs. At present, however, the potential therapeutic value of these compounds for the treatment of cardiovascular disorders appears doubtful when considering many factors such as the lability of the ester group of the molecule, the problem of absorption of many charged quaternary amines and the possible metabolism of acyl carnitine esters to their parent acyl carnitine. It is more likely that any possible benefit of these compounds will derive from their use as pharmacological tools for investigation into the underlying mechanisms of regulation of vascular tone, potentially highlighting further differences between resistance and conduit vessels.

# REFERENCES.

ABE, M., YAMAZAKI, N., SUZUKI, Y., KOBAYASHI, A. & OHTA, H. (1984). Effect of palmitoyl carnitine on $Na^+$, $K^+$-ATPase and adenylate cyclase activity of canine myocardial sarcolemma. *J.Mol.Cell.Cardiol.*, **16**, 239-245.

ADAMS, R.J., COHEN, D.W., GUPTE, S., JOHNSON, J.D., WALLICK, E.T., WANG, T. & SCHWARTZ, A. (1979). *In vitro* effects of palmitylcarnitine on cardiac plasma membrane $Na^+$, $K^+$-ATPase, and sarcoplasmic reticulum $Ca^{2+}$ transport. *J.Biol.Chem.*, **264**, 12404-12410.

ALLELY, M.C. & BROWN, C.M. (1988). The effects of POCA and TDGA on the ischaemia-induced increase in $alpha_1$ adrenoceptor density in the rat left ventricle. *Br.J.Pharmacol.*, **95**, 705P.

AMEZCUA, J.L., PALMER, R.M.J., DE SOUZA, B.M. & MONCADA, S. (1989). Nitric oxide synthesized from L-arginine regulates vascular tone in the coronary circulation of the rabbit. *Br.J.Pharmacol.*, **97**, 1119-1124.

ARMSTRONG, A., DUNCAN, B., OLIVER, M.F., JULIAN, D.G., DONALD, K.W., FULTON, M., LUTZ, W. & MORRISON, S.L. (1972). Natural history of acute coronary heart attacks. A community study. *Br.Heart J.*, **34**, 67-80.

BERRIDGE, M.J. & IRVINE, R.F. (1984). Inositol triphosphate, a novel second messenger in cellular signal transduction. *Nature*, **312**, 315-321.

BEVAN, J.A., BEVAN, R.D., HWA, J.J., OWEN, M.D. & TAYO, P.M. (1986). Calcium regulation in vascular smooth muscle: is there a pattern to its variability within the arterial tree. *J.Cardiovasc.Pharmacol.*, **8**, S71-85.

BHARDWAJ, R. & MOORE, P.K. (1988). Endothelium-derived relaxing factor and the effects of acetylcholine and histamine on resistance blood vessels. *Br.J.Pharmacol.*, **95**, 835-843.

BIGAUD, M. & SPEDDING, M. (1986). Inhibition of the effects of endothelial-derived relaxant factor (EDRF) in aorta by palmitoyl carnitine. *Br.J.Pharmacol.*, **89**, 540P.

BOLTON, T.B. (1979). Mechanisms of action of transmitter and other substances on smooth muscle. *Physiol.Rev.*, **59**, 606-718.

BUSSELEN, P., SERCU, D. & VERDONCK, F. (1988). Exogenous palmitoyl carnitine and membrane damage in rat hearts. *J.Mol.Cell.Cardiol.*, **20**, 905-916.

CAUVIN, C., SAIDA, K. & VAN BREEMEN, C. (1982). Effects of Ca antagonists on Ca fluxes in resistance vessels. *J.Cardiovasc.Pharmacol.*, **4**, S287-S290.

CAUVIN, C., LOUTZENHISER, R., VAN BREEMEN, C. (1983). Mechanisms of calcium antagonist-induced vasodilation. *Ann.Rev.Pharmacol.Toxicol.*, **23**, 373-396.

CAUVIN, C., SAIDA, K. & VAN BREEMEN, C. (1984). Extracellular Ca dependence and diltiazem inhibition of contraction in rabbit conduit arteries and mesenteric resistance vessels. *Blood Vessels*, **21**, 23-31.

CHERRY, P.D., FURCHGOTT, R.F. & ZAWADZKI, J.V. (1981). The indirect nature of bradykinin relaxation of isolated arteries: endothelial dependent and independent components. *Fed.Proc.*, **40**, 689.

CHERRY, P.D., FURCHGOTT, R.F. & ZAWADZKI, J.V. (1983). The endothelium-dependent relaxation of vascular smooth muscle by unsaturated fatty acids. *Fed.Proc.*, **42**, 619.

