SOME EFFECTS OF PROSTAGLANDINS AND RELATED SUBSTANCES, AND OF DRUGS WHICH MODIFY PROSTAGLANDIN BIOSYNTHESIS AND ACTION, ON VASCULAR REACTIVITY IN AN ISOLATED, PERFUSED, VASCULAR BED

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ABSTRACT

Prostaglandins (PGs) were tested over the concentration range 10^{-13} to 10^{-6} M in the perfused rat mesenteric vascular bed (MVB). PGE2, PGF2α and PGA2 enhanced noradrenaline (NA) and potassium (K^+) contractions. PGI2 inhibited but PGE1 and PGA1 at low concentrations potentiated and at high concentrations inhibited NA contractions. K^+ responses were uninfluenced by any of: PGE2, TxB2, PGI2, 6-keto-PGF1α, PGD2, PGB2. 16,16-dimethyl PGE2 had actions which were a mixture of those of PGE2, PGE1 and PGF2α. Indomethacin blocked responses to all stimuli whereas imidazole, 1-benzyl imidazole, nicotinic acid, dipyridamole and 9,11-azoprosta-5,13-dienoic acid preferentially inhibited NA and angiotensin II responses. The effects of both the cyclooxygenase and thromboxane synthetase inhibitors were reversed by exogenous PGE2. RIA analysis of MVB perfusate and incubates of mesenteric artery and arteriole rings combined with TLC analysis identified immunoreactive PGE2, PGF2α, 6-keto-PGF1α and TxB2. Adenosine was found to be a PG antagonist in the MVB and in isolated lymphocytes. Hydralazine preferentially inhibited NA responses, an effect consistent with antagonism of a PG since RIA analysis showed that hydralazine did not stimulate PGI2 biosynthesis.

It has been shown that the MVB synthesized several PGs including TxB2 and that vascular reactivity depended on the modulatory actions these PGs. The compounds tested here appeared to modify MVB reactivity by interfering with PG action and/or biosynthesis.

ABSTRACT

Nous avons étudié l'effet des prostaglandines (PGs) au niveau du territoire mésentérique du rat, à des concentrations allant de 10^{-3}M à 10^{-6}M. PGE_2, $PGE_{2\alpha}$, ainsi que PGA_2 potentialisent l'effet vaso-constricteur de la noradrénaline (NA) et du potassium (K^+). A toutes ces concentrations, PGI_2 inhibe l'effet vaso-constricteur de la noradrénaline; à de faibles concentrations PGE_1 et PGA_1 ont un effet potentialisateur alors qu'à de hautes concentrations elles ont un effet inhibiteur. PGE_2, TxB_2, PGI_2, 6-keto-$PGF_{1\alpha}$, PGD_2, PGB_2 ne modifient pas la réponse du K^+. 16-16-dinéthyl PGE_2 a des actions similaires à PGE_2, PGE_1 et $PGF_{2\alpha}$. L'indométhacine bloque la réponse à tout stimuli, alors que l'imidazole, le 1-benzyl imidazole, l'acide nicotinique, le dipyridamole, ainsi que l'acide 9,11-cizoprosta-5,13-diénoïque inhibent de façon plus spécifique l'effet de la NA et de l'angiotensine II. La PGE_2 exogène inverse l'effet des inhibiteurs de la cyclooxigénase et de la thomboxane synthétase. Des dosages radioimmunologiques et des chromatographies sur couche mince de perfusat de territoire mésentérique, des incubations d'artères mésentériques et de bandes d'artérioles, ont démontré l'immunoréactivité de PGE_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ et de TxB_2. L'adénosine antanogise l'effet des PGs dans le territoire mésentérique et dans les lymphocytes isolés. L'hydralasine inhibe l'effet de la NA, cet effet est compatible avec son rôle d'antagoniste de PG, puisque des dosages radioimmunologiques démontrent que l'hydralazine ne stimule pas la synthèse de la PG_2.

Il a été démontré que le territoire mésentérique synthétise plusieurs PGs incluant la TxB_2 et que la réactivité vasculaire dépend de l'action modulatrice de ces PGs. Les substances que nous avons étudiées semble modifier la réactivité du territoire mésentérique en interférant avec l'action de la PG et/ou sa biosynthèse.

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List of Abbreviations and Symbols

AMP	5'adenosine monophosphate
ADP	5'adenosine diphosphate
ATP	5'adenosine triphosphate
ANGIO	angiotensin II
ANOVA	analysis of variance
AZO	9,11-azo-prosta-5,13-dienoic acid
1-b-IMID	1-benzylimidazole
cAMP	3', 5'-cyclic adenosine monophosphate
cyclic AMP	3', 5'-cyclic adenosine monophosphate
^{o}C	degrees Celsius
dibutryl cAMP	$N^6,O^{2'}$-dibutryl-3', 5'-cyclic Adenosine monophosphate
DIPYR	dipyridamole
ECF	extracellular fluid
g	gram
µg	microgram
mg	milligram
ng	nanogram
pg	picogram
hr	hour
HYD	hydralazine
IMID	imidazole
INDO	indomethacin
L	litre
ml	millilitre
MVB	mesentric vascular bed
min	minute
M	molar
mM	millimolar

μM	micromolar
nM	nanomolar
pM	picomolar
NA	noradrenaline
m	moles
nm	nanometre
PAPAV	papaverine
PG	prostaglandin
SD	standard deviation of the mean
SE	standard error of the mean
sec	second
Tx	thromboxane
α	alpha
β	beta
λ	gamma
ω	omega
≃	approximately
=	equals
X	multiplied by

for gregory whose dreams are our reality

GENERAL INTRODUCTION

Pre-amble

Because of the ubiquitous nature and diverse actions of prostaglandins any introduction must of necessity encompass the chemistry, biosynthesis, regulation of biosynthesis and metabolism. *In vivo*, these factors determine the extent and diversity of prostaglandin actions. Prostaglandins are not stored, thus their release by tissues reflect *de novo* synthesis (*Vane 1978*). Prostaglandins were first identified in the male reproductive accessory glands and their secretions. It is now known that prostaglandins are widely distributed in mammalian tissues and body fluids *e.g.* kidney, lung, thymus, spleen, iris, thyroid, adipose tissue, uterus, placenta, blood vessels (arteries and veins), central nervous system, adrenals, gut, menstrual fluid, amniotic fluid, synovial fluid, etc. One well documented exception is the red blood cell, to date a synthesis capability has not been detected in human, subhuman, mature or immature erythrocytes (*Horrobin 1978, Johnson et al 1974*). The occurrence of prostaglandins is not restricted to mammalian tissue, *Schneider et al (1973)* identified PG like material in coral and more recently three plants were shown to have prostaglandin-like fatty acids (*Cao and Cepero 1976*).

Nomenclature

Since the discovery of PGs in *1935* by *M.S. von Euler*, and the adoption of the now official term "*prostaglandin*" much has changed in methods used to assign names to the prostaglandins.

The letters E and F in prostaglandin designation refer to early observations of separation of prostaglandins (partially) by ether extraction of tissue homogenates. The E prostaglandins were taken up in the ether phase and the F prostaglandins remained in the aqueous buffer phase; acidic or basic treatment of these prostaglandins yeilded derivatives designated A and B respectively (*Hamberg 1973*).

Structure

All prostaglandins contain 20 carbon atoms, a cyclopentane ring (five membered), two side chains and a terminal carboxyl group (-C(=O)-OH) of the parent prostanoic acid. Today classification is based upon the type and position of molecular groups and the degree of unsaturation (double bonded carbons, C = C), in addition all prostaglandins possess a C-13, C-14 double bond and a 15-hydroxyl group which as will be discussed later appear to be essential for PG bioactivity (see Fig. *1* for structure and numbering system). Because of the rigidity imparted by the cyclopentane ring and the existence of double bonds, various isomers of prostaglandins occur.

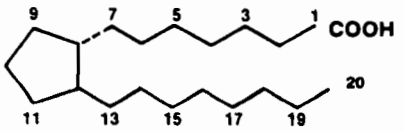


Fig. 1: Structural formula of the hypothetical prostanoic acid.

An alpha/beta (α/β) system is employed to define the stereo-chemical nature of the substituents on the cyclopentane ring. α Substituents are situated on the same side of the ring as the aliphatic side chain (C-7 to C-1) bearing the carboxyl group at C-1. β Substituents are

located on the side of the ring bearing the alkyl side chain (C-13 to C-20).

There are two main natural prostaglandin groups, the 1 series and the 2 series, which have been widely studied. This categorization is based on the degree of unsaturation of a side chain. The 1 series is derived from dihomogammalinolenic acid (DGLA, 8,11,14 eicosatrienoic acid) which contains one double bond side chain and the 2 series, with two double bonds in the side chain is derived from arachidonic acid (AA, 5,8,11,14-eicosatetraenoic acid). There is a 3 series in mammalian systems derived from 5,8,11,14,17 eicosapentenoic acid (*Flower 1977; Raz 1977; Needleman et al 1976, 1979*) which has three double bonds in the side chain. However not much is known about this series.

The structures of the main prostaglandins classified on the basis of substituents on the pentane ring as A,B,C,D,E and F are shown in Fig. 2. These prostaglandins differ from each other in several characteristic ways (*Schneider 1976*).

1. Number of double bonds (C = C)
2. Number of hydroxyl groups (R-C-OH)
3. Presence or absence of keto groups (R-C(R)=O)

Recent advances in both analytical and stabilization techniques have led to the identification by biological action and finally chemical and structural characterization of three other types of compounds.

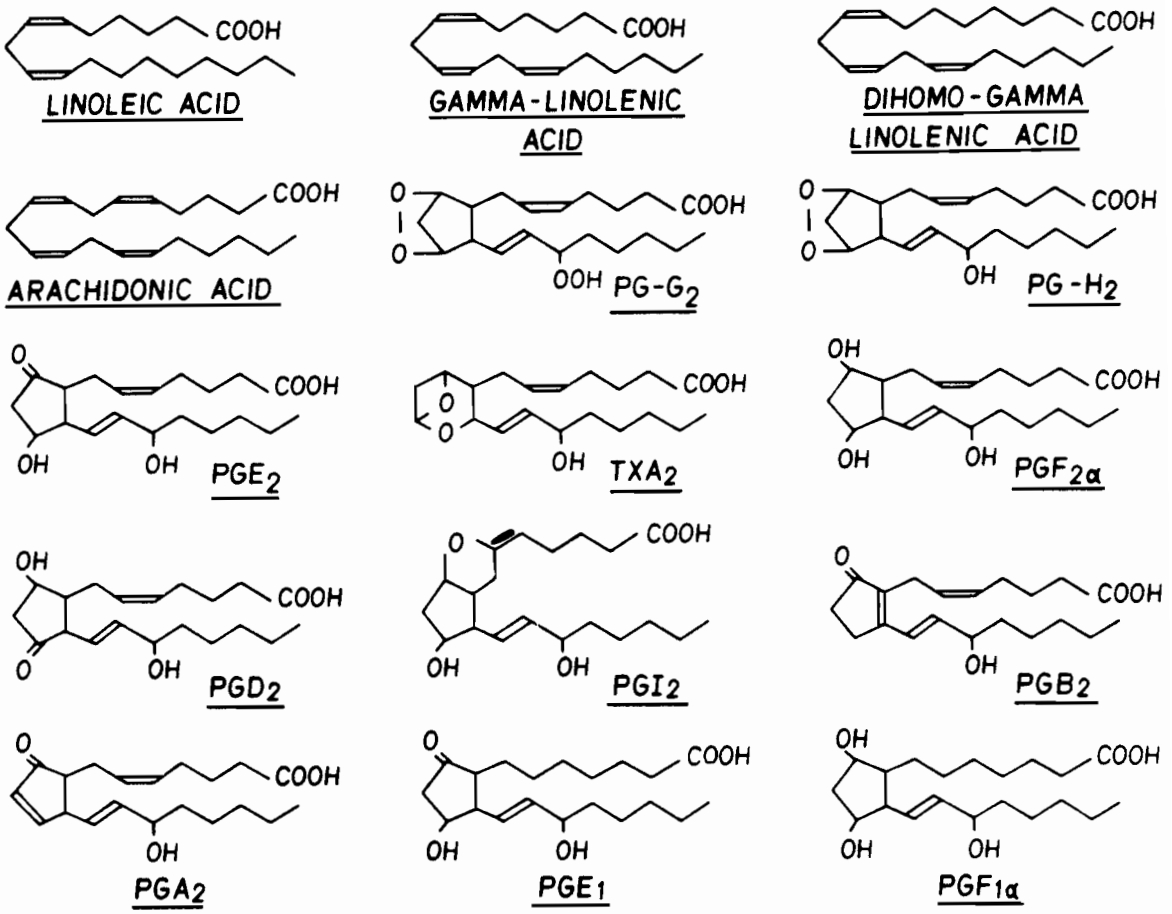


Fig. 2: Structures of the most important essential fatty acids, prostaglandins and related substances.

1. Prostaglandin endoperoxides: They have two oxygen atoms derived from a single oxygen molecule, linking the 9 and 11 positions of the cyclopentane ring, and are formed as intermediates in the biosynthesis of prostaglandins (*Mclouf et al 1977;* Fig. *4*). Prostaglandin (PG)H2 has side chains similar to the primary PGs described above while PGG2 has an extra oxygen between the 15-carbon and its hydroxyl group, this -OOH group is termed a hydroperoxy group, indicating the presence of the two oxygen atoms (Fig. 2).

2. Thromboxanes. These are derived from the endoperoxides and have an oxygen atom inserted into the cyclopentane ring, producing a cyclohexane compound with an oxygen atom bridging carbons 11 and 9 (Fig. 2). These compounds were originally discovered in thrombocytes, hence their name.

3. Prostacyclin (PGI2) formerly termed PGX. In this molecule an oxygen atom links the 9 carbon of the cyclopentane ring to the 6 carbon of the aliphatic side chain (C-7 to C-1, see Fig. 2).

Outline of prostaglandin biosynthesis

The structures of the main prostaglandin compounds are shown in Fig. 2. The prostaglandins of the 1 and 2 series are synthesized from essential fatty acids (EFAs) of the linolenic acid (LA) family while prostaglandins of the 3 series are synthesized from α linoleic acid (Fig. *3*).

Linoleic acid is an 18 carbon acid, with two double bonds and the omega (ω) bond six carbons away from the terminal carbon of the alkyl side chains (C-13 to C-20, Fig. 2). It is therefore designated 18:2ω6.

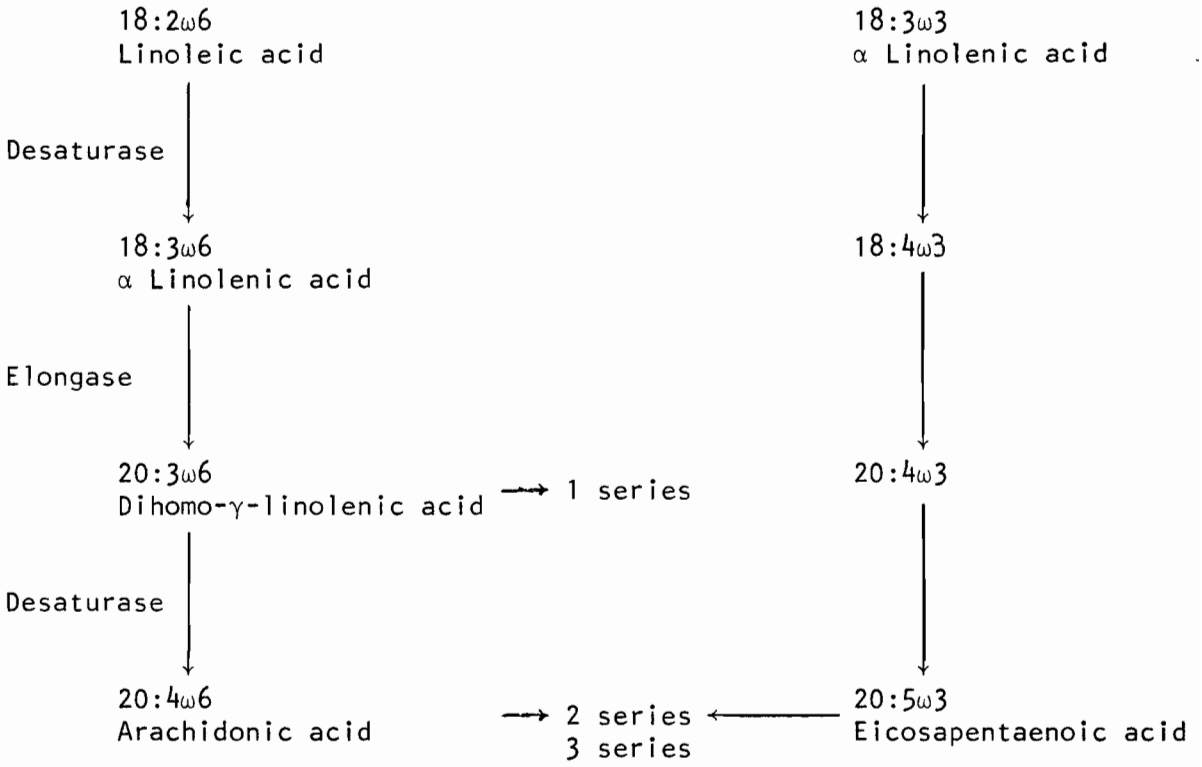
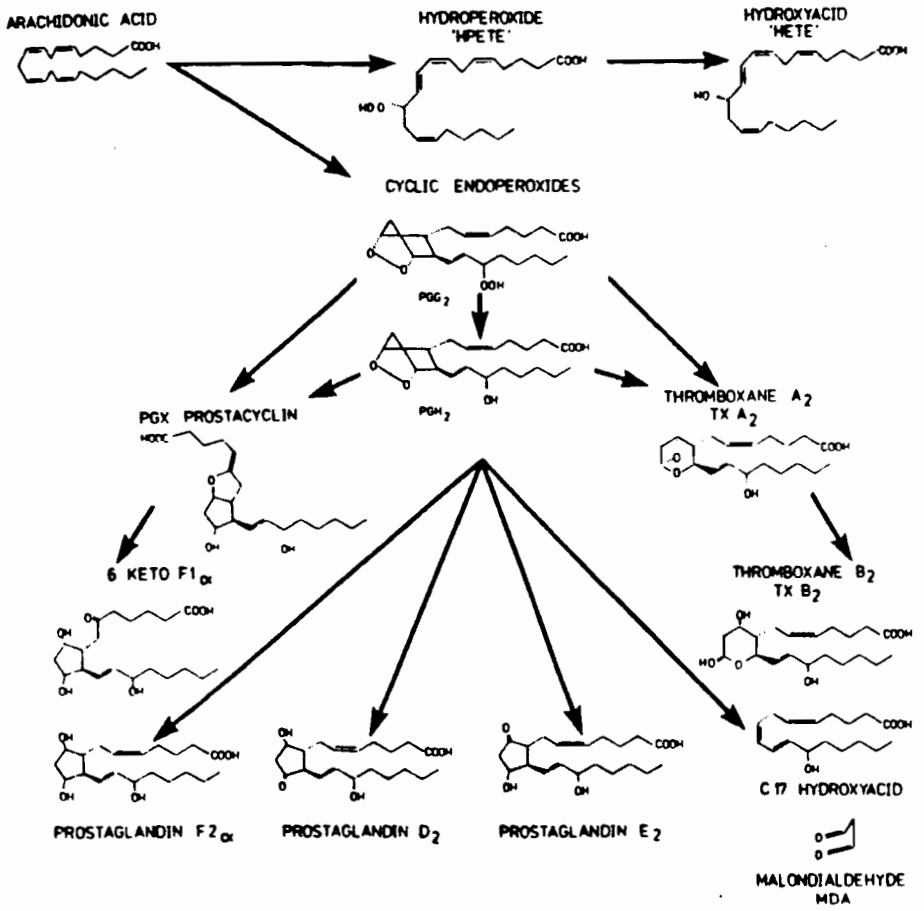


Fig. 3. The metabolism of essential fatty acids by which prostaglandins and related substances are biosynthesized.

Linoleic acid is desaturated (2 hydrogen atoms are lost) to give gamma (γ) linolenic acid (GLA = 18:3ω6) and GLA is elongated (by addition of two carbon atoms) to give dihomogammalinolenic acid (DGLA 20:3ω6). DGLA is the precursor of the 1 series of prostaglandins or can be desaturated to give arachidonic acid (AA, 20:4ω6) the precursor for the 2 series. DGLA and AA are termed polyunsaturated fatty acids and are normally found esterified to phospholipids, to cholesterol and to other neutral lipids (*Curtis Prior 1976*). There are two positions for fatty acids in phospholipid molecules. The first position is usually occupied by a saturated (lacks C=C double bonds) and the second by a polyunsaturated fatty acid (containing more than one C = C double bond).