CORR, P.B., SNYDER, D.W., CAIN, M.E., CRAFFORD Jr. W.A., GROSS, R.W. & SOBEL, B.E. (1981). Electrophysiological effects of amphiphiles on canine Purkinje fibres. Implications for dysrhythmia secondary to ischaemia. *Circ.Res.*, **49**, 354-363.

CORR, P.B., GROSS, R.W. & SOBEL, B.E. (1984). Amphipathic metabolites and membrane dysfunction in ischaemic myocardium. *Circ.Res.*, **55**, 136-154.

DAINTY, I.A., BIGAUD, M., McGRATH, J.C. & SPEDDING, M. (1990). Interactions of palmitoyl carnitine with the endothelium in rat aorta. *Br.J.Pharmacol.*, **100**, 241-246.

DANTHULURI, N.R. & DETH, R.C. (1984). Phorbol ester-induced contraction of arterial smooth muscle and inhibition of $\alpha$-adrenergic response. *Biochem.Biophys.Res.Comm.*, **125**, 1103-1109.

DE MEY, J., CLAEYS, M. & VANHOUTTE, P.M. (1982). Endothelium dependent inhibitory effects of acetylcholine, adenosine triphosphate, thrombin and arachidonic acid in the canine femoral artery. *J.Pharmacol.Exp.Ther.*, **222**, 166-173.

DETH, R. & VAN BREEMEN, C. (1977). Agonist induced $^{45}Ca^{2+}$ release from smooth muscle cells of the rabbit aorta. *J.Memb.Biol.*, **30**, 363-380.

DILLON, P.F., ASKOY, M.O., DRISKA, S.P. & MURPHY, R.A. (1981). Myosin phosphorylation and cross-bridge cycle in arterial smooth muscle. *Science*, **211**, 495-497.

DOWNAR, E., JANSE, M.J. & DURRER, D. (1977). The effect of acute coronary artery occlusion on subepicardial transmembrane potentials in the intact porcine heart. *Circulation*, **56**, 217-224.

DUNCAN, G.P., PATMORE, L. & SPEDDING, M. (1986). Positive inotropic effects of palmitoyl carnitine on embryonic chick heart cell aggregates. *Br.J.Pharmacol.*, **89**, 757P.

EGGERMONT, J.A., VROLIX, M., RAEMAEKERS, L., WUYTACK, F. & CASTEELS, R. (1988). $Ca^{2+}$-transport ATPase of vascular smooth muscle. *Circ.Res.*, **62**, 266-278.

EISTETTER, K. & WOLF, H.P.O. (1986). Etomoxir. *Drugs of the Future.*, **11**, 1034-1036.

EIKENBURG, D.C. (1984). Functional characterisation of the pre- and post-junctional $\alpha_1$-adrenoceptors in the *in situ* perfused rat mesenteric vascular bed. *Eur.J.Pharmacol.*, **105**, 161-165.

ENDO, T, NAKA, M. & HIDAKA, H. (1982). $Ca^{2+}$-phospholipid dependent phosphorylation of smooth muscle myosin. *Biochem.Biophys.Res.Comm.*, **105**, 942-948.

EPAND, R.M. & LESTER, D.S. (1990). The role of membrane biophysical properties in the regulation of protein kinase C activity. *Trends Pharmacol.Sci.*, **11**, 317-320.

FASEHUN, O.A., JENNETT, S.M., McGRATH, J.C. & SPEDDING, M. (1987). Effects of $Ca^{2+}$ modulators on the hypoxia-resistant response of the isolated rat portal vein to potassium chloride. *Br.J.Pharmacol.*, **92**, 533P.

FELETOU, M. & VANHOUTTE, P.M. (1988). Endothelium-dependent hyperpolarisation of canine coronary smooth muscle. *Br.J.Pharmacol.*, **93**, 515-524.

FEUVRAY, D., IDELL-WENGER, J.A. & NEELY, J.R. (1979). Effects of ischaemia on rat myocardial function and metabolism in diabetes. *Circ.Res.*, **44**, 322-329.

FOLTS, J.D., SHUG, A.L., KOKE, J.R. & BITTAR, N. (1978). Protection of the ischaemic dog myocardium with carnitine. *Am.J.Cardiol.*, **41**, 1209-1214.

FORSTERMANN, U., GOPPELT-STRUBE, M., FROLICH, C. & BUSSE, R. (1986). Inhibitors of acyl coenzyme A: lysolecithin acyltransferase activates the production of endothelium derived relaxing factor. *J.Pharmacol.Exp.Ther.*, **238**, 352-359.

FROLICH, J., SECCOMBE, D.W. HAHN, P., DODEK, P. & HYNIE, I. (1978). Effect of fasting on free and esterified carnitine levels in human serum and urine: correlation with serum levels of free fatty acids and $\beta$-hydroxybutyrate. *Metabolism*, **27**, 555-561.