The first step in the biosynthesis of prostaglandins is the conversion of free DGLA or AA to PGG endoperoxides (Fig. *4*). This is accomplished by extraction of hydrogen, the closure of the ring at the sites of hydrogen removal (C-8 and C-12), the formation of an oxygen bridge (between C-9 and C-11) and hydroperoxidation (at C-15). The last step is catalyzed by a lipoxygenase enzyme and the first three by an enzyme complex known as cyclo-oxygenase. The cyclo-oxygenase is now thought to be made up of four subunits, two units have heme bound as part of their structure, the other two units containing non-heme iron (*Hemler and Lands 1977*). In addition there is some evidence that there are two types of cyclo-oxygenase, 'E_b' providing basal prostaglandin levels and 'E_a' which is responsive to stimulation (*Smith and Lands 1972*). The endoperoxide PGG produced by the cyclo-oxygenase can be converted to PGH by an enzyme (PGG-PGH reductase) and appears to play a pivotal role in prostaglandin biosynthesis (*Samuelsson 1976*). *Sun et al* (*1977*) suggested that in lung, 'E_b' provided endoperoxide for prostacyclin production and 'E_a' provided endoperoxide for thromboxane synthesis.



ARACHIDONATE METABOLISM

Fig. 4. An outline of the metabolic pathways by which the prostaglandins and related substances are synthesized from arachidonic acid.

There are a number of compounds which can be formed by appropriate modification of the endoperoxides (PGG and PGH). These are enzymatically converted to a variety of products by an isomerase and perhaps a reductase enzyme to yield prostaglandins of the E, D and F types (*Hamberg et al 1974; Nugteren and Hazelhuf 1973*), by thromboxane synthetase to thromboxane A or by 6,9 oxycyclase into prostaglandin I.

Perhaps the best characterized pathway for prostaglandin biosynthesis is that of the 2 series (Fig. 4). Conversion of arachidonic acid to prostaglandin endoperoxides and finally to prostaglandins and thromboxane is not the only route for arachidonic acid metabolism. A significant portion is converted by a lipoxygenase system, first to 12 L-hydroperoxy-5,8,11,14-eicosatetraenoic acid (HPETE) and then to 12 L-hydroxy-5,8,11,14-eicosatetraenoic acid (HETE) (Fig. 4).

For more detailed descriptions of prostaglandin biosynthesis see *Hamberg and Samuelsson 1967; Christ and Van Dorp 1973; Hamberg et al 1975; Mclouf et al 1977; Yamamoto et al 1977; Sun et al 1977; Pace-Asciak and Rangaraj 1978)*

Prostaglandin interconversion

Prostaglandins can be converted from one class to the other, by enzymatic and in some situations, by non enzymatic reactions. The most important interconversions documented thus far are:

1. The reduction of PGE, 9 keto-(C=O)-group by a NADPH-dependent 9-keto-reductase enzyme producing PGF. This reaction is probably reversible under the appropriate conditions. There are probably several types

of 9-ketoreductase in light of the discovery by *Hassid et al (1977)* of three different isoenzymes in a single tissue.

2. The loss of a H_2O molecule from the PGE, cyclopentane ring to produce PGAs. This reaction may take place enzymatically but can certainly take place nonenzymatically especially during isolation procedures at acidic pH (noted earlier in section on nomenclature). This has resulted in some debate about the real vs apparent existence and importance of the A type prostaglandins.

3. Isomerization of the PGA cyclopentane ring double bond from the 10:11 position to the 8:12 position produces PGBs (see Fig. *2* for structures and Fig. *1* for numbering system). This reaction can take place in the presence of an isomerase (*Jones et al 1974*) or in its absence. Recently PGD was shown to be produced by nonenzymic isomerization of an endoperoxide (*Hamberg and Fredholm 1976*) in addition to enzyme mediated isomerization (*Sun et al 1977*).

Prostaglandin inactivation

Prostaglandins are metabolized to a variety of end products with little or no biological activity (*Anggard and Samuelsson 1966*). A large number of metabolic pathways have been described (*Samuelsson et al 1971; Curtis Prior 1976; Pong and Levine 1977*). The following probably are the most important in inactivating the prostaglandins:

1. Dehydratation of C-15 hydroxyl group of the alkyl side chain (C-13 to C-20) by 15-hydroxy-dehydrogenase to give 15-keto compounds. This enzyme is widely distributed (*Anggard and Samuelsson 1966; Saeed and Roy 1972; Jarabak 1972; Marazzi and Matschinsky 1972; Limas and Cohn*

1973; Kaplan et al 1975; Pace-Asciak and Rangaraj 1978) and requires NAD as a co-factor. It is the main enzyme responsible for the destruction of the biological activity of PG(s) E and F. There is also a second type of dehydrogenase requiring NADP as a co-factor and displaying preference for PGF (*Lee and Levine 1975*).

2. Reduction of the C-13 double bond. This is catalyzed by Δ^{13} reductase which is found in many tissues (*Larsson and Anggard 1970*) and is thought to be a soluble enzyme. This reduction of the C-13 double bond is thought to follow 15 hydroxy-prostaglandin dehydrogenase mediated reactions (*Curtis Prior 1976*).

3. Beta (β)-oxidation. This is the removal of the carbon fragments beginning at the carboxylic end of the molecule (C-1). The products of β-oxidation are dinor - or tetranor - prostaglandin derivatives, indicating the removal of two or four carbons respectively. β-oxidation is the typical fate of many fatty acids (*Bohinsky 1973*).

4. Omega (ω) hydroxylation. A hydroxyl group is substituted on the terminal carbon of the omega end of the molecule (Alkyl side chain). This usually follows the three processes described above.

5. Omega (ω) oxidation. The omega hydroxyl group is converted to an (acidic) carboxyl group.

Metabolism of the E prostaglandins by these five processes in tandem gives 7 α hydroxy-5,11-diketotetra-norprosta-1,16-dioic acid, the main urinary metabolite in man.

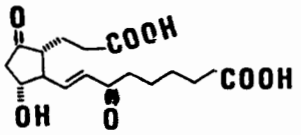


Fig. 5: Main urinary PGE metabolite in man.

While the catabolism of prostaglandins in man appears to be straight forward it is apparently more complicated in the rat. In this species there appears to be three pathways, each leading to a different end product (*Green 1971*).

6. Thromboxane (Tx) A2 is a very labile substance, with a short biological half life (*Samuelsson 1976*). It is apparently nonenzymatically converted to TxB2 (see Fig. 2 for structure), a more stable, biologically inactive product. TxB2 can apparently be metabolized to 2,3 dinor TxB2 by β oxidation by-passing the other catabolic steps. Thus at least in man and monkey, TxB2 undergoes single step β-oxidation (*Roberts et al 1977a, 1977b; Kindahl 1977*).

7. Prostacyclin (PGI2) is also unstable and is converted to 6 keto PGFlα. Unlike E and F prostaglandins, PGI2 is apparently not taken up and inactivated by the lung (*Dusting et al 1978*). However recent studies indicate that PGI2 is a substrate for kidney, lung and smooth muscle catabolic enzyme systems (*McGuire and Sun 1978; Wong et al 1978*). PGI2 appears to be a good substrate for vascular smooth muscle 15 hydroxy prostaglandin dehydrogenase, while 6-keto-PGFlα is not (*Wong et al 1978*).

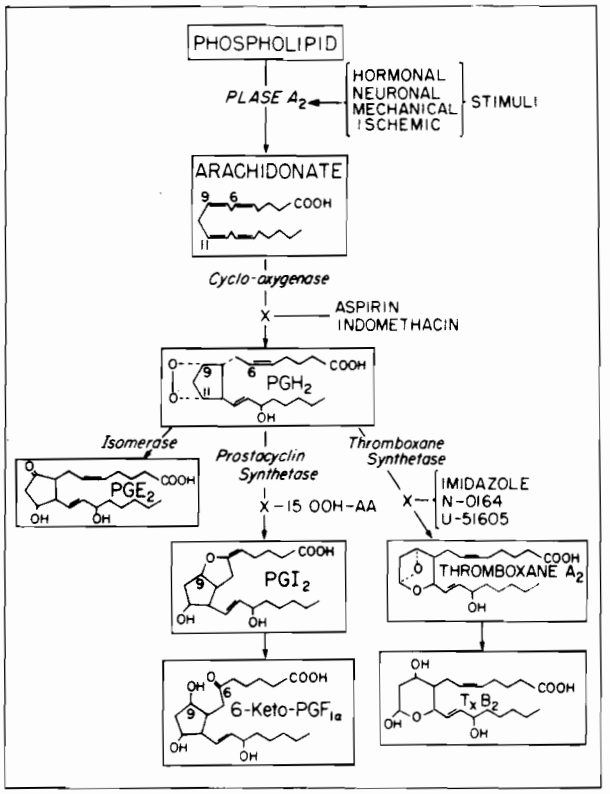


Figure 6 Pathways of Arachidonate Metabolism.
The substrates are boxed in, the enzymes are indicated on the arrows, and the inhibitors are indicated with an X at their site of action.

Control of prostaglandin biosynthesis

The esterified forms of the prostaglandin precursors are not available to the prostaglandin cyclo-oxygenase complex, therefore the unsaturated fatty acids must first be released. This is now thought to be the rate limiting step in prostaglandin biosynthesis (*Samuelsson 1969; Flower and Blackwell 1976; Sun et al 1977*) and is mediated by the phospholipase enzyme (*Blackwell et al 1978*, Fig. 6).

Available evidence does not indicate whether one or several of the phospholipid classes provide precursor for prostaglandin cyclo-oxygenase. Recent data on phospholipase A_2 activity in platelets showed the loss of 1-^{14}C (C-20 4ω-6) arachidonic acid from a variety of phospholipids. *Russel and Deykin (1976)* found that phosphatidylcholine and phosphatidylinositol of human platelet phospholipid stores lost prelabeled arachidonic acid upon stimulation; *Blackwell et al (1977)* and *Bills et al (1976)* demonstrated that rabbit platelets lost arachidonic acid from phosphaticylcholine, phosphatidylinositol and phosphatidylethanolamine fractions. In their study on human platelets *Jesse and Cohen (1976)* concluded that diacyl phosphatidylethanolamine was the primary source of the arachidonic acid released. In attempts to clarify the situation *Russel and Lenard (1977)* and *Russel and Deykin (1976)* determined the distribution of phospholipids in membranes of erythrocytes, viruses and platelets. They found that the phospholipid distribution was assymetric, phosphotidylcholine predominantly in the exterior of the membrane and both phosphotidylserine and phosphatidylethanolamine were located on the surface of the inner membrane (facing the cytosol). Thus if different phospholipases were present, paralleling the distribution of the phospholipids, stimulation of prostaglandin synthesis in discrete locations might be possible. Indeed in liver

and brain tissue differing types of phospholipases have been identified (*Goracci et al 1978*). The possibility then exists that other esterified forms of phospholipids other than those discussed above may play important roles (*Vogt 1978; Dawson and Irvine 1978; Flower 1977*).

The first direct demonstration of the sequence of induced release of polyunsaturated fatty acids from phospholipids by phospholipase A_2 and their conversion to prostaglandins was by *Flower et al (1976,1975)*. Utilizing radiolabeled arachidonic acid, isolated platelets and slices of spleen, they showed that stimulation resulted in the release of arachidonic acid from the prelabelled phospholipid stores. This was paralleled by a significant increase in labeled prostaglandins; because prostaglandins are not stored, their release reflects *de novo* synthesis.

Regulation of phospholipase activity

The phospholipases of plasma membranes and microsomes appear to be absolutely dependent upon calcium ions (*Newkirk and Waite 1973*). The optimal calcium ion concentration for phospholipase A activity of liver plasma membrane is 0.5 mM (*Colard-Torquebiau et al 1975*). In platelets divalent calcium ionophores (A23187 and X537A) are potent stimulators of phospholipase activity and prostaglandin biosynthesis (*Feinstein et al 1977; Oelz et al 1977; Pickett et al 1977*). A23187 also stimulates prostaglandin biosynthesis by the thyroid gland *in vitro* (*Waelbroeck and Boeynaems 1978*) and cultured endothelial cells (*Weksler et al 1978*). In intact cells chelation of the extracellular calcium does not block phospholipase activity suggesting an intracellular location of the calcium sensitive site. This is supported by data from *Colard-Torquebiau et al (1976)* who demonstrated preferential phospholipase A_2 activity on intracellular

phospholipids. Therefore regulation of phospholipase A_2 activity can be effected by alterations in the availability of calcium. Additional support for calcium control of phospholipase A_2 activity comes from the observations that drugs with local anaesthetic activity (mepacrine, chlorpromazine, tetracaine, lidocaine and cocaine) which can displace calcium from binding sites are able to prevent phospholipase activation (*Feinstein et al 1977; Flower and Blackwell 1976; Kunze et al 1976*) at fairly high concentrations (10^{-3} M). While the evidence for calcium control of phospholipase A_2 activity is persuasive, it is also known that phospholipase A_2 of lysosomal origin has no obligatory requirement for calcium (*Waite et al 1976*). It is therefore possible that other controlling factors may prove to be equally as important as calcium.

Stimulators of prostaglandin synthesis

The exact mechanism by which various stimuli activate prostaglandin biosynthesis is at present, poorly understood. In Table *1* a survey of a variety of agents and other stimuli which activate prostaglandin synthesis is shown. It is known that tachyphylaxis to one stimulus does not hinder the ability of another agent to stimulate prostaglandin synthesis. Thus the tachyphylaxis must be at a site other than the phospholipase mediated step (*Needleman et al 1975*). This suggests that these stimuli do not act directly on the phospholipase enzyme.

Co-factors in prostaglandin biosynthesis

Much of what is known about co-factors substances necessary for efficient prostaglandin biosynthesis has been gleaned from *in vitro* enzyme experiments. These co-factors invariably enhanced the production of one or more of the metabolites of arachidonic acid or dihomogammalinolenic acid

TABLE 1

STIMULANTS OF PROSTAGLANDIN BIOSYNTHESIS

Stimulus	*Tissue*	*Reference*	
catecholamine	vascular smooth muscle, spleen	Grodzinska et al	1976
		Gimbrone	1976
		Bedwani and Miller	1975
serotonin	brain, stomach, bovine seminal vesicle, vascular smooth muscle	Coceani et al	1967
		Wolfe et al	1976
		Takeguchi et al	1976
		Alexander and Gimbrone	1976
acetylcholine	stomach, heart	Pace-Asciak et al	1971
		Justand and Wennmalm	1974
TSH	thyroid	Haye et al	1976
ACTH	adrenals	Laychock et al	1976
LH	uterus	Kuehl	1974
pentagastrin	stomach	Shaw and Ramwell	1968
bradykinin	heart, bovine mesenteric artery	Needleman et al	1975
		Juan and Lembeck	1976
angiotensin	spleen, endothelial cells	Peskar and Hertting	1973
		Gimbone and Alexander	1975
vasopressin	kidney	Zusman and Keiser	1977
oxytocin	uterus	Roberts	1976
collagen	platelets	Vincent and Zijlstra	1977
thrombin	platelets	Hamberg et al	1974
calcium ionophores	platelets, endothelial cells	Feinstein et al	1977
		Weksler et al	1978
ATP	heart	Needleman et al	1965
cyclic AMP	3T3 fibroblasts	Lindgren et al	1978
uremia	vascular smooth muscle	Silberhauer	1978

TABLE 1 cont.

Stimulus	*Tissue*	*Reference*	
anaphylaxis	heart	Anhut et al	1977
vibration	vascular smooth muscle	Baenzigner et al	1977
hypotonic solution	intestine	Vogt and Distelkotter	1967
ischemia	kidneys, heart	McGiff et al	1970
		Berger	1977
radiation	skin	Camp et al	1978
homogenization	seminal vesicle	Penneys et al	1977
hemoglobin	seminal vesicle, platelets	Yoshimoto et al	1970
		Hemler and Lands	1977
		Tai et al	1977
heme	platelets, seminal vesicle	Tai et al	1977
		Ho et al	1977
		Hemler and Lands	1977
		Ogino et al	1978

usually only changes in PGE or PGF concentrations were reported because of limited assay capability available at the time. In such a consideration of co-factor requirements, the mechanism(s) of action can only be speculated upon.

Although the cyclo-oxygenase is associated with the membrane fraction of cells, a heat stable co-factor(s) from the soluble fraction was required for good activity (*Samuelsson 1970*). Today, for biosynthetic studies (*in vitro*), this co-factor is replaced by either hydroquinone, epinephrine or some other phenolic compound and reduced glutathione (GSH) (*Lee and Lands 1972; Yoshimoto et al 1970; Van Dorp 1967*). These co-factors have been shown to increase the conversion rate of arachidonic acid to prostaglandins in many tissues (*Takeguchi et al 1971; Kingston, Greaves 1976; Yoshimoto et al 1970*). However in liver microsomes neither hydroquinone nor glutathione (GSH) were able to increase prostaglandin synthesis (*Morita et al 1977*). Recently *Dembinska-Kiec et al (1977)* suggested that the routine addition of co-factors has distorted the profile of PG synthesis in various tissues, leading to the overemphasis of the importance of some prostaglandins, especially PGE_2.

The addition of glutathione appears to favour PGE2 production, because omission of this co-factor results in increased production of other PGs including PGF (*Chen et al 1975; Lee, Lands 1972; Takeguchi et al 1971*). As a result of omission of glutathione no increase in oxygen uptake was seen although PGE2 production was sharply reduced suggesting the diversion of PG endoperoxides from PGE2 isomerase (*Yoshimoto et al 1970*). In human platelet incubates, *Ho et al (1976)* found that glutathione had no effect on TxB2 production whereas hydroquinone appeared to inhibit its production. On the other hand, *Tai and Yuan (1977)* reported that glutathione did

stimulate TxB2 production. In vascular smooth muscle, glutathione inhibited the production of PGI2 whereas in its absence PGI2 levels increased while PGE2 output was reduced (*Dembinska-Kiec et al 1977*). This effect of glutathione is not nonspecific, because other sulphydryl (SH) containing substances have no significant effect on the enzyme (PG synthetase; *Van Dorp 1967; Takeguchi et al 1971*).

Inhibition of prostaglandin biosynthesis

As a result of the unravelling of the biosynthetic pathway there are a number of key steps at which use of an appropriate pharmacological agent could aid immensely in understanding the biological role of prostaglandins *in vivo* or *in vitro*. These stages are shown in a single schematic outline (Fig. 6).

Phospholipase inhibition

As discussed earlier phospholipase A_2 seems to play a crucial role in prostaglandin biosynthesis by making free arachidonic acid available to the cyclo-oxygenase. Thus by inhibiting the phospholipase, substrate is denied and the pathway becomes inactive.

There is abundant evidence that this is exactly how the glucocorticoids regulate prostaglandin biosynthesis (*Dembinska-Kiec et al 1978; Korbut et al 1978; Chandrabose et al 1978; Hong and Levine 1976; Kantrocoitz et al 1975*).

Inhibition of cyclo-oxygenase

The cyclo-oxygenase produces endoperoxides from arachidonic or dihomo-linolenic acid. Agents which inhibit this enzyme can be categorized into four classes: substrate analogues; other fatty acids; end products analogues; and nonsteroidal anti-inflammatory drugs (NSAID).

Lands et al (1972,1973) tested a number of substrate analogues and reported that those fatty acids which did not stimulate oxygen utilization acted as competitive inhibitors. The fatty acids with the greatest degree of unsaturation appeared to be more potent inhibitors. *Ahern and Downing (1970)* reported that acetylenic fatty acids (C = C) irreversibly inhibited prostaglandin synthesis. In general these fatty acids inhibit the production of all the arachidonic acid metabolites (Fig. 5) indicating that they inhibit both the cyclo-oxygenase and lipoxygenase enzymes. This irreversible inhibitory action was dependent on the presence of oxygen and was prevented by glutathione reductase (*Vanderhoek and Lands 1973*) suggesting that these acetylenic fatty acids served as enzyme substrates. Today 5,8,11,14-eicosatetraynoic acid (an acetylenic fatty acid) is widely used as an experimental tool.

A number of different fatty acids in high concentrations inhibit the cyclo-oxygenase. *Pace-Asciak and Wolfe (1968)* described the irreversible inhibitory actions of oleic, linoleic and linolenic, however *Marnett et al (1977)* reported the stimulation of prostaglandin biosynthesis by lipoic acid. Therefore each fatty acid must be evaluated individually for their effects on cyclo-oxygenase activity over a range of concentrations.

Analogues of prostaglandins, 7-oxa, 5-oxa and thiol derivatives inhibit the cyclo-oxygenase presumably at its active site (*McDonald Gibson et al 1977; Ohki et al 1977*, see review by *Lands and Rome 1976*). These compounds are little used in research today.