FURCHGOTT, R.F. & ZAWADZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, **288**, 373-376.

FURCHGOTT, R.F. (1984). Role of endothelium in responses of vascular smooth muscle to drugs. *Ann.Rev.Pharmacol.Toxicol.*, **24**, 175-197.

FURCHGOTT, R.F., MARTIN, W., CHERRY, P.D., JOTHIANANDAN, D. & VILLANI, G.M. (1985). Endothelium-dependent relaxation, photorelaxation and cyclic GMP. In *Vascular Neuroeffector Mechanisms*, Ed. Bevan, J.A. *et al.*, pp. 105-114. Elsevier, Amsterdam.

FURCHGOTT, R.F., KAHN, M.T., JOTHIANANDAN, D. & KAHN, A.S. (1988). Evidence that endothelium-derived factor of rabbit aorta is nitric oxide. In *Vascular Neuroeffector Mechanisms*, Ed. Bevan, J.A. *et al.*, pp. 77-84. IRL Press, Oxford.

FURCHGOTT, R.F. (1990). Studies on endothelium-dependent vasodilation and the endothelium-derived relaxing factor. *Acta.Physiol.Scand.*, **139**, 257-270.

GARCIA, M.L., KING, V.J., SIEGL, P.K.S., REUBEN, J.P. & KACZOROWOSKI, G.J. (1986). Binding of $Ca^{2+}$ entry blockers to cardiac sarcolemmal vesicles. *J.Biol.Chem.*, **261**, 8146-8151.

GODFRAIND, T., MILLER, R. & WIBO, M. (1986). Calcium antagonism and calcium entry blockade. *Pharmacol.Rev.*, **38**, 321-416

GRIFFITH, T.M., EDWARDS, T.H., LEWIS, M.J., NEWBY, A.L. & HENDERSON, A.H. (1984). The nature of endothelium derived vascular relaxant factor. *Nature*, **308**, 645-647.

GROSMAN, N. (1988). Synergistic effect of palmitoylcarnitine and the ionophore A23187 on isolated rat mast cells. *Agents and Actions*, **25**, 277-283.

GRYGLEWSKI, R.J., PALMER, R.M.J. & MONCADA, S. (1986). Superoxide anion is involved in the breakdown of endothelium derived relaxing factor. *Nature*, **320**, 454-456.

HAYASHI, H., SUZUKI, Y., KAMIKAWA, T., KOBAYASHI, A. & YAMAZAKI, N. (1981). Effects of long chain acylcarnitine and L-carnitine on action potential of canine papillary muscle. *J.Mol.Cell.Cardiol.*, **13**(S2), 79.

HEATHERS, G.P., YAMADA, K.A., KANTNER, E.M. & CORR, P.B. (1987). Long-chain acyl carnitines mediated the hypoxia induced increase in alpha$_1$-adrenergic receptors on adult canine myoctes. *Circ.Res.*, **61**, 735-746.

HEKIMIAN, G. & FEUVRAY, D. (1985). Reduction of ischaemia-induced accumulation of acylcarnitine by TDGA and its influence on lactic dehydrogenase release in diabetic hearts. *Diabetes*, **35**, 906-910.

HELENIUS,A. & SIMONS, K. (1975). Solubilization of membranes by detergents. *Biochem.Biophys.Acta*, **415**, 29-79.

HIATT, W.R., NAWAZ, D. & BRASS, E.P. (1987). Carnitine metabolism during exercise in patients with peripheral vascular disease. *J.Appl.Physiol.*, **62**, 2383-2387.

HULSMANN, W.C. (1976). Coronary vasodilatation by fatty acids. *Basic Res.Cardiol.*, **71**, 179-191.

HULSMANN, W.C., DUBELAAR, M.L., LAMERS, J.M.J. & MACCARI, F. (1985). Protection by acyl-carnitines and phenylmethylsulfonyl fluoride of rat heart subjected to ischaemia and reperfusion. *Biochem.Biophys.Acta.*, **847**, 62-66.

IDELL-WENGER, J.A., GROTYOHANN, L.W. & NEELY, J.R. (1978). Coenzyme A and carnitine distribution in normal and ischaemic hearts. *J.Biol.Chem.*, **253**, 4310-4318.

IGNARRO, L.J., BUGA, G.M., WOOD, K.S., BYRNS, R.E. & CHAUDHURI, G. (1987). Endothelium-derived relaxing factor (EDRF) produced and released from artery and vein is nitric oxide. *Proc.Natl.Acad.Sci.*, **84**, 9265-9269.

IKEBE, M., INAGAKI, M., KANAMARU. K. & HIDAKA, H. (1985). Phosphorylation of smooth muscle light chain kinase by $Ca^{2+}$-activated, phospholipid-dependent protein kinase. *J.Biol.Chem.*, **260**, 4547-4550.

INOUE, D. & PAPPANO, A.J. (1983). L-Palmitoylcarnitine and calcium ions act similarly on excitatory ionic currents in avian ventricular muscle. *Circ.Res.*, **52**, 625-634.

ISOMAA, B., ENGBLOM, A.C. & AKERMAN, K.E.O. (1989). Interaction of aliphatic amphiphiles with calmodulin and cyclic nucleotide phosphodiesterase. *Biochem.Biophys.Acta.*, **998**, 131-136.

KAMM, K.E. & STULL, J.T. (1989). Regulation of smooth muscle contractile elements by second messengers. *Ann.Rev.Physiol.,* **51**, 299-313.

KANAIDE, H., SHOGAKIUCHI, Y. & KAKAMURA. (1987). The norepinephrine-sensitive $Ca^{2+}$-storage site differs from the caffeine-sensitive site in vascular smooth muscle of the rat aorta. *FEBS Lett.*, **214**(1), 130-134.

KATOH, N., WRENN, R.W., WISE, B.C., SHOJI, M. & KUO, J.F. (1981). Substrate proteins for calmodulin sensitive $Ca^{2+}$-dependent protein kinases in heart, and inhibition of their phosphorylation by palmitoylcarnitine. *Proc.Natl.Acad.Sci.*, **78**, 4813-4817.

KATZ, A.M. & MESSINEO, F.C. (1981) Lipid-membrane interactions and the pathogenesis of ischaemic damage in the myocardium. *Circ.Res.*, **48**, 1-16.

KNABB, M.T., SAFFITZ, J.E., CORR, P.B. & SOBEL, B.E. (1986). The dependence of electrophysiological derangements on accumulation of endogenous long-chain acyl carnitine in hypoxic rat neonatal myocytes. *Circ.Res.*, **58**, 230-240.

KOMORI, K. & SUZUKI, H. (1987). Electrical responses of smooth muscle cells during cholinergic vasodilatation in the rabbit saphenous artery. *Circ.Res.*, **61**, 586-593.

KONDO, K., SUZUKI, H., OKUNO, T., SUDA, M. & SARUTA, T. (1980). Effects of nifedipine, diltiazem and verapamil on the vasoconstrictor responses to norepinephrine and potassium ions in the rat mesenteric artery. *Arch.Int.Pharmacodyn.Ther.*, **245**, 211-217.

LAMERS, J.M.J., STINIS, J.T., MONTFOORT, A. & HULSMANN, W.C. (1984). Modulation of membrane function by lipid intermediates: a possible role in myocardial ischaemia. In *Myocardial Ischaemia and Lipid Metabolism.* Ed. R. Ferrari *et al.*, pp. 107-125. Plenum, New York.

LEIJTEN, P.A.A. & VAN BREEMEN, C. (1984). The effects of caffeine on the noradrenaline-sensitive calcium store in rabbit aorta. *J.Physiol.*, **357**, 327-339.

LEIJTEN, P.A.A. & VAN BREEMEN, C. (1986). The relationship between noradrenaline-induced contraction and $^{45}Ca$ efflux stimulation in rabbit mesenteric artery. *Br.J.Pharmacol.*, **89**, 739-47.

LETTS, L.G. & PIPER, P.J. (1982). The actions of leukotrienes C4 and D4 on guinea-pig isolated hearts. *Br.J.Pharmacol.*, **76**, 169-176.

LEVITSKY, D.O. & SKULACHEV, V.P. (1972). Carnitine: the carrier transporting fatty acyls into mitochondria by means of an electrochemical gradient of $H^+$. *Biochem.Biophys.Acta.*, **275**, 33-50.

LIEDTKE, A.J., NELLIS, S. & NEELY, J.R. (1978). Effects of excess free fatty acids on mechanical and metabolic function in normal and ischaemic myocardium in swine. *Circ.Res.*, **43**, 652-661.

LIEDTKE, A.J., NELLIS, S.H. & COPENHAVER, G. (1979). Effects of carnitine in ischaemic and fatty acid supplemental swine hearts. *J.Clin.Invest.*, **64**, 440-447.

LOIRAND, G., PACAUD, P., MIRONNEAU, C. & MIRONNEAU, J. (1986). Evidence for two distinct calcium channels in rat vascular smooth muscle cells in short-term primary culture. *Pflugers Arch.*, **407**, 566-568.

LOPASCHUK, G.D., WALL, S.R., OLLEY, P.M. & DAVIES, N.J. (1988). Etomoxir, a carnitine palmitoyltransferase 1 inhibitor, protects hearts from fatty acid-induced ischaemic injury independent of changes in long chain acylcarnitine. *Circ.Res.*, **63**, 1036-1043.