Non steroidal anti-inflammatory agents (NSAID)

The discovery by *Smith and Willis (1971)* and *Vane (1971)* that aspirin (acetylsalicylic acid) and aspirin-like drugs inhibited prostaglandin biosynthesis was a significant development in the area of prostaglandin research. Since then, examination of a variety of nonsteroidal anti-inflammatory drugs have provided evidence for a significant correlation between therapeutic benefits and their potency as cyclo-oxygenase inhibitors (*Vane 1976; see review Flower 1974*). A number of other NSAID which possess anti-inflammatory properties cannot, at therapeutic concentrations, inhibit the conversion of arachidonic acid to prostaglandins. These include chloroquine (*Greaves 1972b*), steroids (*Chandrabose et al 1978; Greaves 1972*), chlorpromazine and a large number of other drugs (*Vane 1978; Flower 1974*). It is becoming apparent that while these compounds (those acting at the cyclo-oxygenase level and those that do not) can inhibit stimulated prostaglandin synthesis, they do not inhibit basal prostaglandin biosynthesis (*Scherer et al 1978; Pace-Asciak et al 1978*).

Mechanism of action of non steroidal anti-inflammatory drugs (NSAID)

Aspirin inhibition of prostaglandin biosynthesis is apparently irreversible (*Flower 1974*); this is apparently due to the acetylation of the amino-terminal serine of the cyclo-oxygenase enzyme (*Roth and Siox 1978*).

Indomethacin is the NSAID most widely used as a research tool in probing the role of prostaglandins. Unlike aspirin it does not appear to irreversibly inactivate the cyclo-oxygenase. In vascular tissue and platelets, its effect is readily reversible upon washout (*Coupar and McLennan 1978; Malik 1978; Manku et al 1976; Crook et al 1976*). Further support for this is the inability to demonstrate covalent binding of indomethacin to

the cyclo-oxygenase (*Stanford et al 1977*). There is some evidence that the availability of arachidonic acid will determine the 50% inhibitory concentration (IC50) of indomethacin in a competitive manner (*Flower 1974*). This then probably accounts for the reported wide variability of indomethacin IC50 concentrations ranging from as low as 5×10^{-9} (*Robinson et al 1978*) to 1.5×10^{-4} M (*Bhattacharee et al 1973*). Variation in the sensitivities of cyclo-oxygenases in different tissues will also determine their susceptibility to inhibition. Some inhibitory concentrations of indomethacin on prostaglandin synthesis are shown in Tables 2 and 3, exemplifying the variation in sensitivity to this NSAID.

Inhibitors of endoperoxide metabolism

In 1973, two groups, *Nugteren et al and Hamberg et al* identified two endoperoxide intermediates in prostaglandin biosynthesis. Other exciting discoveries were those of thromboxane (Tx) A2 (*Hamberg et al 1975*) and prostacyclin (PGI2) (*Bunting et al 1976; Moncada et al 1976; 1977*). Since their identification, pharmacological inhibitors of their respective synthetases have been identified.

Inhibition of thromboxane synthesis

In the case with TxA2, benzydamine (an anti-inflammatory agent) was shown to inhibit TxA2 synthesis at concentrations lower than those needed to inhibit the cyclo-oxygenase (*Moncada et al 1976*). More recently imidazole was identified as a selective inhibitor of TxA2 production (*Needleman et al 1977; Moncada et al 1977*). A number of other agents have been reported to selectively inhibit TxA2 synthesis. These compounds include NO164* (a phenyl phosphate derivative of phloretin phosphate)

* *Sodium p-benz-4-[1-oxo-2-4(4-chlorobenzyl)3-phenyl-propyl] phenyl phosphate.*

TABLE 2

INDOMETHACIN INHIBITORY POTENCY ON PROSTAGLANDIN BIOSYNTHESIS
(*Enzyme Preparations*)

Enzyme Preparation		*PG Assayed*	*Indo. IC50* (μM)	*Reference*	
guinea pig	lung homotenate	F2α	0.75	Vane	1971
dog	spleen microsomes	F2α	0.3	Sykes and Maddox	1972
		E2	0.17	Flower et al	1972
rabbit	brain homogenates	E2	3.6	Flower and Vane	1972
rabbit	kidney microsomes	E2	3.9	Blackwell et al	1973
rabbit	renal microsomes	E2	0.62	Tachizawa et al	1977
bovine	seminal vesicles	E2	7.0	Tomlinson et al	1972
		F2α	30	Flower et al	1973
		E2	38	Flower et al	1973
sheep	seminal vesicles	E2	14	Moncada et al	1976
aortic	microsomes	12	2.77×10^3	Gryglewski et al	1976

TABLE 3

INHIBITION OF PROSTAGLANDIN BIOSYNTHESIS IN ISOLATED TISSUE BY INDOMETHACIN

Preparation	*PG Assayed*	*Indo. Concentration (μM)*	*% Inhibition*	*References*
human platelets	E2	0.17	50	Smith and Willis 1971
cat spleen (whole)	E2	0.84-14.0	100	Ferreira et al 1971*a*
dog spleen (whole)	E2,F2α	1	60	Ferreira et al 1971*b*
rabbit spleen (slices)	E	2.8	70	Gryglewski and Vane 1972
rabbit heart (whole)	E2	1.43	100	Minkes et al 1973
rabbit jejunum (whole)	E2	2.8-28.0	100	Ferreira et al 1972
rat uteri (whole)	E2,F2α	2.8-11.2	100	Vane and Williams 1972
rabbit leukocytes	E1	84.0	100	McCall and Youlten 1973
human stomach	E2	10	50	Peskar et al 1977
rabbit retina	E2	150	50	Bhattacharee et al 1973
smooth muscle cells	E2	5	50	Baenziger et al 1977
synovial culture	E2	0.005	50	Robinson et al 1978

(*Kulkarni and Eakins 1976; Eakins and Kulkarni 1977; Needleman et al 1977*), 3-isopropyl-3-nicotinyl indole (L8027) (*Gryglewski et al 1977*), dipyridamole (*Best et al 1978; Ally et al 1977*), benzydamine (*Moncada et al 1976*), 9,11 azoprosta-5,13,dienoic acid (U51605) (*Gorman et al 1977; Fitzpatrick and Gorman 1978*), nicotinic acid (*Vincent and Zijlstra 1978*). At high concentrations these compounds have other actions including inhibition of the cyclo-oxygenase, prostacyclin synthetase, 15 hydroxyprostaglandin dehydrogenase and phosphodiesterase enzymes.

Of these compounds, imidazole appears to be the most selective TxA2 synthetase inhibitor (*Needleman and Bryan 1978; Sun et al 1977; Nijkamp et al 1977*) and appears to compete with the substrate PGH2 for the active site on Tx synthetase (*Tai and Yuan 1978*).

Recent kinetic studies indicate that the Tx synthetase reaction is bimolecular, suggesting that two molecules of PGH2 are utilized in the production of one molecule each of TxA2 and HHT (*Anderson et al 1978*).

Inhibitors of prostacyclin synthesis

The compound prostacyclin (PGI2) was first suggested as a short lived intermediate in the formation of 6-keto-PGF1α in rat stomach (*Pace-Asciak 1971; 1977*). The structure of PGI2 was elucidated by *Johnson et al (1976)* and confirmed this earlier prediction (*Pace-Asciak 1971*, Fig. 2).

Selective pharmacological inhibition of PGI2 synthesis by 15-hydroperoxy arachidonic acid (*Gryglewski et al 1976*) and 13-hydroperoxy-linoleic acid (*Salmon et al 1978*) has been shown. Another PGI2 synthetase inhibitor tranylcypromine (*Gryglewski et al 1976*), was less potent when compared with the substrate analogues *in vitro*. Recently, *Dusting et al (1978)* presented evidence suggesting that the inactivity of 15-hydroxyperoxy arachidonic acid *in vivo* is a result of its metabolism to a PGI2 precursor. The

prostaglandin analogue U51605, in addition to inhibiting TxA2 synthetase, appears to be equipotent in inhibiting PGI2 synthesis (*Sun et al 1977; Needleman et al 1977*).

Inhibitor of PGH to E isomerase

Van Evert et al (1978) found that 5,8,11 eicosatrienoic acid which accumulates during essential fatty acid deficiency inhibited the conversion of endoperoxide to E type prostaglandins. This compound can be made from oleic acid in the absence of prostaglandin precursors.

At the present time pharmacological modification of the prostaglandin system is limited to the use of inhibitors of enzymatic synthesis. There is an acute necessity for the development of prostaglandin receptor antagonists (*Needleman 1978*). In the following section the available prostaglandin antagonists and their actions are covered.

Antagonists of prostaglandins

The 7-oxa derivatives of prostynoic acid were developed by *Freid et al (1968)*. They showed that 7 oxa-prostynoic acid (7OPA) inhibited prostaglandin induced contractions of the guinea pig ileum and gerbil colon. This compound has been tested in many preparations and has been shown to possess mixed antagonist properties or no effect. Structure activity studies (*Freid et al 1969; 1971*) established the independence of antagonist activity from the size of the prostynoic acid ring, however there appears to be a requirement for the acetylenic bond (C ≡ C) in investing specificity against prostaglandins (*Ford and Freid 1968*). More recent studies have established that 7OPA is a partial agonist; receptor binding studies demonstrated its affinity for the prostaglandin receptor (*Gorman and Miller 1973*) and studies in blood vessels established its intrinsic activity (*Ozaki et al 1978*).

Recent efforts along the same line have produced 8-ethoxycarbonyl 10,11 dihydro-PGA (*Scholkens et al 1975*) which did not appear to display specificity in its actions (*Adaikan et al 1977*). Other members of the prostaglandin family have been modified to produce antagonists. Two examples of these are 11,15-bisdeoxy PGE1 (*Tolman et al 1977*) and N-dimethylamino PGF2α (*Fitzpatrick et al 1978*). The latter compound N-dimethylamino PGF2α was a potent and effective antagonist of PGF2α (*in vitro* and *in vivo*).

Other compounds bearing little structural resemblance to prostaglandins have been reported to antagonize the actions of prostaglandins. Amongst these compounds the dibenzoxapine derivatives and the polyphloretin phosphate esters are the most widely tested.

There are a number of other agents which appear to possess prostaglandin antagonist actions *in vitro*. However these substances have been inadequately investigated to determine the nature of their antagonism. These substances include: morphine (*Jacques 1969; Collier et al 1974*), fenamates (*Collier et al 1968; Sanner 1976*), prostaglandins themselves (*Sanner and Eakins 1976; Manku et al 1978*), ouabain (*Kadar et al 1969; Greenberg et al 1974; Horrobin et al 1977*), progesterone (*Chang 1973*), estradiol (*Gutnecht et al 1972*), quercetin (*Ramaswamy 1971*), lithium (*Mendels and Fraser 1974*), diphenylhydantoin (*Greenberg et al 1974*).

Prostaglandins and vascular smooth muscle

Goldblatt (*1933; 1935*) and *von Euler* (*1935*) described what were perhaps the first effects of extracted prostaglandins on vascular smooth muscle. The latter investigation clearly demonstrated association of the biological

activity with the lipid soluble acid fraction. Since these early studies numerous other studies have confirmed and extended the observation of profound effects of prostaglandins on vascular smooth muscle.

The *in vivo* and *in vitro* studies on the biological actions of prostaglandins show considerable variability depending on the species, vascular tissue, existing tone and the concentrations of prostaglandins used. Prostaglandins may have potentiating, inhibitory, biphasic or no effect on vascular smooth muscle (*Altura and Altura 1976*). They appear to participate in the normal and pathophysiological functioning of the circulatory system and might be extremely important as determinants of vascular tone and reactivity (*McGiff et al 1976; Manku et al 1977; 1978*). These actions are summarized in Tables *4*, *5* and *6*.

Vascular biosynthesis of prostaglandins

Prostaglandin biosynthesis has been reviewed earlier. These substances are synthesized by virtually every tissue in the body including blood vessels. There does not appear to be any evidence for storage of prostaglandins; as a result, their appearance is indicative of *de novo* synthesis in response to stimulation. This can be brought about by a variety of stimuli (see Table *1*) including vasoactive agents such as noradrenaline, angiotensin II and vasopressin. *In vivo* very little prostaglandin can be found in the arterial circulation presumably because of the efficient uptake and degradatory mechanism in the lung. Thus with the possible exception of prostacyclin, it is probable that the prostaglandins are "local" hormones. Very little thromboxane (Tx) A2 is synthesized by vascular smooth muscle (*Tuvemo 1978; Needleman et al 1977*), although thromboxane A2 is the most potent constrictor of vascular smooth muscle formed during arachidonic acid metabolism (*Tuvemo 1978*).

TABLE 4

SOME EFFECTS OF PROSTAGLANDINS IN VASCULAR BEDS

Species	*Bed*	*Prostaglandin (PG)*	*Effect*	*Reference*	
rabbit	pulmonary	E1,E2	dilation	Hauge et al	1967
	kidney	E2,E1,A2	dilation	Malik and McGiff	1976
	coronary	I2	biphasic	Karmazyn et al	1978
rat	mesenteric	F2α	constriction	Manku et al	1977
	mesenteric	E1	dilation	Messina et al	1974
	coronary	I2	biphasic	Karmazyn et al	1978
	kidney	E2	biphasic	Malik	1978
	spleen	TXB2	constriction	Malik	1978
		F2α	constriction	Malik	1978
		E2	dilation	Malik	1978
guinea pig	coronary	A2	constriction	Schror and Krebs	1977
	coronary	I2	biphasic	Dusting et al	1977
cat	skeletal muscle	F2α,I2	dilation	Smith et al	1978
	pancreas	I2	dilation	Smith et al	1978
	stomach	E1,E2,I2	dilation	Smith et al	1978
	pulmonary	E2	dilation	Smith et al	1978
	kidney	E1,E2,F2α,I2	no effect	Smith et al	1978
dog	coronary	I2	dilation	Armstrong et al	1977
	skeletal muscle	I2	dilation	Horri et al	1978
	uterus	E1,E2,A1	dilation	Clark et al	1977
	kidney	F2α	no effect	McGiff and Itskovitz	1976
dog	cerebral	F2α	constriction	White et al	1971
	pulmonary	PGH2,E2,F2α,D2	constriction	Kadowitz and Hyman	1977
				Hyman et al	1977
		F2α	constriction	Fitzpatrick et al	1978
				Wicks et al	1976
		TXB2	constriction	Wasserman and Griffin	1977
	mesenteric	E1,E2,A2	dilation	Lee	1968
		H2	dilation	Dusting et al	1978
				Chapnick et al	1977

TABLE 5

EFFECT OF PROSTAGLANDINS ON RESPONSES TO VASOACTIVE AGENTS

Species	*Tissue*	*Vasoactive Agent*	*Prostaglandin (PG)*	*Effect*	*Reference*	
rabbit	kidney	NA	F2α	potentiation	Malik and McGiff	1975
			E1,E2,A2	inhibition		
	aorta	angio II	A1,E1,E2,F2α	potentiation	Strong and Bohr	1967
		KCl	A1,E1,E2,F2α	potentiation	Strong and Bohr	1967
		angio II	A2	no effect	Lee et al	1965
		NA	A2	no effect	Lee et al	1965
	renal	NA	E1	potentiation	Rioux et al	1978
	mesentery	NA	E1,E2,F2α	inhibition	Malik and McGiff	1976
rat	mesocaecal	NA	E2	inhibition	Viguera and Sunahara	1969
	artery	angio II	E2	no effect		
	crewmaster	NA	E1	inhibition	Kaley and Weiner	1968
		angio II	E1	inhibition	Weiner and Kaley	1969
		vasopressin	E1	inhibition	Messina et al	1974
		5HT	E1	no effect	Messina et al	1974
	mesentery	NA	E1	inhibition	Manku et al	1977
					Malik and McGiff	1975
		angio II	E1	inhibition	Weiner and Kaley	1969
		vasopressin	E1	inhibition	Messina et al	1974
		5HT	E1	inhibition	Messina et al	1974
		NA	A1,A2,E2,F2α	potentiation	Manku et al	1977
					Coupar and McLennan	1978
		NA	I2	inhibition	Ally et al	1978
		NA				
	spleen	NA	TXB2,F2α	potentiation	Malik	1978
		NA	E2	inhibition	Malik	1978
	skeletal muscle	NA	E2	inhibition	Messina et al	1975
cat	spleen	NA	E2	biphasic	Hedqvist and Brudin	1969
	skeletal muscle	NA	E1,E2	inhibition	Chu et al	1974
		angio II	E1,E2	inhibition	Holmes et al	1963
dog	mesentery artery	NA	E1,E2,A2,F2α	potentiation	Greenberg et al	1973
	spleen	NA	E1,E2	no effect	Davies and Withrington	1971
		angio II	E1	no effect		
	hind paw	NA	E1	inhibition	Kadowitz et al	1971
		angio II	E1	inhibition		
		5HT	E1	potentiation		
	saphenous vein	NA	F2α,C2,D2	potentiation	Jeffery et al	1977

TABLE 6

DIRECT EFFECT OF PROSTAGLANDINS ON ISOLATED VASCULAR SMOOTH MUSCLE

Species	*Tissue*	*Prostaglandin (PG)*	*Effect*	*Reference*	
rabbit	mesentery	TXA2	contraction	Bunting et al	1976
	celiac	TXA2	contraction	Bunting et al	1976
	mesentery	I2	relaxation	Bunting et al	1976
	celiac	I2	relaxation	Bunting et al	1976
	aorta	TXA2	contraction	Needleman et al	1976
		E1,E2,A1	contraction	Strong and Bohr	1967
	renal	F2α	contraction	Malik and McGiff	1975
		E1	contraction	Rioux et al	1978
		E1,E2,A2	relaxation	Malik and McGiff	1975
	thoracic	PGH1	contraction	Needleman et al	1977
rat	mesentery	F2α,A2	contraction	Manku et al	1977
	aorta	E1,E2,A1,A2	contraction	Altura and Altura	1976
		F2α	contraction	Altura and Altura	1976
	renal	E1,E2,A2,F2α	contraction	Malik and McGiff	1975
	pancreatic	E1,B1,B2,F2α	contraction	Saunder and Moser	1972a,b
	portal vein	E1,E2,A1,A2,F2α	contraction	Altura and Altura	1976
dog	mesentery	A1,E2,E1,F1α	biphasic	Strong and Bohr	1967
		F2α	no effect	Greenberg et al	1973
	cerebral	F2α	contraction	Pennink et al	1972
human	umbilical	E1	relaxation	Hiller and Karim	1968
		E1,F2α,TXA2	contraction	Tuvemo	1978
		I2	biphasic	Pomerantz et al	1977
bovine	coronary	I2	relaxation	Dusting et al	1977
	cerebral	TXA2	contraction	Ellis et al	1977
swine	coronary	TXA2	contraction	Svensson and Hamberg	1976
		H2	contraction	Svensson et al	1976
sheep	ductus arteriosus	E1,E2,A1,A2	relaxation	Starling et al	1976
	umbilical	F2α	little effect	Novy et al	1974
		E2	constriction	Rankin and Phernetton	1978
		E1	constriction	Rudolph and Hyman	1978

Vascular effects of prostaglandins

Direct actions

Prostaglandins of the E series generally tend to decrease blood pressure whereas the F type prostaglandins either decrease or increase blood pressure depending on the species studied (*Horton 1969; Altura and Altura 1976*). Prostaglandins appear to affect vascular smooth muscle at sites not susceptible to blockade by adrenergic, cholinergic, antihistaminic or ganglionic blocking drugs but sensitive to agents such as 7-oxa prostynoic acid, polyphoretin phosphate and SC19220 (*Nakano et al 1971; Adaikan and Karim 1974; Park and Dyer 1972*).

Prostaglandins of the E type decrease peripheral vascular resistance in almost every vascular bed studied, with PGE1 generally being more potent than PGE2 (*Malik, McGiff 1976; Nakano 1973*). One notable exception is the pulmonary vascular bed which is constricted by PGE2 (*Kadowitz et al 1975; Mathe et al 1977*). Direct split screen microscopy measurement of exteriorized rat mesocaecal circulation have shown that E type prostaglandins dilate the arterioles (*Messina et al 1974*).

F type prostaglandins cause marked increase in pulmonary vascular resistance (*Kadowitz and Hyman 1977; Fitzpatrick et al 1978*) and have also been shown to have venoconstrictor action in the same vascular bed (*Ducharme et al 1968*), whereas PGF2 caused dilation in skeletal muscle at concentrations lower than those used in the above studies(*Smith et al 1978*). In the perfused spleen, PGF2α caused constriction of the renal vasculature whereas PGE1 and PGE2 caused vasodilation at the same concentration (*Malik 1978*). Although the enzyme 9 keto reductase is present in many rat tissues (*Leslie and Levine 1973*), conversion of PGE2 to PGF2α during perfusion of the spleen was insufficient to produce a constriction. These actions of

prostaglandins are thought to be the result of a direct action on the resistance vessels (*Malik and McGiff 1976*). *Samovo (1972)* demonstrated that PGE1 and PGE2 inhibited pressor responses to infusions of angiotensin II, vasopressin and noradrenaline in the spinal cat, revealing the direct actions at the arteriolar level.