MARTIN, W., VILLANI, G.M., JOTHIANANDAN, D. & FURCHGOTT, R.F. (1985). Selective blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation by haemoglobin and by methylene blue in the rabbit aorta. *J.Pharmacol.Exp.Ther.*, **232**(3), 708-716.

MARTIN, W., SMITH, J.A. & WHITE, D.G. (1986). The mechanisms by which haemoglobin inhibits the relaxation of rabbit aorta induced by nitrovasodilators, nitric oxide or bovine retractor penis inhibitory factor. *Br.J.Pharmacol.*, **89**, 563-571.

MARTIN, W., DRAZAN, K.M. & NEWBY, A.C. (1989). Methylene blue but not changes in cyclic GMP inhibits resting and bradykinin-stimulated production of prostacyclin by pig aortic endothelial cells. *Br.J.Pharmacol.*, **97**, 51-56.

MATSUI, K., NAKAZAWA, M., TAKEDA, K. & IMAI, S. (1985). Effects of *l*-carnitine chloride and its acetyl derivative on the electrophysiological derangement induced by palmitoyl-*l*-carnitine in isolated canine ventricular muscle. *Jpn.J.Pharmacol.*, **39**, 263-270.

MESSINEO, F.C., PINTO, P.B. & KATZ, A.M. (1982). Effects of palmitic acid and palmityl carnitine on calcium sequestration by rabbit skeletal sarcoplasmic reticulum vesicles. *Adv.Myocardiol.*, **3**, 407-415.

MOLAPARAST-SALESS, F., LIEDTKE, A.J. & NELLIS, S.H. (1987). Effects of the fatty acid blocking agents, oxfenicine and 4-bromocrotonic acid, on performance in aerobic and ischaemic myocardium. *J.Mol.Cell.Cardiol.*, **19**, 509-520.

MOORE, P.K., AL-SWAYEH, O.A., CHONG, N.W.S., EVANS, R.A. & GIBSON, A. (1990). L-$N^G$-nitro arginine (L-NOARG), a novel, L-arginine-reversible inhibitor of endothelium-dependent vasodilatation *in vitro*. *Br.J.Pharmacol.*, **99**, 408-412.

MULVANY, M.J., NILSSON, H. & FLATMAN, J.A. (1982). Role of membrane potential in the response of rat small mesenteric arteries to exogenous noradrenaline stimulation. *J.Physiol.*, **332**, 363-373.

MOUSLI, M., BUEB, J., BRONNER, C., ROUOT, B. & LANDRY, Y. (1990). G protein activation: a receptor-independent mode of action for cationic amphiphilic neuropeptides and venom peptides. *Trends Pharmacol.Sci.*, **11**, 358-362.

NAKAYA, H. & TOHSE, N. (1986). Electrophysiological effects of acetylglycerylethеrphosphorylcholine on cardiac tissues: comparison with lysophosphatidylcholine and long chain acyl carnitine. *Br.J.Pharmacol.* **89**, 749-757.

NICHOLS, A.J. & HILEY, C.R. (1985). Identification of adrenoceptors and dopamine receptors mediating vascular responses in the superior mesenteric arterial bed of the rat. *J.Pharm.Pharmacol.*, **37**, 110-115.

NISHIZUKA, Y. (1984). The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature*, **308**, 693-698.

OWEN, M.P. & BEVAN, J.A. (1985). Acetylcholine induced endothelial-dependent vasodilation increases as artery diameter decreases in rabbit ear. *Experentia*, **41**, 1057-1058.

OWENS, K., KENNETT, F.F. & WEGLICKI, W.B. (1982). Effects of fatty acid intermediates on $Na^+$-$K^+$-ATPase activity of cardiac sarcolemma. *Am.J.Physiol.*, **242**, H456-461.

PALMER, R.M.J., FERRIGE, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327**, 524-525.

PALMER, R.M.J., ASHTON, D.S. & MONCADA, S. (1988a). Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature*, **333**, 664-666.

PALMER, R.M.J., REES, D.D., ASHTON, D.S. & MONCADA, S. (1988b). L-arginine is the physiological precursor for the formation of nitric oxide in endothelium-dependent relaxation. *Biochem.Biophys.Res.Comm.*, **153**, 1251-1256.

PANDE, S.V. (1975). A mitochondrial carnitine acylcarnitine translocase system: carnitine acylcarnitine transport/exchange diffusion/acyl(+)carnitine inhibition/fatty acyl transport. *Proc.Natl.Acad.Sci.*, **72**, 883-887.

PAULSON, D.J., SCHMIDT, M.J., ROMENS, J. & SHUG, A.L. (1984). Metabolic and physiological differences between zero-flow and low-flow myocardial ischaemia: effects of L-acetylcarnitine. *Basic Res.Cardiol.*, **79**, 551-561.