Though the net effect of prostaglandins on selected vascular beds and mean blood pressure are known, little is known of prostaglandin action on total systemic blood flow distribution. In a recent study *Smith et al (1978)* examined the effects of PGE1, PGE2, PGF2α and PGI2 on blood flow *in vivo* to heart, lung, kidney, adrenal, skeletal muscle, stomach, jejunum, pancreas, liver and spleen. They found that PGE1 significantly increased gastric blood flow, PGE2 increased cardiac output, lung and gastric blood flow and PGF2α increased skeletal muscle and gastric blood flow. PGI2 increased cardiac output, skeletal muscle, gastric, jejunal and pancreatic blood flow. None of the prostaglandins increased or decreased blood flow to the liver. These data clearly demonstrated that *in vivo* the same prostaglandin exerts a differential vasodilatory action on vascular beds. The reduced systemic blood pressure seen was not a result of global vasodilation. The only prostaglandin showing consistent vasodilation was PGI2. Further eivdence for the potent vasodilatory action of PGI2 is its potent antihypertensive actions in acute (noradrenaline and angiotensin infusion) hypertension and that caused by the release of renal occlusion (*Scholkens 1978*).

Thromboxane A2, the proaggregatory metabolite of prostaglandin endoperoxides in thrombocytes, contracts all blood vessels (*Tuvemo 1978*). It is not presently known if it affects microvessel blood flow *in vivo*, nor if it is released by blood vessels other than the umbilical artery (*Tuvemo 1978*).

Recently *Malik (1978)* showed that in the perfused spleen TxB2 (the stable metabolite of TxA2) potentiated vasoconstrictor responses to injected noradrenaline and sympathetic nerve stimulation and increased the basal perfusion pressure. PGE2 infused in a similar manner produced opposite effects and was inhibited by simultaneous infusion of TxB2. On a mass basis 200 times more TxB2 was required to inhibit PGE2 actions (see Tables 4 and 5).

Indirect effect

Exogenously administered prostaglandins have the ability to act directly on vascular smooth muscle or to potentiate or inhibit the actions of vasoactive substances (*Altura and Altura 1976;* see Table 5). In the rat mesentery or cremaster muscle (*in vivo*, *Messina et al 1974*) and rat spleen (*in vitro*, *Malik 1978*) PGE1 depressed arteriolar responses to a number of vasoconstrictors including epinephrine and norepinephrine. In the rat mesentery (*in vitro*) PGE1, PGE2, PGF2α, PGA1, PGA2 potentiated vasoconstriction to noradrenaline and adrenergic nerve stimulation (*Coupar and Mclennan 1978; Manku and Horrobin 1977; Malik and McGiff 1976*) whereas in the rabbit mesentery (*in vitro*) vascular responsiveness to these stimuli were attenuated by these prostaglandins (*Malik and McGiff 1976*).

Prostaglandins can exhibit biphasic effects on vascular smooth muscle; *Strong and Bohr (1967)* observed that low concentrations of prostaglandins relaxed and high concentrations contracted renal and mesenteric arteries. Recently *Pomerantz et al (1977)* reported a similar biphasic effect of PGI2 on umbilical artery (*in vitro*). In the *in vitro* perfused heart *Dusting et al (1977)* and *Karmazyn et al (1978)* observed that low concentrations of PGI2 increased vascular tone and high concentrations caused vasodilation. In the perfused mesenteric vascular bed (*in vitro*) PGE1 displayed a biphasic

effect on vascular reactivity to noradrenaline, low concentrations potentiating and high concentrations inhibiting pressor responses (*Manku et al 1977*). PGI2 on the other hand produced a monophasic depression of noradrenaline responses in a similar preparation (*Ally et al 1978;* see Table 5).

The discovery (*Smith and Willis 1971; Vane 1971*) and the development of inhibitors of prostaglandin synthesis such as indomethacin and aspirin facilitated studies on the role of endogenous prostaglandins on vascular reactivity and the regulation of vascular tone. In isolated perfused vascular beds indomethacin either inhibited or potentiated vascular responsiveness to vasoconstrictory agents. In the perfused rat mesenteric bed (*Manku and Horrobin 1976*) and perfused rat spleen (*Malik 1978*), indomethacin produced opposite effects on noradrenaline responses, potentiation in the spleen and inhibition in the mesenteric bed. Inhibitors of prostaglandin synthesis (indomethacin and 5,8,11,14 eicosatetraynoic acid, ETA) superfused on the rat cremaster muscle enhanced the vasoconstrictor responses of arterioles to locally infiltrated angiotensin and norepinephrine (*Messina et al 1975*). In the rat mesenteric vascular bed indomethacin inhibited responses to both these vasoconstrictors (*Manku et al 1976*). Thus within one specie there are dramatic differences in the contribution of endogenous prostaglandins to vascular reactivity. An additional complication is that differences exist between species *e.g.* indomethacin which inhibits vascular reactivity in rat mesentery has the opposite effect in rabbit mesentery (*Malik et al 1976*). *In vivo* prostaglandin synthesis inhibitors do not appear to alter arteriolar diameter in vascular beds (*Hintze and Kaley 1977; Altura 1978; Messina et al 1977*). However the use of anaesthetics in these studies might be a contributing factor because if the arterioles were maximally dilated, no further dilation would be possible.

Vascular smooth muscle (VSM)

VSM display a heterogeneity of ultrastructural, physiological and pharmacological properties. This mosaic depends on the species and the part of the vasculature examined. Much of what is known of the properties of VSM has been obtained using easily obtained vessels such as the aorta and other large arteries (*Somlyo and Somlyo 1976*).

Types of smooth muscle

The classical division of vascular smooth muscle is into two groups, 'multi-unit' and 'single-unit' smooth muscle. The 'multi-unit' smooth muscle consists of functionally independent cells with a paucity of cell to cell contacts between them. In contrast 'single-unit' smooth muscle is thought to have close opposition of the adjacent cell membranes. These are termed nexi and are presumed to form the structural links for cell to cell propagation of electrical impulses and for the inherent rhythmicity within the tissue (*Somlyo and Somlyo 1968*). Most large blood vessels behave, under physiological conditions as 'multi-unit' systems whereas in the micro-circulation the precapillary resistance vessels (meta-arterioles) are classified as 'single-unit' smooth muscle. On the other hand, some smooth muscle exhibiting rhythmicity have no morphological evidence of cell to cell contacts (*Henderson et al 1971*).

Other differences do exist between these two types of smooth muscle. In rhythmic smooth muscle, 'single-unit', the contractions are triggered by a fast spike depolarization (cf action potential) usually superimposed on a rhythmic oscillation in membrane potential. In contrast 'multi-unit' smooth muscle display a stable membrane potential and contracts in response to depolarizing stimuli or neurogenic or other vasoactive substances.

General arrangement of smooth muscle cells

Smooth muscle cells have a variable longitudinal dimension. In large vessels *e.g.* aorta, smooth muscle cells are over 100 μm long and about 3-5 μm wide, whereas in the smallest arterioles, they are about 40 μm long and about 3-5 μm wide (*Somlyo and Somlyo 1970,1976*). In large blood vessels there is a large amount of elastic tissue interspersed amongst the circularly oriented smooth muscle. The presence of collagen fibers, in addition to the elastic tissue, protect the mechanical integrity of these blood vessels. The amount of both elastin and collagen decrease with the size of the arteries and is very sparse in the arterioles (*Devine 1978*).

The smooth muscle cell itself is very similar to other cells in that it possesses a nucleus, numerous mitochondria, golgi apparatus, microtubules and sarcoplasmic reticulum.

Ultrastructure

Plasma membrane

Electron microscopy of sections and fractions of the plasma membrane show numerous small invaginations of the cell membrane. The invaginations have been given many names *e.g.* pinocytotic vesicles, microvesicles, surface vesicles (*Somlyo and Somlyo 1968,1970*). The actual function of these surface vesicles is unknown, however mitochondria and sarcoplasmic reticulum are often seen in close proximity to the surface vesicles (*Somlyo and Somlyo 1976; Somlyo et al 1972; Devine et al 1972*). It is possible that these vesicles increase the surface area of the cell thus facilitating diffusion of various substances, in addition to providing binding sites for various cations.

The distance separating cell membranes in the small mesenteric arteries of the rat is of the order of 10 nm, whereas in the rabbit pulmonary artery the distance is 2-3 nm (*Devine 1978*). The muscle cells have a well defined, easily visualized, basement membrane (*Somlyo and Somlyo 1968*). Beyond the basement membrane collagen fibers are found in an apparently random manner among the muscle cells. In the larger blood vessels elastic tissue is associated with the collagen in the intercellular matrix.

Sarcoplasmic reticulum (SR)

This term is used to describe the intracellular tubular system forming conduits with invaginations of the striated muscle cell membrane. This term is used in smooth muscle terminology to describe the diffuse SR normally seen. In many cases smooth muscle SR is associated with microsomal ribosomes and is termed rough SR as opposed to smooth SR (no associated microsomes). The volume of the smooth muscle cell occupied by the SR ranges from 2% to 6% of the cell volume (*Devine 1978*). The SR does not open directly to the extracellular fluid, but forms close relationships with the surface vesicles in the plasma membrane (*Devine et al 1972*).

Role of SR in muscle contractility

In striated muscle the SR is presumed to be the site of activator calcium involved in excitation contraction coupling (*Fabiato and Fabiato 1977; Sandow 1970*). The role of the SR in some smooth muscle appears to be of a similar nature (*Somlyo and Somlyo 1970*). The ability of the smooth muscle SR to accumulate calcium has been demonstrated by *Somlyo and Somlyo (1971)*. These workers noted mitochondrial uptake of calcium perhaps implicating them in vascular contractility. These results have been confirmed with microsomal fractions from rabbit aorta (*Fitzpatrick et al 1972;*

Sands et al 1977) and bovine aorta (*Sands et al 1977*). In a related study *Yamashita et al (1976)* examined calcium uptake, release and Mg ATPase activity of SR from arterial smooth muscle. They found SR uptake of calcium ions was ATP dependent, required the presence of magnesium and was readily released into calcium free buffer. Interestingly the mitochondrial poison, azide, inhibited the SR Mg ATPase enzyme but not its calcium accumulating activity. The optimal conditions for calcium release were the presence of ATP and magnesium at physiological pH. It is therefore possible that SR in aorta and other vascular smooth muscle play an important role in the regulation of muscle activity in association with other intracellular organelles such as the mitochondria (*Vallières et al 1975*).

Contractile proteins in vascular smooth muscle

As in skeletal muscle the contractile machinery of the smooth muscle is made up of thick and thin filaments. The myosin (thick) and actin (thin) filaments extracted from vascular smooth muscle will form a contractile aggregate *in vitro* upon the addition of ATP. *In vitro* this rate of formation of the actin-myosin complex is slower than that with skeletal muscle actin and myosin (*Sparrow et al 1970*). This could be related to a correspondingly slower ATPase activity of the contractile proteins similar to the slow ATPase rate of aortic SR (*Yamashita et al 1976*).

Characteristics of the contractile proteins

Vascular actin is indistinguishable from skeletal actin, both in amino acid composition (*Gosselin-Reye et al 1969*) and its ability to activate skeletal myosin (*Schirmer 1965*). In contrast arterial myosin appears to be structurally quite distinct from skeletal myosin but similar to myosin from other smooth muscles (*Hamoir 1973*). The ATPase activity of vascular

contractile proteins has been localized to S_1 subfragments of the myosin molecule. These subfragments are presumably the points of attachment in the formation of actin and myosin cross bridges (see *Paul and Ruegg 1978* for detailed biochemical explanation of these events).

Activation of actomyosin system

The requirement for ionized calcium by vascular smooth muscle is best demonstrated in leaky smooth muscle cells. The plasma membrane is rendered permeable to calcium by glycerination or chelation of membrane calcium. Porcine carotid arteries prepared by these procedures contract in response to added ATP only if the free ionized calcium available is greater than 10^{-7} M, with maximal activation at 10^{-5} M (*Paul and Ruegg 1978*). Recently *Ashley et al (1978)* using an intracellular calcium sensitive electrode determined that the resting intracellular (cytoplasmic) fluid ionized calcium was 10^{-7} M and rose to 10^{-5} M during maximal contractile responses of skeletal muscle fibres. The close agreement between these data strongly suggest that the calcium requirements of smooth and skeletal muscle are similar.

Calcium sensitive regulatory site

In skeletal muscle the site of calcium interaction is at the troponin-tropomyosin level (*Fabiato and Fabiato 1977*). The picture is not quite as clear with regards to vascular smooth muscle. *Drake* and *Hartshorne (1975)* suggested that the calcium regulatory site was located on the actin filaments and possibly linked to a troponin like substance. To date no direct evidence for the presence of troponin in vascular smooth muscle has been documented (*Sparrow and Brocksmer 1972; Paul and Ruegg 1978*). Evidence for a myosin linked calcium regulated site has been presented by *Mrwa* and *Ruegg (1975)*. This site has been localized to the light molecular

weight chains of myosin. In the absence of calcium ions the interaction of actin and myosin is unlikely, the binding of calcium to the myosin molecule appears to be crucial for actomyosin complex formation.

In skinned smooth muscle (glycerol or EDTA treated), calcium antagonists lack any inhibitory effect when the contractile system was directly activated by ATP and calcium (*Weder and Grim 1973*). These data indicate that the calcium sensitive site(s) differ from more peripheral calcium binding sites. In a comparative study using skinned skeletal, cardiac and smooth muscle, it was established that calcium sensitivity differed between muscle types (*Nakahata 1978*).

A model for smooth muscle contraction was proposed by *Hewman (1971)*. He suggested that the thick and thin filaments were arranged in parallel bundles. The thin filaments (attached at one end to a dense body) presumably slide and interdigitate between the thick filaments during a contraction. Some morphological evidence for this arrangement was provided by *Crooke* and *Fay (1972a,b)*.

Mechanism(s) of relaxation

Contraction is associated with an increase in ionized calcium; conversely the obvious mechanism for initiating relaxation is to either decrease cytosolic calcium or alternatively decrease the sensitivity of the contractile proteins to calcium (*Somlyo and Somlyo 1970*). In terms of simply calcium movement, relaxation can be effected by inhibition of calcium influx, resequestration of cytosolic calcium and/or extrusion of ionized calcium across the plasma membrane.

It has been suggested by *Anderson (1972)* that increased levels of cyclic AMP are responsible for relaxation in smooth muscle. Use of phosphodiesterase inhibitors in various preparations have provided indirect support for this suggestion (*Poch and Kukovetz 1972; Ferrari 1974*). In studies in which cAMP levels were measured and correlations made with the time course of relaxation no such relationship could be substantiated in both vascular and nonvascular smooth muscle (*Herlihy et al 1976; Ally 1976; Collins and Sutter 1975; Inatomi et al 1975; Diamond and Hartle 1974; Diamond and Holmes 1974*). In an earlier study by *Al Tai and Graham (1972)*, theophylline and papaverine (in concentrations which inhibit cyclic AMP phosphodiesterase) antagonized rather than enhanced PGE induced relaxation of perfused rabbit ear artery. Recently *Schror et al (1979)* reported that PGI2 relaxation of coronary blood vessels (rat and bovine) was associated with a significant decrease in intracellular cyclic AMP levels. In another model of excitation — contraction coupling, made up of a homogeneous cell population (platelets) *Wang et al (1978)* measured cyclic AMP levels, adenylate cyclase activity and phosphodiesterase activity in response to aggregating stimuli and inhibitors. These workers concluded that no correlation could be found between either the activation or the inhibit of platelet aggregation and levels of cyclic AMP.

METHODS AND MATERIALS

Male Wistar strain rats (180-220 g) were housed in group wire cages at the Clinical Research Institute of Montreal, animal care services where light was regulated into 12 hours on, 12 hours off. Food (Purina Laboratory Rat Chow) and water were allowed *ad libitum*. These animals were used for all pharmacological studies. In experiments in which prostaglandin synthesis was determined, Wistar rats (150-320 g) similarly housed at the animal care facilities of the University of Freiburg, West Germany, were used.

Preparation of the perfused mesenteric vascular bed

The rats were anaesthetized with ether and the abdominal cavity exposed. The superior mesenteric artery was cannulated (P-50 or P-60 cannula) and perfused using a Watson-Marlow flow inducer with a modified Krebs-Henseleit buffer of the following composition: mM: 150 sodium, 4.3 potassium, 1.0 magnesium, 2.5 calcium, 0.85 phosphate, 25 bicarbonate, remaining anion chloride, 5.5 glucose, bubbled with a mixture of 95% oxygen and 5% carbon dioxide, final pH 7.4 at a constant temperature of 30°C. The ileal artery was ligated and the vascular bed dissected out along its margin with the intestine. The external surface of the vascular bed was washed clean with buffer and transferred to a PT-10 (Canlab Montreal) flow stream divider and suspended in a jacketed muscle bath (30°C). Perfusion pressure was recorded via a side arm off the stream divider using a Bell and Howell blood pressure transducer (sensitivity 0-250 mm Hg, 1 mm Hg = 133.3 Pascals). The signals from the transducer were recorded on a thermal paper recorder (Devices, England) calibrated for a 100 mm Hg full scale

deflection, with an input of 2.5 mVolts. Flow rate was adjusted to give a baseline perfusion pressure of 25-30 mm Hg. Using preparations from 180-220 g rats, this was found to be 3 ml/min. A sample trace showing changes in perfusion pressure during the dissection is shown in Fig. 7 (flow rate 3 ml/min, 180 g ♂ rat). The segiments of the tracing are: (a) the *in situ* perfusion pressure (23.3 ± 2.0 mm Hg, n = 10, M ± SD) upon cannulation of the artery, (b) *in situ* perfusion pressure upon transection of the ileal artery (26.66 ± 2.1 mm Hg, n = 6, (c) *in situ* perfusion pressure upon ligation of the ileal artery (28.17 ± 2.4 mm Hg, n = 6), (d) *in vitro* perfusion pressure after severing the small arteries to the gut (27.16 ± 3.7 mm Hg, n = 6). Any alteration in the procedures for the isolation of the vascular bed, for a particular experiment, is indicated in the appropriate section of the results.

After an equilibration period of 1 hour, vascular reactivity was tested by an intra-arterial injection of a pressor agent (100 µl bolus). In this study pressor substances used were, noradrenaline bitartrate (1 µg/100 µl) and potassium ions (43×10^{-3} moles). These pressor agents caused a transient vasoconstriction and was recorded as an increase in perfusion pressure (see Fig. 29 for an example of noradrenaline and potassium contractions).

At the start of each experiment 4 test injections of the appropriate agent were administered at 5 minute intervals while the preparation was being perfused with buffer; the mean of these responses was taken as 100%. The substance (synthetic or natural) to be investigated was then added to the buffer reservoir and the effects on the response to the same dose of pressor agent were investigated. Each substance was investigated over a range of concentrations added cumulatively to the buffer reservoir, unless

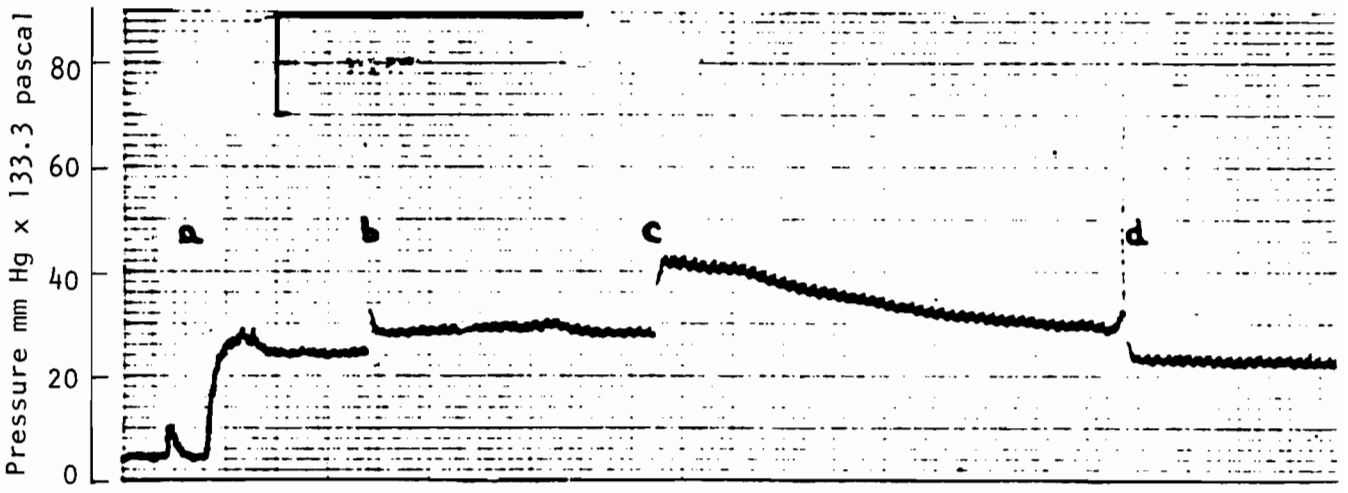


Fig. 7. Pressure recording of changes in rat mesenteric vascular resistance during a typical dissection and preparation of the vascular bed.

a Cannulation of the mesenteric artery;

b Section of the ileal-colon artery;

c Ligature of ileal-colon artery;

d Dissection of vasculature from border with gut.

Flow rate 3 ml/min, rat ♂ 180 g, horizontal bar indicates 5 min., vertical bar 20 mm Hg (1 mm Hg = 133.3 Pa).

noted otherwise in the results. The pressor amplitude (mm Hg) concentration response curves to intra-arterial injections of noradrenaline and potassium ions are shown in Fig. 8.

Expression of experimental data

Inhibition or potentiation of pressor responses to a particular pressor agent are expressed as a percentage of the mean pressor response amplitude (mm Hg) obtained prior to exposure to the test substance. The normal pressor response amplitude to bolus injections of noradrenaline (3×10^{-9} moles) was 41.5 ± 2 mm Hg (M ± SEM, n = 25) and that to potassium ions (43×10^{-3} moles) was 62.5 ± 3 mm Hg (M ± SEM, n = 28). Responses were pooled and the data used to construct log dose-response lines. Ordinate: responses as a % of control; Abscissa: concentration of the compound in the buffer. In some cases graphical analyses of the data by the method of *Hofstee (1952)* and *Dowd and Riggs (1965)* was carried out.

Periarterial nerve stimulation

In some experiments the adrenergic nerves innervating the arteries of the mesenteric vascular bed were stimulated. Platinum electrodes were placed 2-4 mm below the tip of the cannula and the nerves stimulated at supramaximal voltage with biphasic rectangular pulses, 0.5 msec duration, 10 Hz, for 15-25 seconds every 5 minutes, using a Grass SDII stimulator.

Statistical analysis

Statistical evaluation was performed using a single classification analysis of variance (ANOVA) for unequal sample size (*Sokal and Rohlf 1966*) allowing comparison of corresponding points in dose response curves. The homogeneity of variance was determined prior to each ANOVA by the F max test

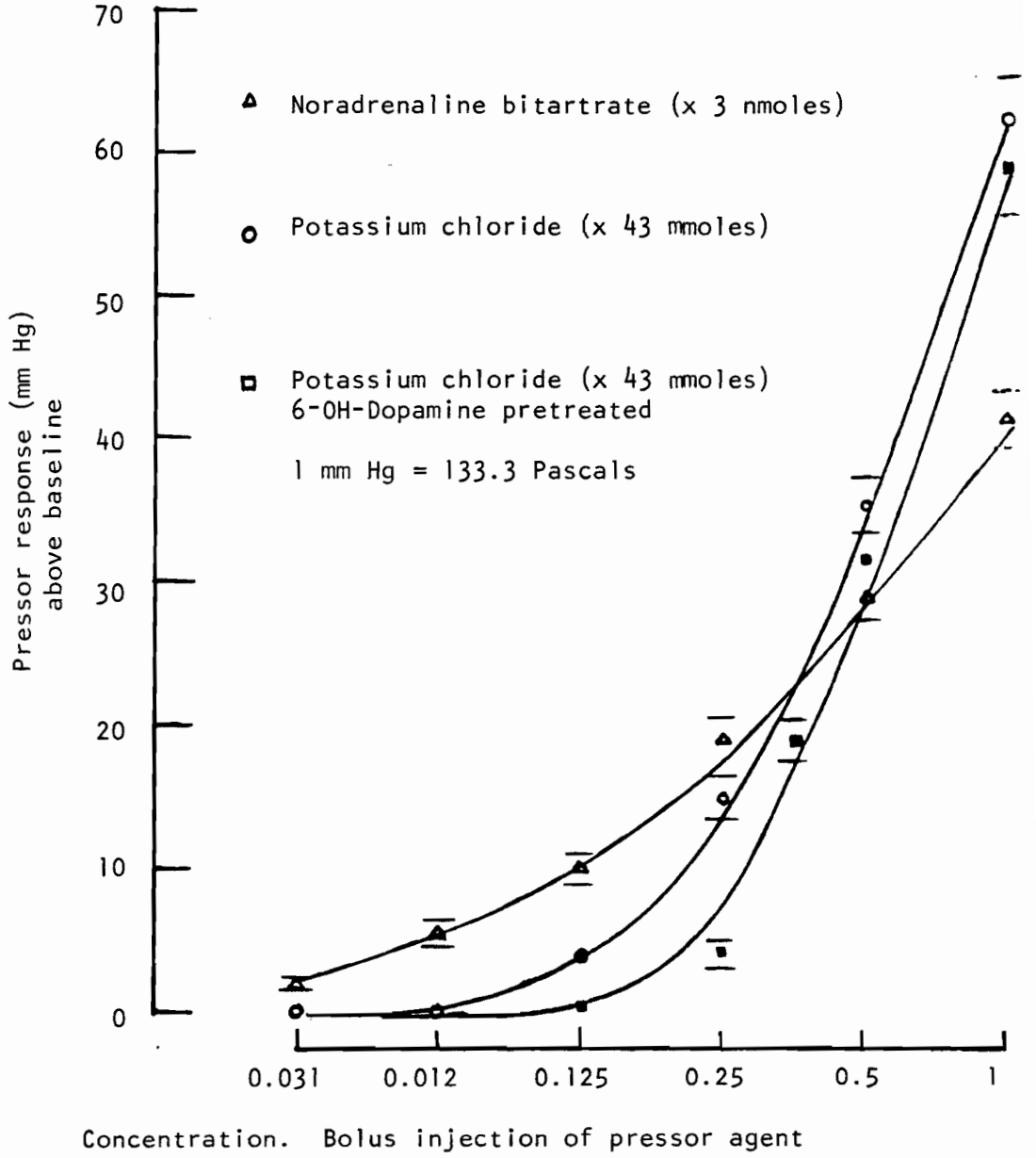


Fig. 8. The effect of noradrenaline and potassium ions injected intra-arterially as a 100 µl bolus into the isolated perfused rat mesenteric vascular bed. Δ noradrenaline, ○ K^+, □ K^+ (6 hydroxy-dopamine pretreated, 50 mg/kg IP 2 days).

Ordinate: pressor responses mm Hg (1 mm Hg = 133 Pa).

Abscissa: Amount of noradrenaline or potassium ions injected as a 100 µl bolus.

Each point represents the mean ± SE for six experiments. (1 mm Hg = 133.3 Pascals, Flow rate = 3 mls/min).

(*Sokal and Rohlf 1966*). The computation of the ANOVA was routinely carried out using a Hewlett-Packard programmable calculator and a program from the same source. The reliability of the program was checked using sample data from *Sokal* and *Rohlf (1966)*. A p value < 0.05 was considered significant.

Other preparations used

Rat Fundic Strip: Male Wistar rats were anaesthetized with ether, the abdomen opened and the grey fundal part of the stomach cut away, irrigated with buffer (composition as above), cut into strips and mounted in a 50 ml muscle bath. Complete details are given in the appropriate results section. The fundic strip was contracted with PGE2 (10 ng/ml, 2.8×10^{-8} M). Dose response lines for papaverine were constructed using several concentrations of this drug.

Raji cells: These experiments were carried out by Dr. R.A. *Karmali*. Briefly, an established lymphoblastoid line of Raji cells (*Menezes et al 1976*) were suspended in 5 ml of growth medium RPMI 1640 + 10% heat inactivated fetal calf serum; 100 U/ml penicillin, 100 µg/ml streptomycin, 10 µg/ml garamycin, 0.25 µg fungizone) at an initial concentration of 2×10^5 cells/ml. The media contained either no adenosine or 1×10^{-4} or 2×10^{-4} M adenosine. At each adenosine concentration incubations were carried out in the presence of PGE2 (0.1, 10, 10^2, 10^3, 10^4 pg/ml; 2.8×10^{-12} to 2.8×10^{-8} M). 8 sets of each culture were prepared and incubated at 37° C for 72 hours. All cultures were harvested at the same time and numbers of cells/ml counted using a Coulter cell counter.

Arterial rings: Briefly, the mesenteric artery was cannulated as described above and perfused continuously. Using a pair of fine blunt forceps the tissue adherent to the blood vessels of the mesenteric vascular

bed was stripped away. Once this had been completed the mesenteric artery and its attendant blood vessels were transferred to a vial containing pre-chilled buffer ($4^{o}C$). From these arteries, rings were prepared and used in experiments to determine the effect of noradrenaline and various drugs on prostaglandin biosynthesis. Complete details are given in the appropriate results section.

Prostaglandin assay

Theory: The assay for prostaglandins is based on competition between a fixed quantity of radiolabelled prostaglandin (^{3}H PG) and varying amounts of unlabelled prostaglandin for binding sites on rabbit antibodies raised against a specific prostaglandin. Thus there is an inverse relationship between the amount of labelled prostaglandin-antibody complex and the amount of unlabelled prostaglandin present in the assay mixture. The quantity of unlabelled prostaglandin in the sample was estimated by determining the antibody bound radioactivity. Separation of the antibody bound prostaglandin from the unbound prostaglandins was accomplished by charcoal adsorbtion. After centrifugation, the supernatant was decanted and the amount of radio-active prostaglandin bound to the antibody determined by liquid scintillation spectrometry. The concentration of unlabelled prostaglandin in the sample was calculated from a standard curve for the particular radioimmunoassay.

Assay materials

1. Assay buffer. The buffer was prepared by combining 16.2 ml of 0.5 M Na_2HPO_4, 3.8 ml of 0.5 M NaH_2PO_4 and 100 ml of 1.5 M NaCl stock solutions. The mixture was then diluted to 1 L with double distilled water and 1 g of gelatin added. This phosphate-buffered-saline mixture was stored at 4^oC.

2. Charcoal slurry. This was prepared by adding 10 g of charcoal to 0.5 L of assay buffer prepared as described above but omitting the gelatin. The slurry was prepared at least 24 hours before its use, and was also stored at 4^oC.

3. Radio-labelled prostaglandins. ^3H-TxB2 was prepared and purified as described by *Granstrom et al (1976)* using washed human platelets as enzyme source and 5,6,8,9,11,12,14,15-^3H-arachidonic acid (New England Nuclear Co., specific activity 64 Ci/mmole) as substrate*. ^3H PGE2 was bought from the same source; ^3H-6-keto PGF1α was prepared from labelled arachidonic acid using vascular smooth muscle and purified by thin layer chromatography*.

4. Unlabelled prostaglandins, TxB2, PGE2, 6-keto-PGF1α.

5. Anti-sera to TxB2, PGE2 and 6-keto-PGF1α.

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* Prepared by technical staff in laboratories of Drs. B.A. *Peskar* and B.M. *Peskar*. Rabbits were immunized with prostaglandin-albumin conjugate. Antisera were harvested as described by *Anhut et al (1977)*.

Prostaglandin assay

Each standard binding reaction was conducted in a total volume of 2.0 ml. This was equilibrated overnight at 4°C. The components of the incubation mixture for a TxB2 assay were added to a 14 x 100 mm plastic disposable test tube in the following order:

1. 1.4 ml of assay buffer containing ^{3}H prostaglandin of the desired CPM.

2. 0.5 ml of either perfusate, incubate, unlabelled prostaglandin or assay buffer.

3. 0.1 ml of an appropriate titer of anti-sera which bound 40-50% of ^{3}H prostaglandin in the absence of unlabelled prostaglandins.

Upon the addition of the antisera the test tubes were each vortexed for 20 seconds, thoroughly mixing the contents. The mixture was allowed to equilibrate overnight at 4°C. A group of no more than 20 tubes were selected and 0.2 ml of the charcoal slurry (see assay materials) was added and mixed immediately. Within 2-3 min the test tubes were placed in the centrifuge and centrifuged at 3,000 RPM (Sorval RC-3, swinging bucket rotor, 4°C) for 10 min. The supernatant was decanted into a 10 ml plastic scintillation counting vial containing 7.0 ml of Scintigel(R) cocktail. The vial was capped and the contents thoroughly mixed. The radioactivity present was counted in a liquid scintillation spectrometer. Assay blanks were prepared as described but omitting the 0.1 ml of antisera, substituting buffer or buffer containing the drug being used in its place. This blank value was used to correct all raw counts per minute (CPM) obtained in the assay. For 6-keto-PGF1α assays a smaller volume of sample (0.1 or 0.2 ml) was used and the volume of assay buffer adjusted to compensate, ensuring that the final incubation volume remained a constant 2 mls.

Specificity of antisera

The 6-keto-PGF1α and PGE2 antisera showed negligible crossreactivity with most prostaglandins. In contrast the TxB2 antisera showed significant cross reactivity with PGD2 and negligible cross reactivity with the other prostaglandins tested (*Anhut et al 1977*).

Calculation of assay results

The concentration of prostaglandin in each sample or unknown was calculated using a programmable desk top calculator (Hewlett Packard). The program used was written by Dr. B.A. *Peskar*, the data for the standard curve was processed to generate an equation describing a logit plot for the percentage inhibition of ^{3}H prostaglandin (TxB2, PGE2 or 6-keto-PGF1α) to the antisera versus the logarithm of the quantity (pg) of standard (unlabelled TxB2, PGE2 or 6-keto-PGF1α). The line of best fit (least squares method of regression analysis) for the standard curve (now transformed to a line by the transformation of the data to logit plot) allows the rapid calculation of the amount of prostaglandin present in each unknown. The corrected counts per minute (raw counts per minute — blank counts per minute = corrected counts per minute [CPM]) for each unknown was calculated and was then expressed as % inhibition of binding of ^{3}H PG to the antisera. This latter value was then substituted into the equation describing the standard curve (line), the solution of which yielded the amount of prostaglandin present in the aliquot of unknown. Each standard curve was determined using 15 concentrations of either TxB2, PGE2 or 6-keto-PGF1α.

The program automatically rejected all values which were less than the 10% confidence limit of the standard curve. This limit is the smallest amount of prostaglandin which can be reliably determined by the radioimmuno-

assay. An alternative way of terming this is "the limit of sensitivity". The upper (90%) and lower (10%) confidence limits for the three radioimmunoassays are shown in Table 8.

Table 7 Confidence limits (pg) of the TXB2, PGE2 and 6-keto-PGF1α radioimmunoassays

PROSTAGLANDIN	CONFIDENCE LIMIT (pg)		ANTISERA
	10%	90%	Lot
TXB2	26	1440	146-4
PGE2	7.9	299	32-7
6-keto-PGF1α	132	3650	176-5

Expression of data

Perfusate: Prostaglandin concentrations in the perfusates were expressed as picogram (pg, 10^{-12} g) per milliliter (pg/ml). To convert the release of prostaglandins to pg/min multiply the values shown as pg/ml by a factor of 3 [*i.e.* pg/ml x 3 ml/min (flow rate) = pg/min].

Incubates: The amount of prostaglandin synthesized during the 10 minute incubation period of the arterial rings were standardized by converting the values obtained per incubate to nanogram (10^{-9} g) prostaglandin per gram tissue (wet weight) per 10 minute (ng x g^{-1} x 10 min^{-1}). This conversion or standardization facilitated comparison of different experiments.

Thin layer chromatography (TLC)

Perfusate: For TLC, 2 x 30 mls of perfusate from vascular beds (350 g ♂) was acidified to pH 3.2 with 0.1 N HCl and extracted twice with 2 volumes of ethyl acetate. After separation and centrifugation, the ethyl acetate phases were combined, evaporated (rotary evaporator 40°C), the residues (unknown) solubilized with 1 ml ethanol and transferred to a small vial and the volume was reduced to 100µl ; an aliquot was spotted onto a TLC plate (Merck, Kieselgel 60, Darmstadt FRG) along with authentic TXB2, PGE2, PGF2α, 6-keto-PGF1α and PGD2 in parallel channels. After development the marker spots were visualized by spraying with phosphomolybdate reagent (Merk FRG) and heating in an oven (120°C) for 1 minute. The sample channel was then divided into thirteen (1 cm) zones from origin to solvent front. The silica gel in each 1 cm section was scraped off the plate and the prostaglandins eluted overnight into 1.0 ml of methanol. The methanol was decanted, evaporated and the residue redissolved in 1.0 ml of assay buffer. Aliquots of this were analyzed in the radioimmunoassay for TXB2. Two different solvent systems were used for thin layer chromatographic separation of prostaglandins; these were: diethyl ether: methanol: acetic acid (180:1:2, volume:volume), and ethylacetate:ethanol:acetic acid (100:1:1, volume:volume).

Arterial rings

Approximately 2.4 ml of arterial ring incubates (0.3 ml x 8 rats) was acidified to pH 3.3 with 0.1 N HCl and extracted with 10 ml of ethyl acetate. The extract was then processed as described above for extracts of perfusates. The zones of the TLC plate was assayed for TxB2 like activity in the TxB2 radioimmunoassay. The TLC plate was developed in the solvent system ethyl acetate:ethanol:acetic acid (100:1:1, volume:volume).

Materials

Drugs were obtained from the following sources:

Aldrich Chemical Company, Montreal - aminophylline, 1-benzylimidazole, 1-benzylinosine, 2'deoxyadenosine, 3'deoxyadenosine, imidazole-4-acetic acid, isobutylmethylxanthine;

Boehringer-Ingleheim, Montreal - dipyridamole;

Eli Lilly and Company, Toronto - A23187;

Hoffman-La Roche, Montreal - verapamil, X537A;

Linz Pharmaceutical, Austria - hexobendine;

Nelson Research Company, California - N0164;

Norwich Pharmaceutical, New York - dantrolene;

Rougier Incorporated, Montreal - diprophylline;

Sigma Chemical Company, St Louis - adenine, adenosine, 5'adenosine monophosphate, 5'adenosine diphosphate, 5'adenosine monophosphate, 2-chloroadenosine, 3',5'-cyclic adenosine monophosphate, $N^6,O^{2'}$-dibutyryl-3',5'-cyclic adenosine monophosphate, 6-N-methyladenosine, angiotensin II, arachidonic acid, caffeine, dopamine, dopamine sulphate, ethylenediamine tetraacetic acid, guanosine, 5'guanosine monophosphate, hydaralazine, 6-hydroxydopamine, imidazole, indomethacin, inosine, mefenamic acid, nicotinic acid, noradrenaline bitartrate, noradrenaline HCl, papaverine, phenoxybenzamine, phentolamine, reserpine, theobromine. theophylline, tyramine, 8-chloroxanthine, hypoxanthine, xanthine;

Upjohn Company, Kalamazoo - prostaglandin (PG) A1, PGA2, PGB2, PGD2, PGF1α, PGF2α, 6-keto-PGF1α, PGI2, TxB2, 16,16-dimethyl-PGE2;

All other chemicals were reagent grade from various sources.

The following compounds were gifts from individuals:

lidoflazine - Ms V. Bartlett, Queens University, Kingston;

theophylline-7-acetic acid - Dr. K. Nakatsu, Queens University, Kingston;

9,11,azo-prosta-5,13-dienoic acid - Dr. B. Peskar, University of Freiburg.

SECTION 1: PROSTAGLANDINS

RESULTS

Preliminary Experiments

Effect of periarterial nerve stimulation and exogenous pressor stimuli on perfusion pressure in isolated perfused rat mesenteric arterioles: chemical sympathectomy of adrenergic nerves.

Isolated rat mesenteric blood vessels perfused with physiological salt solution maintained a steady basal perfusion pressure for up to six hours. Stimulation of periarterial nerves or injection of noradrenaline or potassium ions into the arterial cannula via a side arm constricted the mesenteric blood vessels, detected as a transient increase in the perfusion pressure. The vasoconstrictor response of the mesenteric vascular bed to periarterial nerve stimulation but not those to injected noradrenaline or potassium ions were abolished by pretreatment of the animals with reserpine (2.5 mg/kg, intraperitoneally for 1 day) or 6-hydroxydopamine (50 mg/kg, intraperitoneally for 3 days, Fig. *8*). These drugs are known to cause selective depletion of the adrenergic nerve terminals (*Goodman and Gilman 1974*).

Thus the periarterial nerves to the mesenteric arterioles appear to be postganglionic adrenergic. The pressor response to potassium ion depolarization did not appear to depend on the liberation of noradrenaline from the nerve endings.

In two experiments each, neither tyramine (1 mg/ml) dopamine or dopamine sulphate (100 µg/ml, respectively) injected intraarterially produced a response (contraction or relaxation).

Effect of α adrenoreceptor blockers on pressor responses

The reversible α blocker phentolamine (10^{-8} - 10^{-6} M) and the alkylating irreversible α blocker phenoxybenzamine (10^{-6} M) were both effective in blocking responses to periarterial nerve stimulation and injected noradrenaline but not those to potassium ion depolarization (3 experiments).

Calcium dependence of contractile responses

After equilibration of the isolated perfused mesenteric vascular bed with normal buffer switching to a Ca free buffer (essentially the same as the normal buffer but with the calcium omitted) did not result in any alteration in baseline perfusion pressure (six preparations). This was not the case with pressor stimuli, noradrenaline pressor response amplitude decreased to 70-80% of normal and potassium ion depolarization was ineffective within ten minutes. In the presence of a calcium free Krebs buffer containing 10 mM EDTA both noradrenaline and potassium pressor responses disappeared (two preparations). Analysis* of the calcium free buffer (made by omitting the calcium chloride) revealed the presence of low concentrations ($\approx 10^{-6}$ M) of ionized calcium ions. This extracellular calcium concentration was however insufficient to initiate contraction upon membrane depolarization (by intraarterial injection of potassium ions).