PAULSON, D.J., NOONAN, J.J., WARD, K.M., STANLEY. H., SHERRATT, A. & SHUG, A.L. (1986). Effects of POCA on metabolism and function in the ischaemic rat heart. *Basic Res.Cardiol.*, **81**, 180-187.

PATMORE, L., DUNCAN, G.P. & SPEDDING, M. (1989). Interaction of palmitoyl carnitine with calcium antagonists in myocytes. *Br.J.Pharmacol.*, **97**, 443-450.

PEARSON, J.D. & GORDON, J.L. (1985). Nucleotide metabolism by the endothelium. *Ann.Rev.Physiol.*, **47**, 617-627.

PHILIPSON, K.D., LANGER, G.A. & RICH, T.L. (1985). Charged amphiphiles regulate heart contractility and sarcolemma-$Ca^{2+}$ interactions. *Am.J.Physiol.*, **248**, H147-150.

PIPER, M.H., SEZER, O., SCHWARTZ, P., HUTTER, J.F., SCHWEICKHARDT, C. & SPIECKERMANN, P.G. (1984). Acyl-carnitine effects on isolated cardiac mitochondria and erythrocytes. *Basic Res.Cardiol.*, **79**, 186-198.

PITTS, B.J.R., TATE, C.A., VAN WINKLE, B., WOOD, J.M. & ENTMAN, M.L. (1978). Palmitylcarnitine inhibition of the calcium pump in cardiac sarcoplasmic reticulum: a possible role in myocardial ischaemia. *Life Sci.*, **23**, 391-402.

QUAST, U. (1988). Inhibition of the effects of the $K^+$ channel stimulator cromakalim (BRL34915) in vascular smooth muscle by glibenclamide and forskolin. *Naunyn-Schmiedeberg's Arch.Pharmacol.*, **337** (suppl.), R.72, Abstr. 288.

RAPOPORT, R.M. DRAZNIN, M.B. & MURAD, F. (1983). Endothelium-dependent relaxation in rat aorta may be mediated through cyclic GMP-dependent protein phosphorylation. *Nature*, **306**, 174-176.

RASMUSSEN, M., FORDER, J., KOJIMA, I. & SCRIABINE, A. (1984). TPA-induced contraction of isolated rabbit vascular smooth muscle. *Biohem.Biophys.Res.Comm.*, **122**, 776-784.

RASMUSSEN, H., TAKUWA, Y. & PARK, S. (1987). Protein kinase C in the regulation of smooth muscle contraction. *FASEB J.*, **1**, 177-185.

REGITZ, V., PAULSON, D.J., NOONAN, J., FLECK, E. & SHUG, A.L. (1987). Protection of the ischaemic myocardium by propionylcarnitine taurine amide. Comparison with other carnitine derivatives. *Z.Kardiol.*, **76**(S5), 53-58.

REGOLI, D., RHALEB, N., DION, S. & DRAPEAU, G. (1990). New selective bradykinin receptor antagonists and bradykinin $B_2$ receptor characterisation. *Trends Pharmacol.Sci.*, **11**, 156-161.

ROTH, D.M. & LEFER, A.M. (1983). Studies on the mechanism of leukotriene induced coronary artery constriction. *Prostaglandins*, **26**(4), 573-581.

RUBANYI, G.M. & VANHOUTTE, P.M. (1986). Superoxide anions and hyperoxia inactivate endothelium derived relaxing factor. *Am.J.Physiol.*, **250**, H222-227.

RYAN, U.S., RYAN, J.W. & WHITAKER, C. (1976). Localization of angiotensin converting enzyme (kininase II) II. Immunocytochemistry and immunofluorescence. *Tissue Cell*, **8**, 125-145.

SAGIURA, M., INAGAMI, T., HARE, G.M.T. & JOHNS, J.A.J. (1989). Endothelin action: inhibition by a protein kinase c inhibitor and involvement of phosphoinositols. *Biochem.Biophys.Res.Comm.*, **158**(1), 170-176.

SAIDA, K. & VAN BREEMEN, C. (1984). Characteristics of norepinephrine-sensitive $Ca^{2+}$ store in vascular smooth muscle. *Blood Vessels*, **21**, 43-52.

SANDERMANN Jr., H. (1978). Regulation of membrane enzymes by lipids. *Biochem.Biophys.Acta*, **515**, 209-237.

SHUG, A.L. & SUBRAMANIAN, R. (1987). Modulation of adenine nucleotide translocase activity during myocarial ischaemia. *Z.Kardiol.*, **76**(S5), 26-33.

SHIKANO, K., LONG, C.J., OHLSTEIN, E.H. & BERKOWITZ, B.A. (1988). The comparative pharmacology of endothelium-derived relaxing factor and nitric oxide. *J.Pharmacol.Exp.Ther.*, **247**, 873-881.

SIBLEY, D.R., PETERS, J.R., NAMBI, P., CARON, M.G. & LEFKOWITZ, R.J. (1984). Desensitization of turkey erythrocyte adenylate cyclase. *J.Biol.Chem.*, **259**, 9742-9749.

SILIN, P.J., STRULOWITZ, J.A., WOLIN, M.S. & BELLONI, F.L. (1985). Absence of a role for superoxide anion, hydrogen peroxide and hydroxyl radical in endothelium mediated relaxation of rabbit aorta. *Blood Vessels*, **22**, 65-73.

SMITH, J.M., SANCHEZ, A.A. & JONES, A.W. (1986). Comparison of rubidium-86 and potassium-42 fluxes in rat aorta. *Blood Vessels*, **23**, 297-309.

SPEDDING, M. & MIR, A. (1987). Direct activation of $Ca^{2+}$ channels by palmitoyl carnitine, a putative endogenous ligand. *Br.J.Pharmacol.*, **92**, 457-468.

SPEDDING, M., ANDERSON, A.J. & PATMORE, L. (1989). Definition of the interaction of acyl carnitines, Bay K 8644 and diphenylalkylamine calcium-antagonists. *Proceedings of the 4th Int.Symposium on Calcium-antagonists*, Florence: Giovanni Lorenzini, p. 207.

SPEDDING, M. (1990). Acyl carnitines and myocardial ischaemia. *J.Mol.Cell.Cardiol.*, **22**(S3), L52.

SUBRAMIAN, R., PLEHN, S., NOONAN, J., SCHMIDT, M. & SHUG, A.L. (1987). Free radical-mediated damage during myocardial ischaemia and reperfusion and protection by carnitine esters. *Z.Kardiol.*, **76**(S5), 41-45.

TAYLOR, S.G., SOUTHERTON, J.S., WESTON, A.H. & BAKER, J.R.J. (1988). Endothelium-dependent effects of acetylcholine in rat aorta: a comparison with sodium nitroprusside and cromakalim. *Br.J.Pharmacol.*, **94**, 853-863.

THOMAS, G. & RAMWELL, P.W. (1988). Peptidyl arginine deiminase and endothelium dependent relaxation. *Eur.J.Pharmacol.*, **153**, 147-148.

THOMAS, G., COLE, E.A. & RAMWELL, P.W. (1989a). $N^G$-Monomethyl L-arginine is a non-specific inhibitor of vascular relaxation. *Eur.J.Pharmacol.*, **170**, 123.

THOMAS, G., HECKER, M. & RAMWELL, P.W. (1989b). Vascular activity of polycations and basic amino acids: L-arginine does not specifically elicit endothelium-dependent relaxation. *Biochem.Biophys.Res.Comm.*, **158** (1), 177-180.

THOMSEN, J.H., SHUG, A.L., YAP, V.U., PATEL, A.K., KARRAS, T.J. & DeFELICE, A.L. (1979). Improved pacing tolerance of the ischaemic human myocardium after administration of carnitine. *Am.J.Cardiol.*, **43**, 300-306.

UGWU, A.C., McGRATH, J.C. & SPEDDING, M. (1987). Comparison of the effects of palmitoyl carnitine and Bay K 8644 on the calcium-sensitivity of the rat tail artery. *Br.J.Pharmacol.*, **92**, 552P.

VAN BREEMEN, C. (1969). Blockade of membrane calcium fluxes by lanthanum in relation to vascular smooth muscle contractility. *Int.Arch.Physiol.Biochem.*, **77**, 710-717.

VAN BREEMEN, C. & McNAUGHTON, E. (1970). The separation of cell membrane calcium transport from extracellular calcium exchange in vascular smooth muscle. *Biochem.Biophys.Res.Comm.*, **39**, 567-574.

VAN BREEMEN, C. AARONSON, P. & LOUTZENHISER, R. (1979). $Na^+$, $Ca^{2+}$ interactions in mammalian smooth muscle. *Pharmacol.Rev.*, **30**, 167-208.

VAN BREEMEN, C. & SIEGEL, B. (1980). The mechanism of $\alpha$-adrenergic activation of the dog coronary artery. *Circ.Res.*, **46**, 426-429.

VAN BREEMEN, C. & SAIDA, K. (1989). Cellular mechanisms regulating $[Ca^{2+}]_i$ smooth muscle. *Ann.Rev.Physiol.*, **51**, 315-329.