Actions of exogenous prostaglandins in the mesenteric vascular bed. Effect of prostaglandins E1 and E2 on noradrenaline and potassium pressor responses.

In preliminary experiments prostaglandin E1 added to the buffer reservoir in a cumulative manner over the concentration range 10^{-13} - 10^{-11} M

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* *Atomic absorbtion spectrometry by Dr A. Lipton.*

produced a biphasic effect on noradrenaline pressor responses. Low concentrations of PGE1 (10^{-13} - 10^{-11} M) caused a potentiation of noradrenaline pressor responses whereas high concentrations (> 10^{-11} M) caused a progressive inhibition (Fig. 9). In contrast PGE1 had no effect (at any concentration tested) upon potassium pressor responses. To test further whether this was a general property of prostaglandins, the effect of PGE2 (over a similar concentration range) was examined. PGE2 potentiated both noradrenaline and potassium pressor responses with maximum potentiation at 10^{-7} M of about 60%. The potentiations were noradrenaline (24 mm Hg x 133 Pa/mm Hg) and potassium (36 mm Hg x 133 Pa/mm Hg) respectively (Fig. 10).

Comparison of the effects of prostaglandins E1, E2, A1, A2 and F2α on noradrenaline pressor responses

Having established that the actions of prostaglandins are unique, in that PGE1 and PGE2 did not produce the same effects, it was decided to examine the effects of other naturally occurring primary prostaglandins. The effects of prostaglandins E1, E2, A1, A2 and F2α on noradrenaline pressor responses are shown in Fig. 11. As can be seen both PGA1 and PGF2α potentiated noradrenaline pressor responses, an action similar to that of PGE2. On the other hand PGA2 potentiated at low concentrations (10 pg/ml) and inhibited at higher concentrations (100 pg/ml to 10 ng/ml). This latter compound behaving somewhat like PGE1. However unlike PGE1, PGA2 potentiated potassium pressor responses (3 experiments).

Effect of PGA1, PGA2 and PGF2α on potassium pressor responses

In three experiments each, the effects of PGA1, PGA2 and PGF2α on potassium pressor responses were evaluated. All three prostaglandins potentiated pressor responses in a concentration dependent manner (10^{-12} - 10^{-6} M).

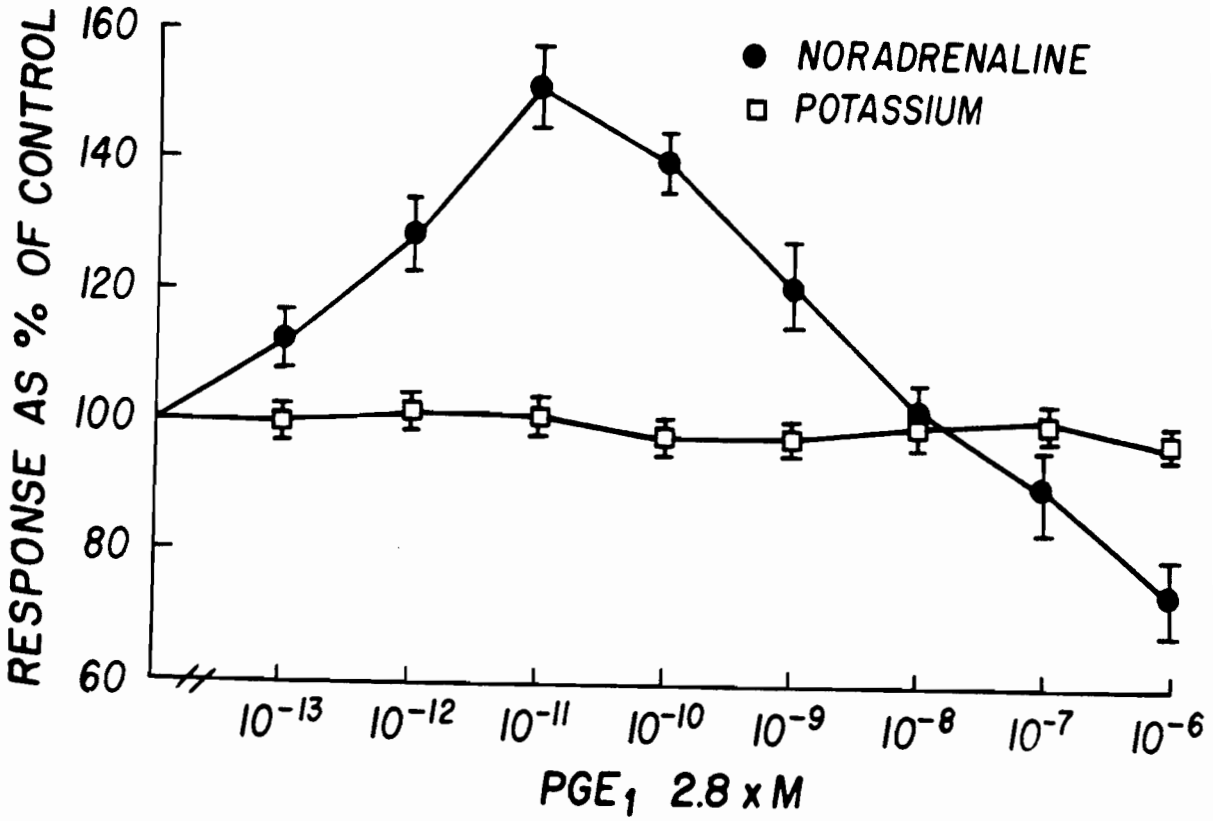

Fig. 9. Influence of PGEl on noradrenaline and potassium pressor responses. Each pressor agent was injected as a 100 µl bolus into the mesenteric artery and the change in resistance, as a result of vasoconstriction, monitored as an increase in perfusion pressure. Preparations were equilibrated for 1 hour before beginning the experiment and test injections of the pressor agent made every 4 or 5 minutes. The mean pressor response of the four contractions prior to the addition of PGEl was taken as 100 % and all subsequent responses expressed as a % of this.

Data shown represent the mean ± SE for six experiments.

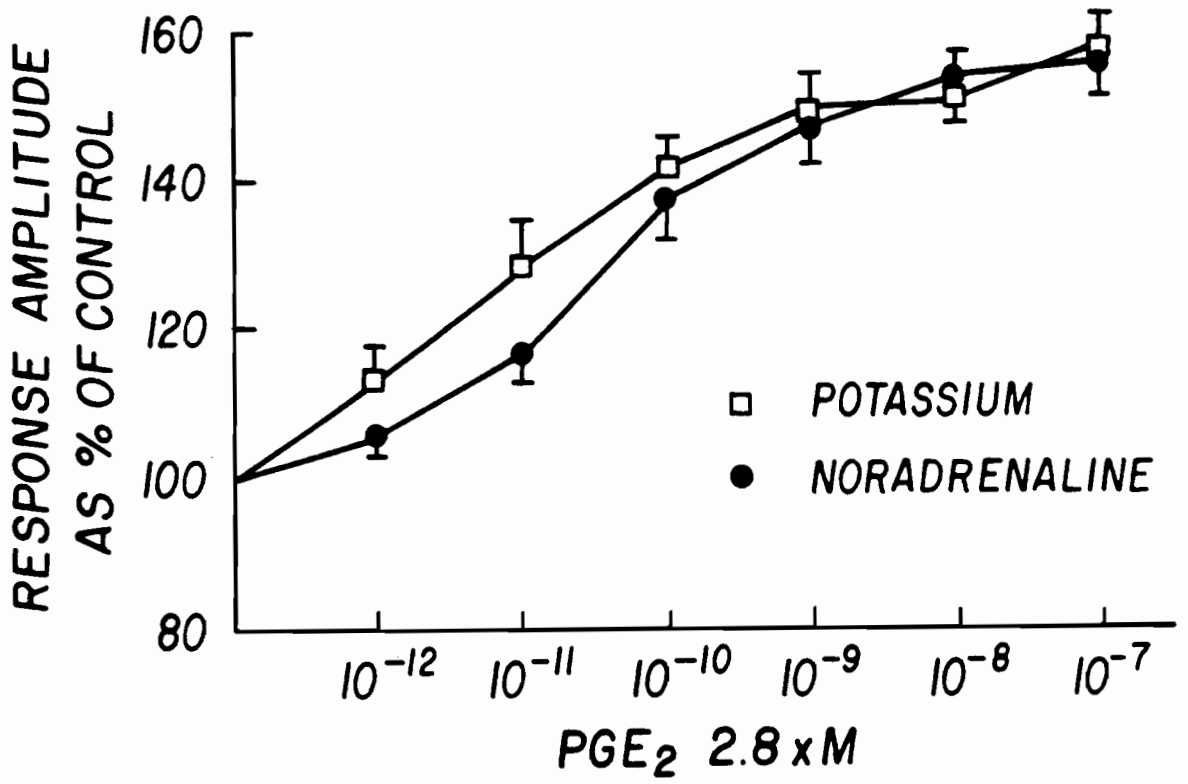

Fig. 10. Influence of PGE2 on noradrenaline and potassium pressor responses as described in Fig. 9. Each point represents the mean ± SE for six experiments.

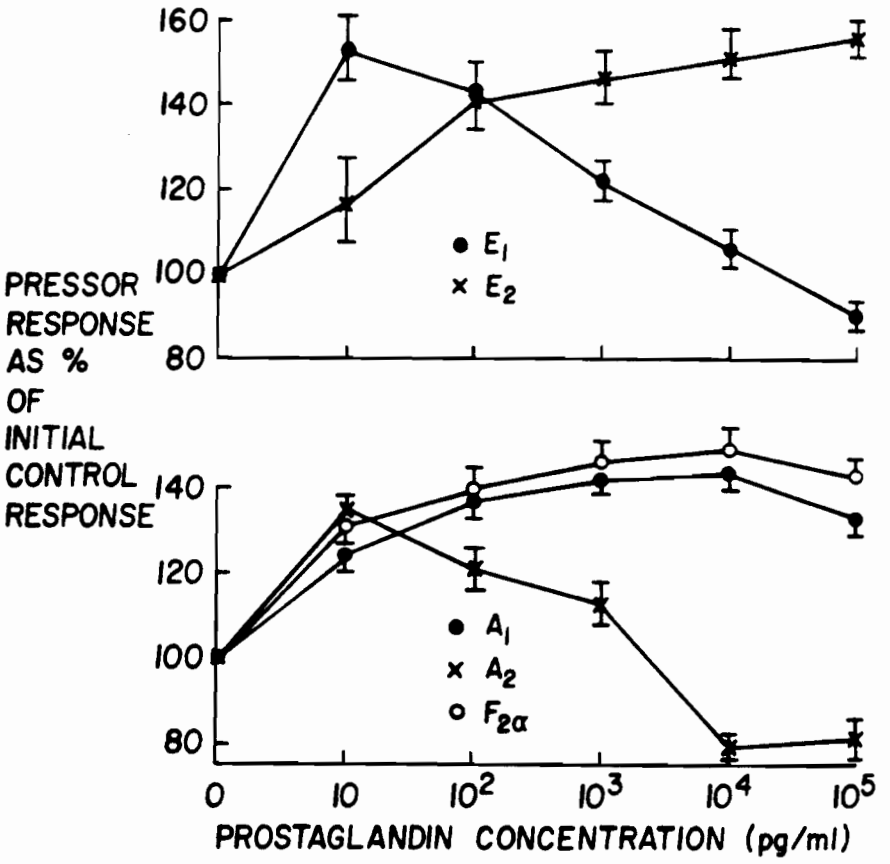

Fig. 11. Influence of prostaglandins (PG) E1, PGE2, PGA1, PGA2 and PGF2α on noradrenaline pressor responses. PGE2, PGE1 and PGF2α potentiated noradrenaline responses in a concentration dependent manner whereas PGE1 and PGA2 had a biphasic effect, potentiating at low concentrations and inhibiting at high concentrations (10 pg/ml of prostaglandin is approximately equal to 2.8 x 10^{-11} M). Each point represents the mean ± SE for six experiments.

Effect of PGE1, PGE2, PGA1, PGA2 and PGF2α on baseline perfusion pressure

Neither PGE1 nor PGE2 caused any alteration in baseline perfusion pressure whereas PGA1, PGA2 and PGF2α produced increases in baseline perfusion pressure.

These increases at the highest concentrations tested (10^{-7} M) were PGA1 (+ 4 mm Hg), PGA2 (+ 6 mm Hg), PGF2α (+ 8 mm Hg). Phentolamine added to the perfusate did not alter the increase in baseline perfusion pressure. This indicated that release of noradrenaline from the nerve endings was not responsible for the increase seen.

Effect of prostacyclin (PGI2) on noradrenaline and potassium pressor responses

Because of its short biological half life each concentration of PGI2 tested was made up immediately prior to use. Over the concentration range 1×10^{-10} M to 10^{-8} M PGI2 inhibited noradrenaline pressor responses whereas potassium pressor responses were unaffected (Fig. *12*). PGI2, thus had actions similar to that of PGE1 (Fig. 9). The concentrations of PGI2 shown in Fig. *12* may be higher than the actual concentration which caused an inhibition because of its continual degradation to 6-keto-PGF1α in aqueous solutions. 6-keto-PGF1α itself had no effect on vascular responsiveness over a similar concentration range. PGI2 did not alter baseline perfusion pressure at any concentration tested. The concentration of PGI2 depressing noradrenaline pressor responses by 50% was 2×10^{-9} M (determined from the Figure shown).

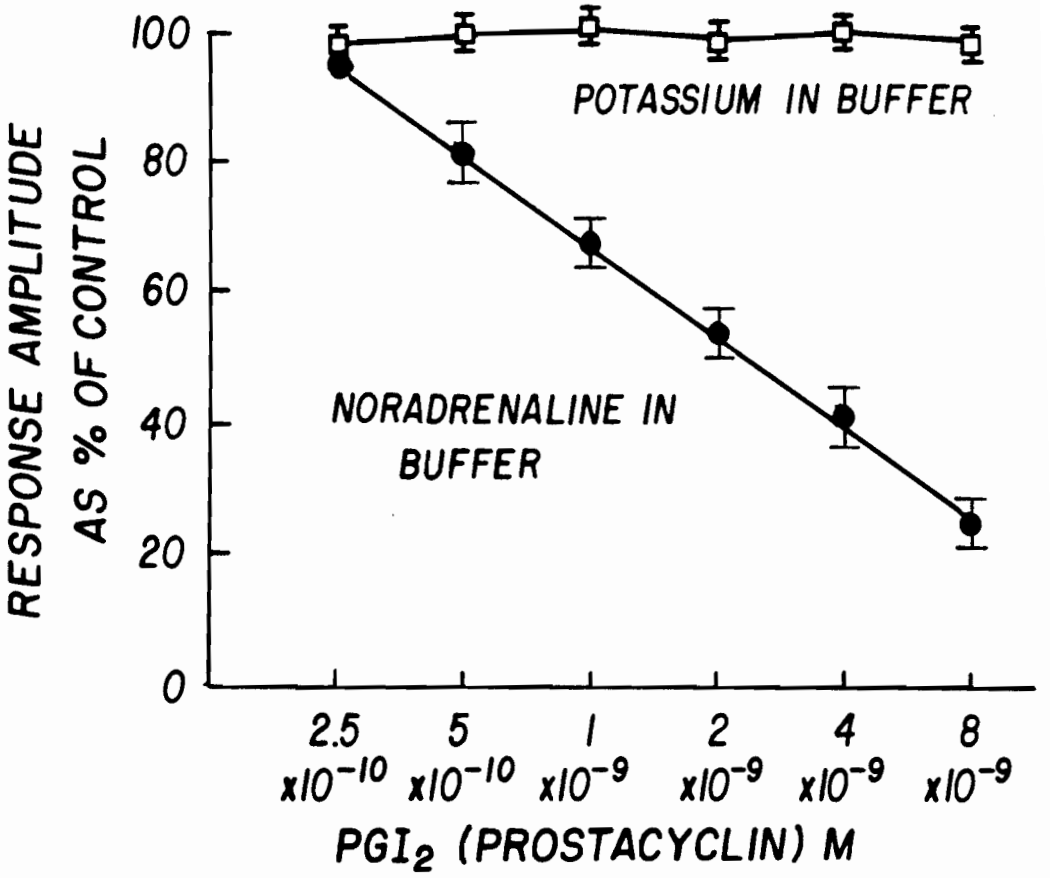

Fig. 12. Influence of prostacyclin (PGI2) on noradrenaline and potassium responses. Each concentration of PGI2 was maintained for at least 15 minutes before the concentration was increased. PGI2 inhibited only noradrenaline responses. Other conditions were as described in Fig. 9 and results.

Effect of thromboxane B2 on noradrenaline and potassium pressor responses

Thromboxane A2 is extremely labile and no stable analogue was available for use therefore the action of its metabolite TXB2 was examined. TXB2 over the concentration range 10^{-9} to 10^{-7} M partially inhibited noradrenaline pressor responses. The threshold concentration being approximately 3×10^{-9} M (Fig. *13*) The maximum inhibition seen was approximately twenty percent. Increasing the TXB2 concentration to 10^{-5} M produced no further inhibition.

TXB2 did not have any apparent effect on potassium pressor responses at any concentration tested (maximum concentration tested 10^{-5} M, Fig. *13*). TXB2 unlike PGI2 or 6-keto-PGF1α caused a transient increase in baseline perfusion pressure however this elevation was not statistically significant (Fig. *13*).

Effect of PGD2 on pressor responses to noradrenaline and potassium

PGD2 (10^{-13} to 10^{-6} M) added to the perfusion reservoir in a cumulative fashion caused no discernable effects on baseline perfusion pressure or pressor responses to either noradrenaline or potassium ions (three preparations).

Effects of prostaglandins on angiotensin II pressor responses

Prostaglandins E1, E2, and F2α potentiated angiotensin pressor responses whereas both PGI2 and TXB2 caused inhibition (2 preparations each).

Effect of arachidonic acid on pressor responses

Two concentrations of arachidonic acid 10 pg/ml to 1.0 ng/ml inhibited noradrenaline pressor responses by 30 percent (mean 3 experiments) whereas higher concentrations 50 ng/ml - 800 ng/ml potentiated vascular

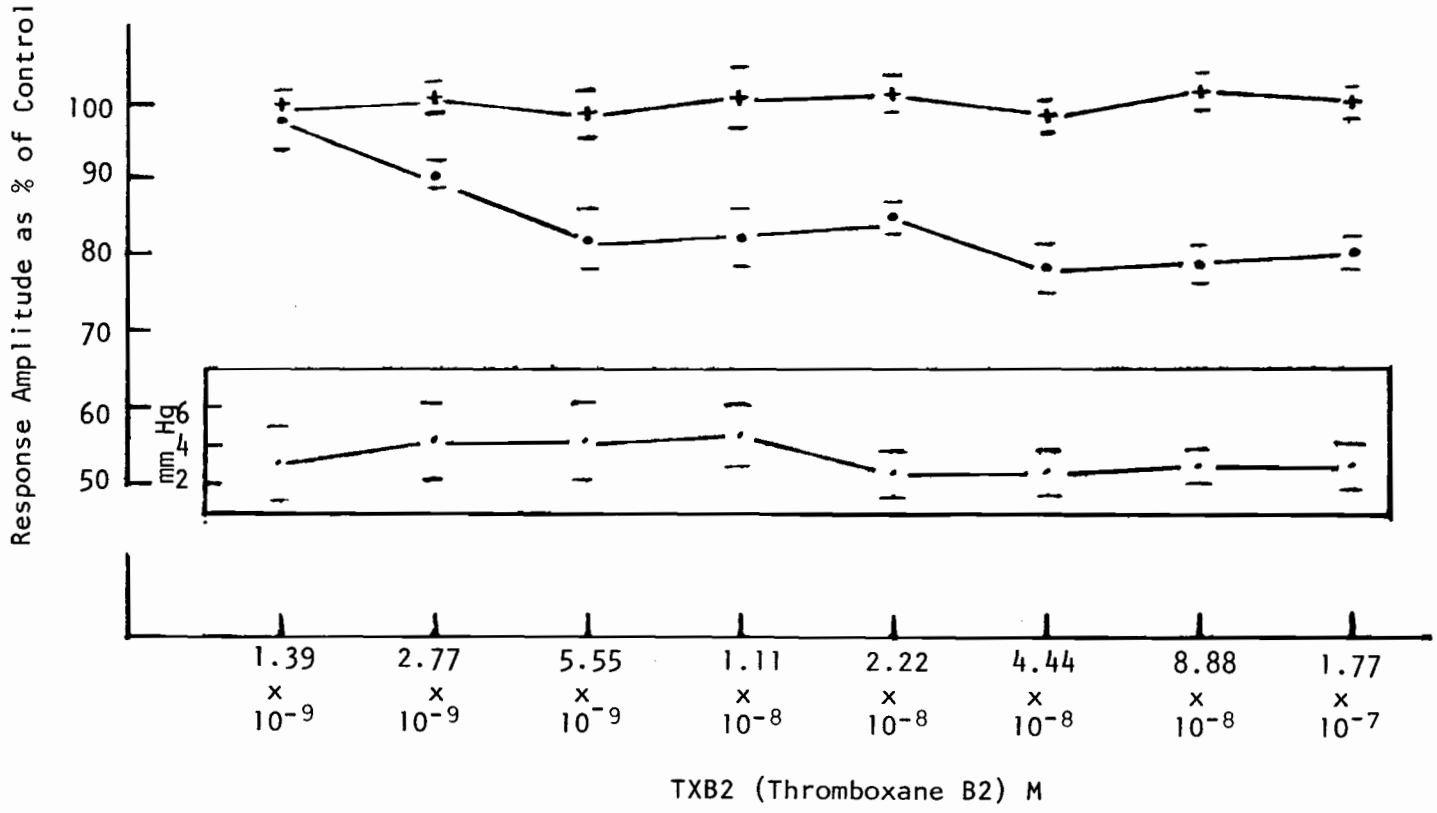

Fig. 13. Influence of thromboxane (TX) B2 (the stable product of thromboxane A2) on vascular responses to bolus intraarterial injections of noradrenaline and potassium. Inset shows effect of TXB2 on basal perfusion pressure. Each point represents the mean ± SE for six experiments Note the weak inhibitory effect of TXB2 on noradrenaline contractions (·) and its lack of effect on potassium contractions (+).

responses (3 experiments). In contrast potassium pressor responses were unaffected by low concentrations of arachidonic acid but were potentiated by high concentrations (2 experiments).

Arachidonic acid did not alter basal perfusion pressure at any concentration tested.

Effect of a long lasting PGE2 analogue 16,16 dimethyl PGE2 (16,16 diMePGE2)

This analogue of PGE2 is resistant to metabolism by 15-hydroxy prostaglandin dehydrogenase and as a result has a prolonged *in vivo* biological half life. It was tested in the mesenteric vascular bed to evaluate its effects relative to those of PGE2.

Effect of 16,16 diMePGE2 on noradrenaline and potassium pressor responses

The addition of 16,16 diMePGE2 to the buffer in concentrations ranging from 10^{-12} to 10^{-7} M caused a progressive increase in response amplitude to injections of potassium ions (Fig. *14*). When preparations were stimulated with noradrenaline 16,16 diMePGE2 produced a biphasic effect on noradrenaline pressor responses. Two concentrations of 16,16 diMePGE2 (2.6×10^{-12} - 2.6×10^{-11} M) enhanced noradrenaline pressor responses, this effect being stable for up to forty minutes.

Increasing the concentration of 16,16 diMePGE2 caused a progressive inhibition of noradrenaline responses, plateauing at concentrations greater than 10^{-9} M (Fig. *15*).

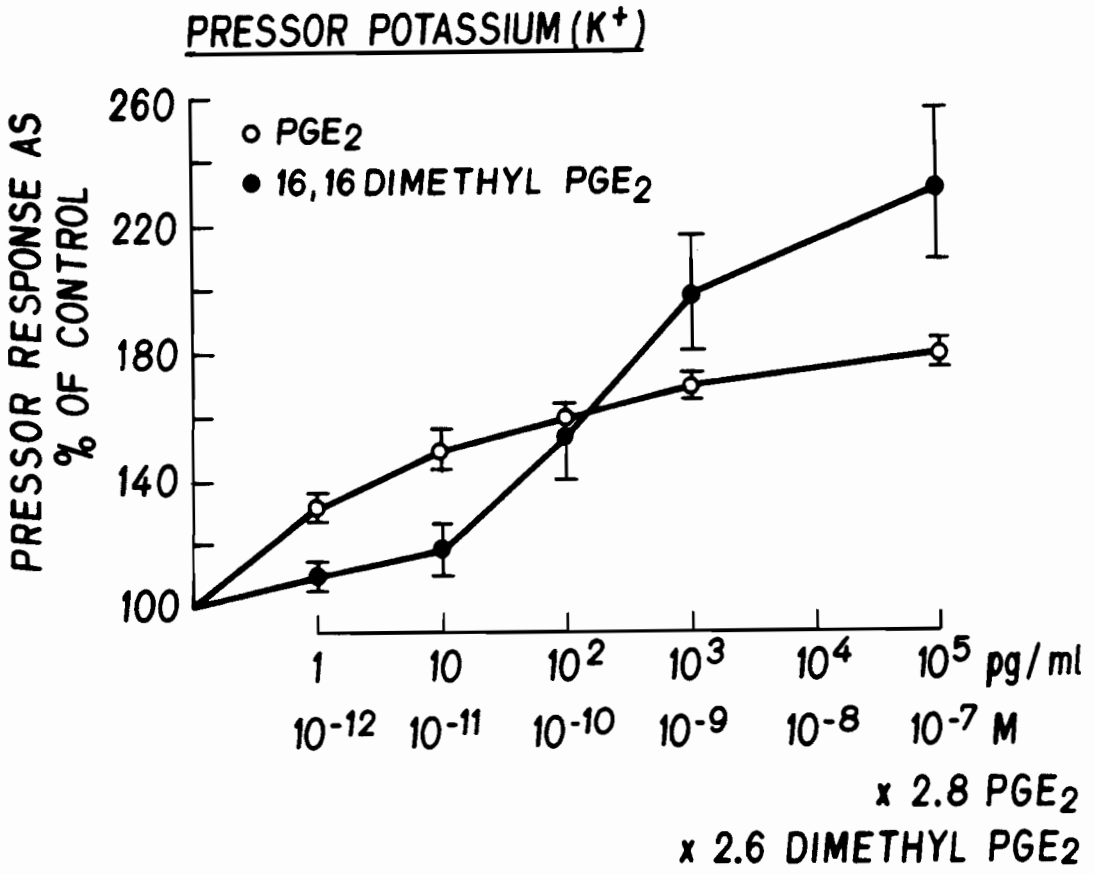

Fig. 14. Influence of PGE2 (o) and 16,16 dimethyl PGE2 (•) on potassium responses in the mesenteric vascular bed. As can be seen both prostaglandins potentiate potassium pressor responses. Each point represents the mean ± SE, n = 5 or 6.

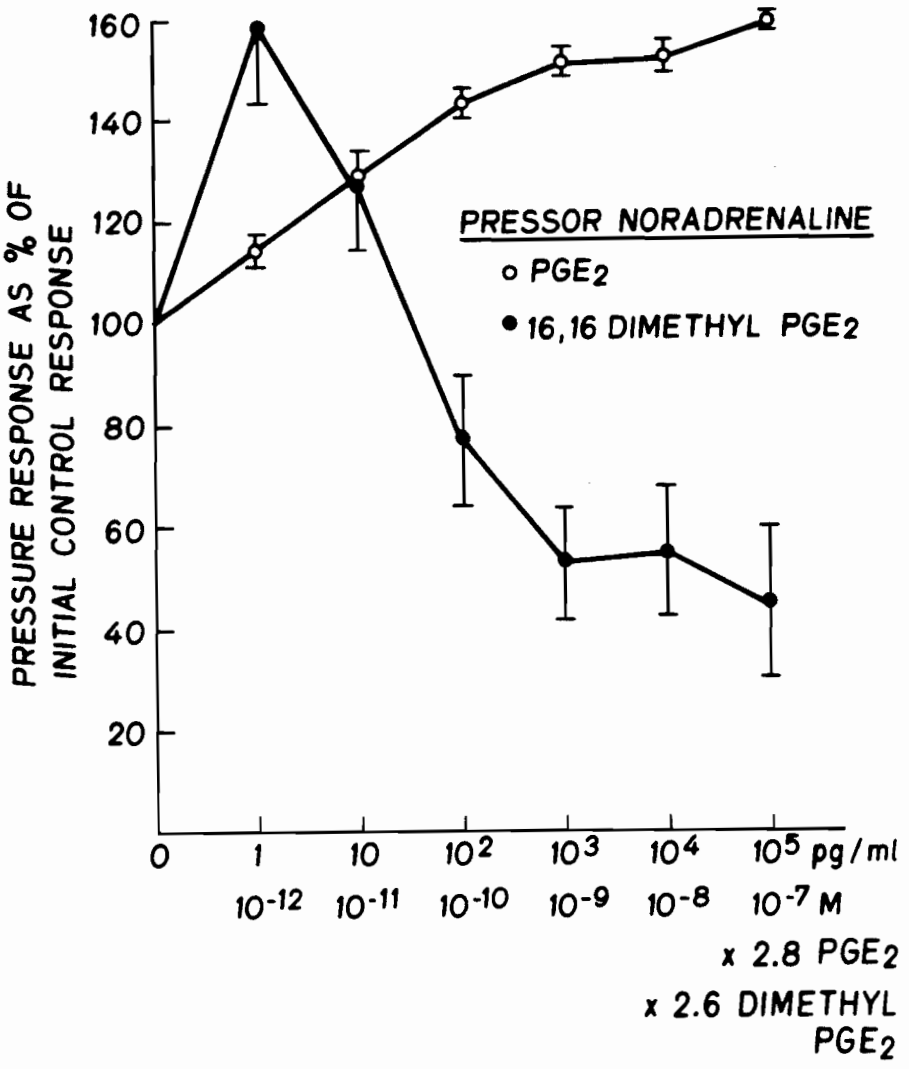

Fig. 15. Influence of PGE2 (o) and 16,16 dimethyl PGE2 (•) on noradrenaline pressor responses of the mesenteric vascular bed. As can be seen 16,16 dimethyl PGE2 had a biphasic effect on pressor responses whereas PGE2 had a monophasic, concentration dependent effect. The data shown for each prostaglandin represents the mean ± SE, n = 6.

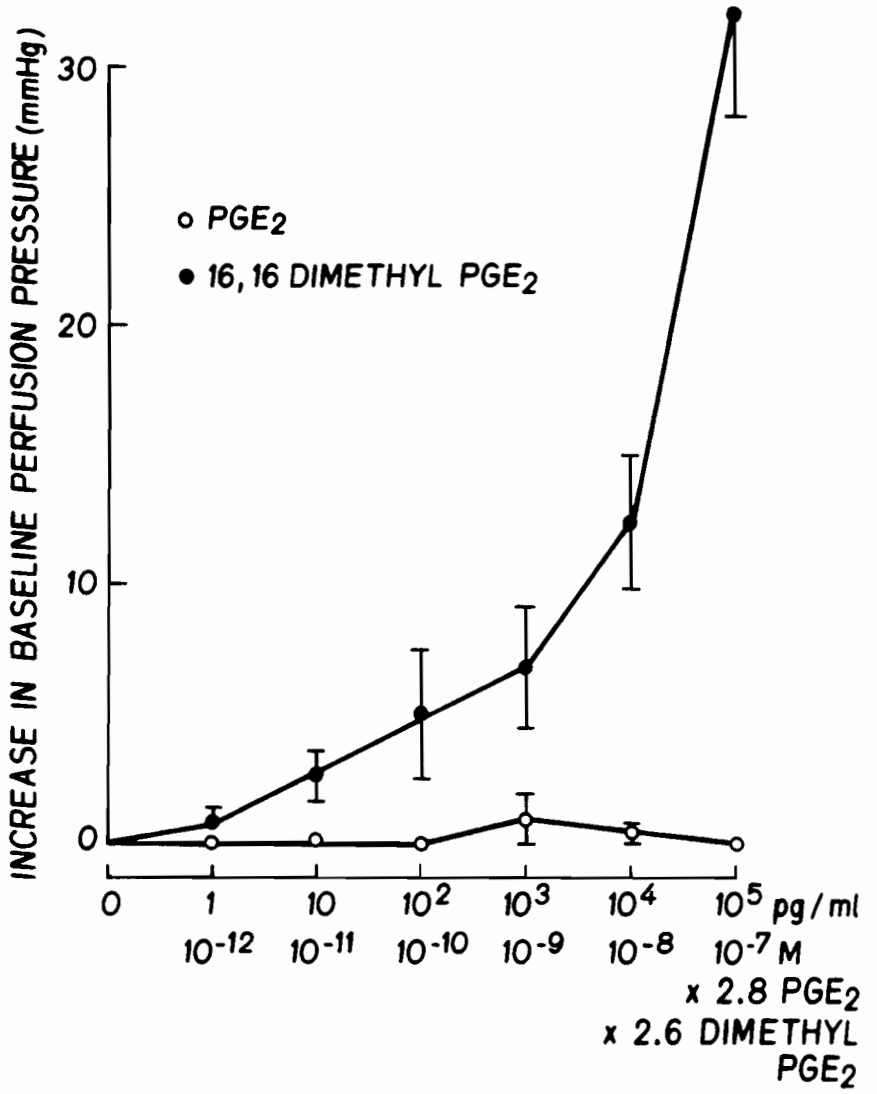

Fig. 16. Direct effects of PGE2 (o) and 16,16 dimethyl PGE2 (•) on vascular smooth muscle tone as indicated by changes in base line perfusion pressure. Each point represents the mean ± SE, n = 6.

Abscissa: concentration of prostaglandins (M).

Ordinate: base line perfusion pressure (mm Hg, 1 mm Hg = 133.3 Pascals).

Effect of 16,16 diMePGE2 on baseline perfusion pressure

16,16 diMePGE2 appeared to have a direct effect on the arteriolar smooth muscle. Over the concentration range tested (10^{-12} to 10^{-7} M) this prostaglandin significantly elevated the baseline perfusion pressure (Fig. *16*). This effect was not dependent on the release of endogenous catecholamine stores, because it persisted in preparations from rats pre-treated with 6 hydroxydopamine (see Methods).

Effect of 16,16 diMePGE2 in low calcium buffer

In preparations perfused with low calcium (2×10^{-6} M) 16,16 diMePGE2 (2.6×10^{-11} M) potentiated noradrenaline pressor responses within five minutes to 240% of control, however, this potentiation was not stable and declined with time. At 40 minutes, pressor responses had fallen to 90% of control and at 60 minutes was approximately 40% of control; these values are the means of two experiments. In normocalcium buffer no decay of 16,16 diMePGE2 (2.6×10^{-11} M) potentiation of noradrenaline pressor responses was seen (6 experiments, pressor response 160 ± 28%, M ± SE) over a similar time span.

Reversal of indomethacin effect on noradrenaline perfused preparations by supraphysiological concentrations of prostaglandins

In the previous experiments the effects of low concentrations of prostaglandins on vascular reactivity to pressor agents were described. For the experiment to be described, the mesenteric artery was cannulated and the vascular bed dissected out as described in methods. Preparations were equilibrated for 1 hour, and then continuously perfused with buffer containing noradrenaline bitartrate (400 ng/ml 1.25×10^{-6} M) and ascorbic acid (50 ng/ml, 2.8×10^{-6} M to prevent the oxidation of the noradrenaline).

The noradrenaline increased the basal perfusion pressure; indomethacin (8 µg/ml, 2.2 x 10^{-5} M) was added and the perfusion pressure fell to the pre-noradrenaline level.

Effect of PGE2

Under these conditions stimulation by depolarizing concentrations of either potassium chloride (43 mmoles) or calcium chloride (25 mmoles) injected intraarterially cannot cause a contraction (Fig. 17). In contrast bolus injections of PGE2 produced a rapid increase in pressure as a result of contraction of the smooth muscle. The contraction declined to baseline in two phases: a fast phase of approximately 1 minute, and a slower phase of approximately 6 minutes (Fig. 18). The amount of PGE2 injected as a 100 µl bolus was extremely high (50 µg, 1.40 x 10^{-7} moles) and probably accounts for the slow secondary relaxation phase. Normally the total duration of a pressor response to noradrenalinewas approximately 1 minute (see Fig. 29).

Effect of TXB2

In a similarly prepared preparation TXB2 injected intraarterially caused a rapid contraction which lasted for about 1 minute, with no similar slow phase as seen with PGE2 (Fig. 19). The amount of TXB2 injected as a 100 µl bolus was of the same order of magnitude as PGE2 (1.4 x 10^{-7} moles).

Effect of PGI2

Injection of PGI2 (1.4 x 10^{-7} moles) into the arterial cannula of a similarly prepared preparation caused a biphasic contraction (Fig. 20). An initial fast contraction, followed by a slower contraction (Fig. 18). Thus PGI2 was distinctly different in its effect.

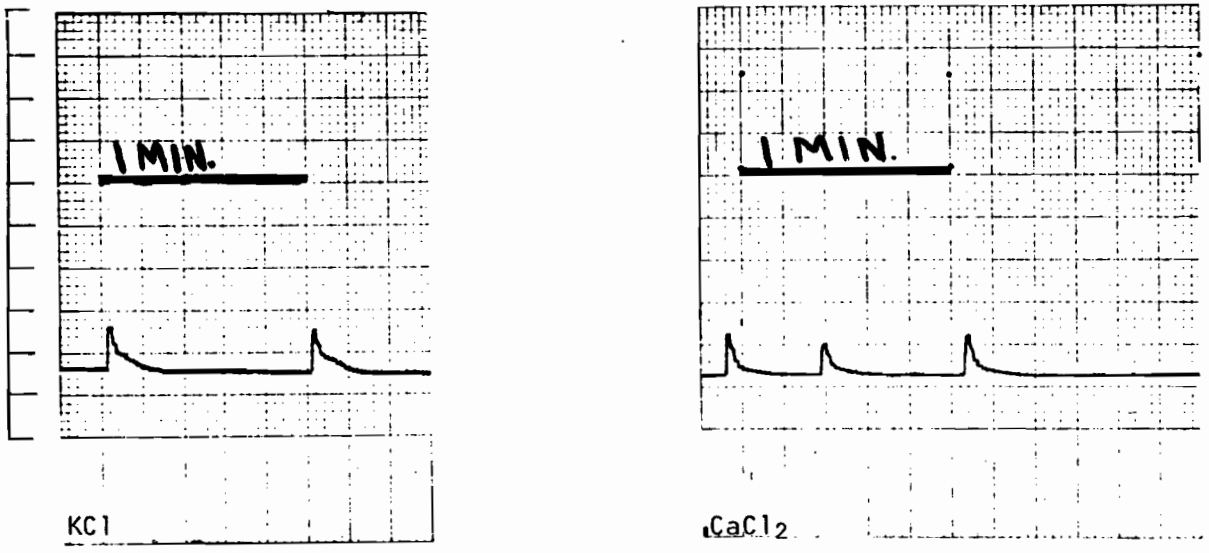

Fig. 17. Response of the mesenteric vascular bed perfused with noradrenaline and indomethacin to bolus intra-arterial injections of potassium or calcium ions. Note that only the injection artifact is seen with no detectable contraction of the vascular smooth muscle. See results for further details. Each division of the vertical scale represents 10 mm Hg (1 mm Hg = 133.3 Pascals). Horizontal bar represents 1 minute.

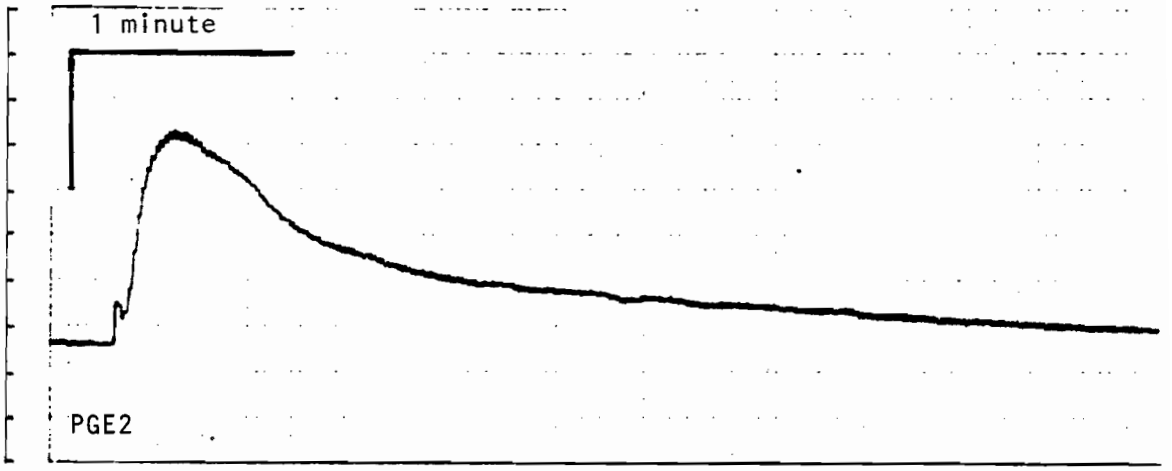

Fig. 18. Pressor response of vascular bed to bolus intra-arterial injection of PGE2. Conditions as in Fig. *17*. Each division of the vertical scale equals 10 mm Hg (1 mm Hg = 133.3 pascals). Horizontal bar represents 1 minute.