VON LOH, D. & BOHR, D.F. (1973). Membrane potentials of smooth muscle cells of isolated resistance vessels. *Proc.Soc.Exp.Biol.Med.*, **144**, 513-516.

WOLKOWICZ, P.E., POWNALL, H.J. & McMILLIN-WOOD, J.B. (1982). (1-pyrenebutyryl)carnitine and 1-pyrenebutyryl coenzyme A: fluorescent probes for lipid metabolite studies in artificial and natural membranes. *Biochemistry*, **21**, 2990-2996.

WALLNOFER, A., CAUVIN, C. & RUEGG, U. (1987). Vasopressin increases $^{45}Ca^{2+}$ influx in rat aortic smooth muscle cells. *Biochem.Biophys.Res.Comm.*, **148**, 273-278.

WALLNOFER, A., WEIR, S., RUEGG, U. & CAUVIN, C. (1989). The mechanism of action of endothelin-1 as compared with other agonists in vascular smooth muscle. *J.Cardiovasc.Pharmacol.*, **13**(S5), S23-31.

WATANABE, H., KOBAYASHI, A., HAYASHI, H. & YAMAZAKI, N. (1989). Effects of long-chain acyl carnitine on membrane fluidity of human erythrocytes. *Biochem.Biophys.Acta*, **980**, 315-318.

WEIR, S.W. & WESTON, A.H. (1986). The effects of cromakalim and nicorandil on electrical and mechanical activity and on $^{86}Rb$ efflux in rat blood vessels. *Br.J.Pharmacol.*, **88**, 121-128.

WEKSLER, B.B., ELDOR, A. & FALCONE, D. (1982). Prostaglandins and vascular endothelium. In *Cardiovascular Pharmacology of the Prostaglandins*. Ed. Herman A.G. *et al.* pp. 137-148. New York, Raven.

WISE, B.C. & KUO, J.F. (1983). Modes of inhibition by acylcarnitines, adriamycin and trifluoperazine of cardiac phospholipid-sensitive calcium-dependent protein kinase. *Biochem.Pharmacol.*, **32**, 1259-1265.

YAMAMOTO, R., KAWASAKI, H. & TAKASAKI, K. (1984). Postsynaptic $\alpha$-adrenoceptor populations in several vascular systems of the anaesthetized rat. *J.Auton.Pharmac.*, **4**, 231-239.

YOKOTA, S., HIRONAKA, Y. & OHARA, N. (1989). Effects of *l*-carnitine on membrane potential derangements induced by palmitoylcarnitine and anoxia in isolated superfused guinea-pig papillary muscle. *Res.Comm.Chem.Path.Pharm.*, **66**(2), 179-190.

ZAWADZKI, J.V., CHERRY, P.D. & FURCHGOTT, R.F. (1980). Comparison of endothelium-dependent relaxation of rabbit aorta by A23187 and by acetylcholine. *The Pharmacologist*, **22**, 271.

ZSCHAUER, A., SCOTT-BURDEN, T., BUHLER, F.R. & VAN BREEMEN, C. (1987). Vasopressor Peptides and depolarization stimulated $Ca^{2+}$-entry into cultured vascular smooth muscle. *Biochem.Biophys.Res.Comm.*, **148**(1), 225-231.

# PUBLICATIONS.

CRIDDLE, D.N., HIGGINS, A.J. & WOODWARD, B. (1987). Effects of three acyl carnitines on the isolated coronary and mesenteric vascular beds of the rat. *BrJ.Pharmacol.,* **92**, 758P.

CRIDDLE, D.N., HIGGINS, A.J. & WOODWARD, B. (1987). Effects of palmitylcarnitine on the isolated rat heart and thoracic aorta. *J.Physiol.,* **391**, 47P.

CRIDDLE, D.N., GWILT, M. & WOODWARD, B. (1988). Interactions between palmitoyl carnitine and vasoconstrictors in the mesenteric vascular bed and aorta of the rat. *BrJ.Pharmacol.,* **98**, 851P.

CRIDDLE, D.N., DEWAR, G.H., WATHEY, W.B. & WOODWARD, B. (1990). The effects of novel vasodilator long chain acyl carnitine esters in the isolated perfused heart of the rat. *BrJ.Pharmacol.,* **99**, 477-480.

CRIDDLE, D.N., DEWAR, G.H., WATHEY, W.B. & WOODWARD, B. (1990). Effects of palmitoyl carnitine and a novel ester derivative on the isolated perfused rat heart. *J.Mol.Cell.Cardiol.,* **22**(SIII), PS73.

CRIDDLE, D.N. & WOODWARD, B. (1991). On the mechanism of action of $P1P^{i}$, a novel vasodilator long chain acyl carnitine ester, in the isolated perfused heart and mesenteric vascular bed of the rat. (Submitted to *Br.J.Pharmacol.*).