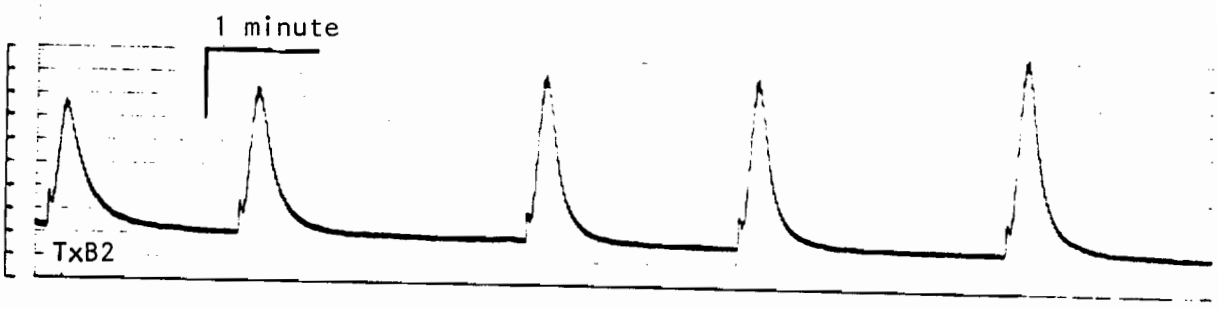

Fig. 19. Pressor response of vascular bed to bolus intra-arterial injection of TxB2. Conditions as in Fig. *17*. Each division of the vertical scale equals 10 mm Hg (1 mm Hg = 133.3 pascals). Horizontal bar represents 1 minute.

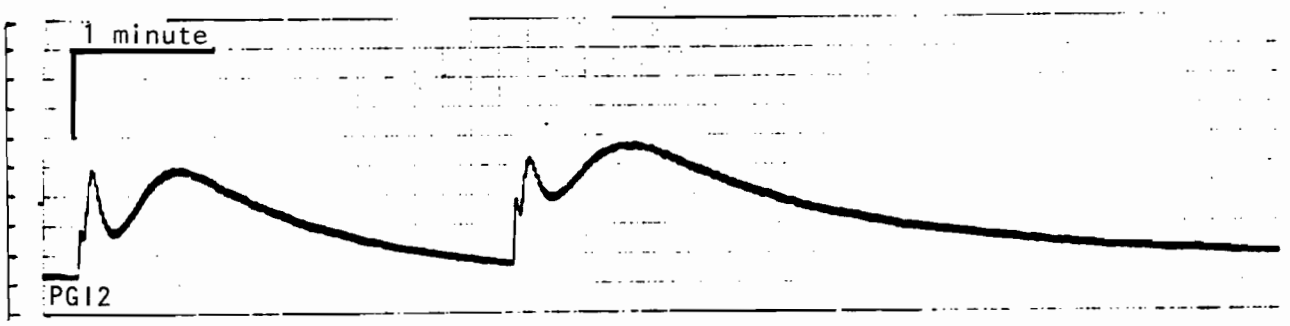

Fig. 20. Pressor response of vascular bed to bolus intra-arterial injection of prostacyclin (PGI2). Conditions as in Fig. *17*. Each division of the vertical scale equals 10 mm Hg (1 mm Hg = 133.3 pascals). Horizontal bar represents 1 minute.

Effect of supraphysiological concentrations of prostaglandins in buffer perfused preparations

It was now established that prostaglandins PGE2, PGI2 and TXB2 produced different effects on vascular responses in preparations perfused simultaneously with indomethacin and noradrenaline. It was therefore of interest to repeat this experiment using normal preparations. The objective being to separate the modulatory effect on noradrenaline actions from the direct contractile actions of the prostaglandins.

Prostaglandins were injected into the arterial cannula of buffer perfused preparations and their direct pressor effects recorded. Prostaglandins E1 and E2 both contracted the blood vessels however the contraction had a duration of 2 minutes (Fig. 21) and that of PGE1 was approximately 1.5 minutes (Fig. 22). TXB2 the stable breakdown product of TXA2 caused a pressor response similar in time course to that of PGE1 (duration 1 minute, Fig. 23). PGI2 produced a distinctive pressor effect, the total duration of the contraction was the longest measured, approximately 3 minutes (Fig. 24). The shape of the PGI2 contraction was unique: a fast phase followed by a brief decay, then a slower contraction; 6-keto-PGF1α the spontaneous breakdown product of PGI2 did not cause a contraction resembling that of PGI2 (Fig. 25). The contraction caused by 6-keto-PGF1α had a duration of $\approx$ 2 minutes and an amplitude smaller than that of PGI2. PGF2α on the other hand produced a fast contraction which decayed slowly back to baseline, the total duration of the contraction was $\approx$ 3 minutes (Fig. 26). The PGF2α contraction was similar to the slow phase of the PGI2 contraction. PGD2 caused a weak contraction with the shortest duration recorded, approximately 20 seconds (Fig. 27).

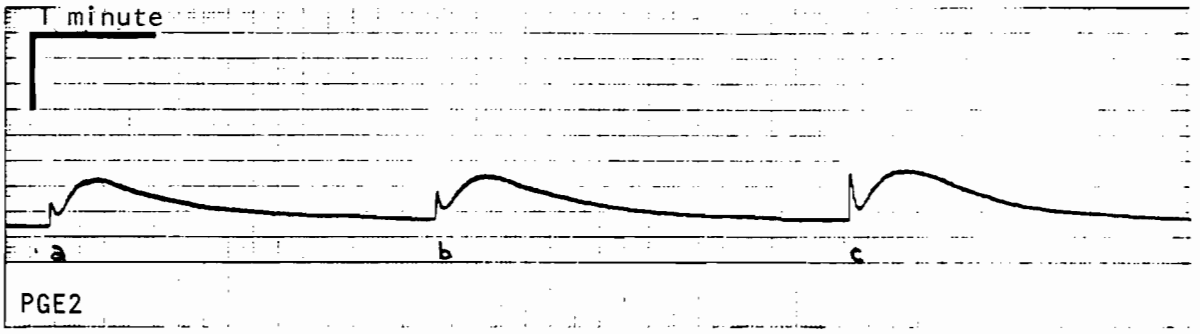

Fig. 21. Contraction of a buffer perfused rat mesenteric vascular bed by bolus intra-arterial injections of PGE2 (a 2.8×10^{-7} m, b 2.8×10^{-7} m, c 5.6×10^{-7} m). The horizontal bar represents 1 minute and the vertical bar 30 mm Hg (1 mm Hg = 133.3 Pa). Concentrations given in same order as responses shown in Figure.

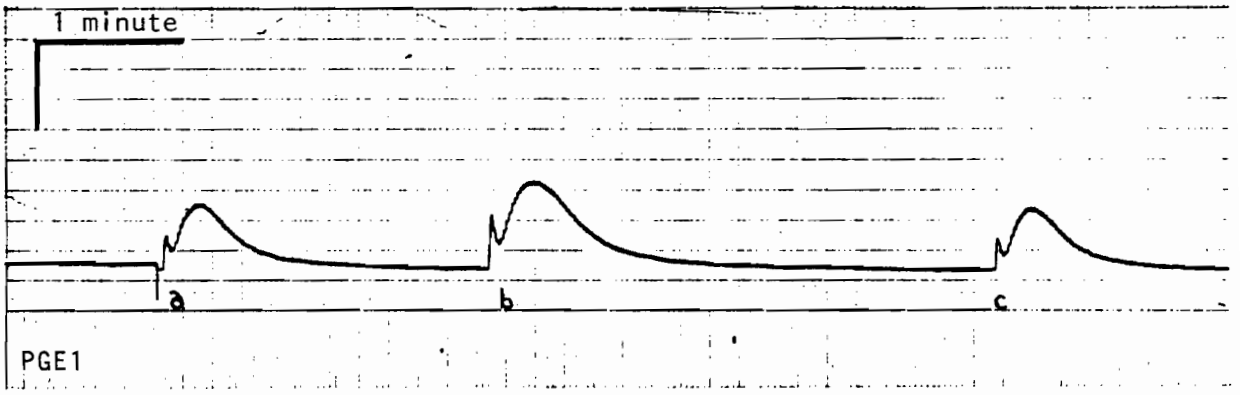

Fig. 22. Contraction of a buffer perfused rat mesenteric vascular bed by bolus intra-arterial injections of PGE1 (a 2.8×10^{-7} m, b 5.6×10^{-7} m, c 2.8×10^{-7} m). The horizontal bar represents 1 minute and the vertical bar 30 mm Hg (1 mm Hg = 133.3 Pa). Concentrations given in same order as responses shown in Figure.

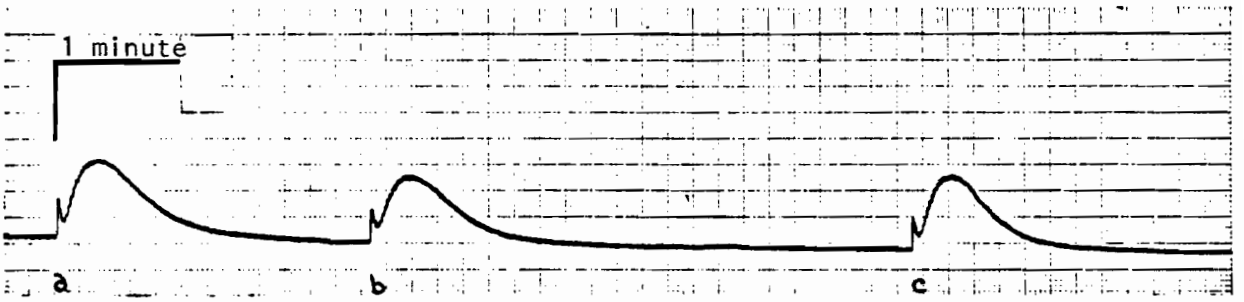

Fig. 23. Contraction of a buffer perfused rat mesenteric vascular bed by bolus intra-arterial injections of TxB2 (a 5.6×10^{-7} , b 2.8×10^{-7} , c 2.8×10^{-7}). The horizontal bar represents 1 minute and the vertical bar 30 mm Hg (1 mm Hg = 133.3 Pa). Concentrations given in same order as responses shown in Figure.

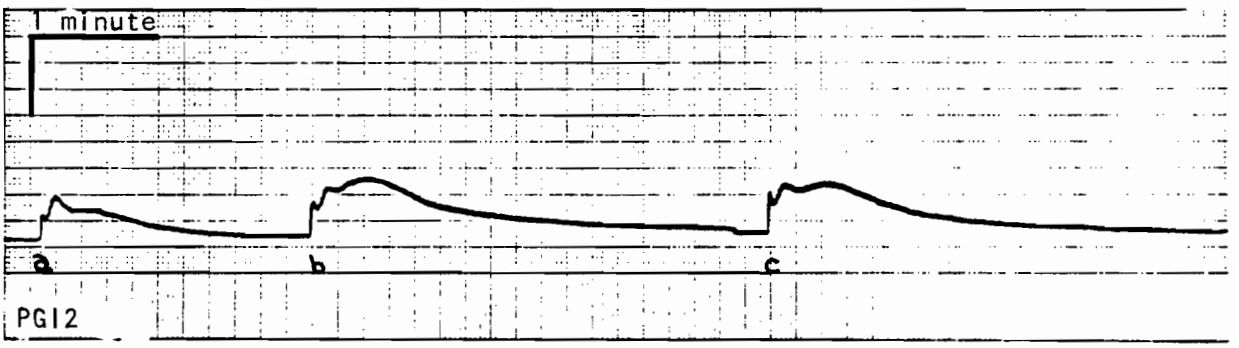

Fig. 24. Contraction of a buffer perfused rat mesenteric vascular bed by bolus intra-arterial injections of PGI2 (a 2.5×10^{-8} m, b 5.0×10^{-8} m, c 5.0×10^{-8} m). The horizontal bar represents 1 minute and the vertical bar 30 mm Hg (1 mm Hg = 133.3 Pa). Concentrations given in same order as responses shown in Figure.

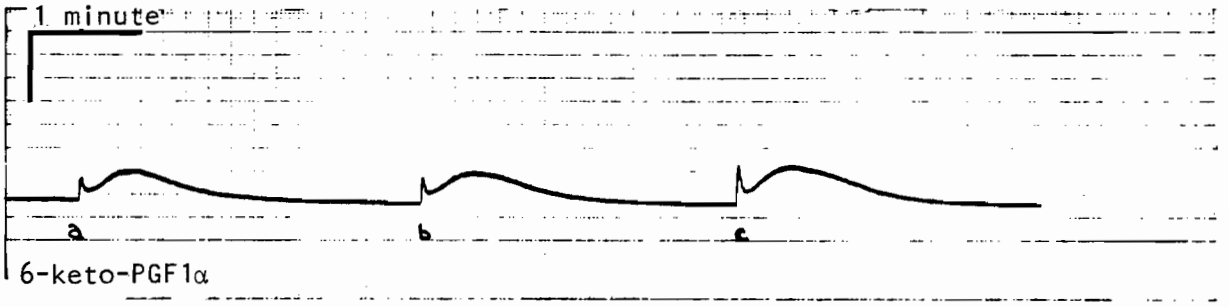

Fig. 25. Contraction of a buffer perfused rat mesenteric vascular bed by bolus intra-arterial injections of 6-keto-PGF1α (a 2.8×10^{-7} m, b 2.8×10^{-7} m, c 5.6×10^{-7} m). The horizontal bar represents 1 minute and the vertical bar 30 mm Hg (1 mm Hg = 133.3 Pa). Concentrations given in same order as responses shown in Figure.

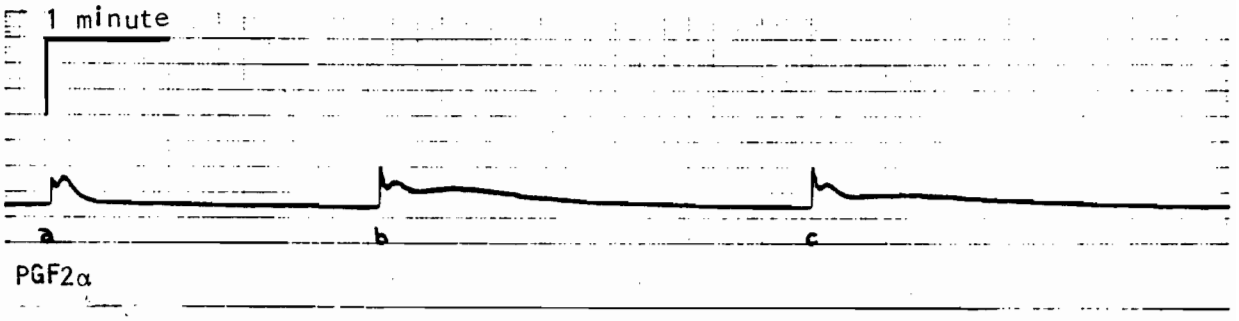

Fig. 26. Contraction of a buffer perfused rat mesenteric vascular bed by bolus intra-arterial injections of PGF2α a 2.8×10^{-7} m, b 5.6×10^{-7} m, c 5.6×10^{-7} m). The horizontal bar represents 1 minute and the vertical bar 30 mm Hg (1 mm Hg = 133.3 Pa). Concentrations given in same order as responses shown in Figure.

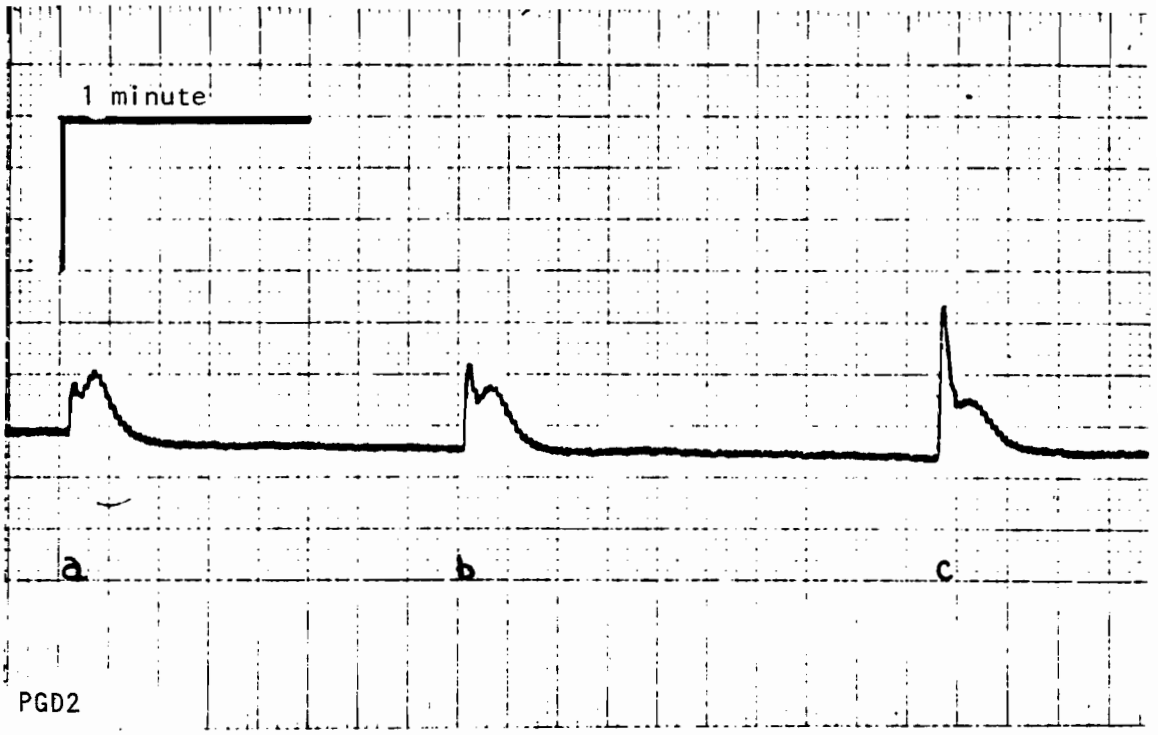

Fig. 27. Contraction of a buffer perfused rat mesenteric vascular bed by bolus intra-arterial injections of PGD2 (a 2.8×10^{-7} m, b 5.6×10^{-7} m, c 1.12×10^{-7} m). The horizontal bar represents 1 minute and the vertical bar 30 mm Hg (1 mm Hg = 133.3 Pa). Concentrations given in same order as responses shown in Figure.

DISCUSSION

Of the eight primary prostaglandins tested only PGA1, PGA2 and PGF2α had any direct contractile effect on the vasculature. Each prostaglandin had a distinct and unique effect on vascular reactivity if responses to both noradrenaline and potassium ions are considered.

PGE1 had a biphasic effect, low concentrations potentiating noradrenaline pressor responses and higher concentrations being inhibitory. A toxic effect of the high concentrations of PGE1 can be ruled out because potassium pressor responses were not similarly inhibited (Fig. 9). Indeed similar biphasic effects of prostaglandins on vascular reactivity have been previously described in the cat spleen (PGE2, *Hedqvist and Brudin 1969*) as have their direct biphasic contractile effects on the dog mesentery (PG(S) A1, A2, E1, Flα, *Strong and Bohr 1967*), human umbilical artery (PGI2, *Pomerantz et al 1977*), rat coronary vascular bed (PGI2 , *Karmazyn et al 1978*) and guinea pig coronary vascularture (PGI2, *Dusting et al 1977*).

PGE2 potentiated vascular responses to both noradrenaline and potassium to approximately the same degree (Fig. *10*), as did PGA1, and PGF2α. In contrast PGA2 had a biphasic effect on noradrenaline responses, low concentrations of PGA2 potentiated and high concentrations inhibited contractility. Thus PGA2 actions at first glance appeared to be similar to those of PGE1; upon examining its effects on potassium responses itwas clear that they are different. PGA2 potentiated potassium responses whereas PGE1 had no effect. In addition PGA2 directly increased smooth muscle tone, a property neither PGE1 nor PGE2 possessed.

The mechanism(s) whereby PGE2, PGA1, PGF2α and low concentrations of PGE1 and PGA2 potentiate noradrenaline responses is not presently known. Some possibilities are: 1. the prostaglandins increase the sensitivity of the contractile proteins; 2. prostaglandins increase the availability of calcium to the contractile proteins; 3. prostaglandins inhibit the neuronal uptake and termination of noradrenaline action ; 4. prostaglandins alter the events of excitation contraction coupling.

Points No. 1 and 2 can be dispensed with because PGE1 at concentrations which potentiated noradrenaline responses had no effect on potassium responses nor did it increase vascular tone. Any sensitization of the contractile proteins should have resulted in the simultaneous facilitation of pressor responses to both stimuli. If the cytosolic calcium concentration increased it would be expected that vascular tone should be increased; this was not seen. In contrast PGA2 (low concentrations) potentiated noradrenaline and potassium pressor responses as did PGE2, PGF2α and PGA1, yet only three of these (PGA1, PGA2 and PGF2α) increased vascular tone. The data obtained with PGA2 showed that concentrations which inhibited noradrenaline responses, had a significant stimulatory effect on the vasculature increasing the basal perfusion pressure. In contrast PGE1 at concentrations causing maximal inhibition of noradrenaline responses had no direct stimulatory effect on the vasculature. Again these contrasting data argue against any simple blockade of calcium uptake or action at a single site.

In considering point No. 3, recall that potassium pressor responses do not depend on the release of noradrenaline from endogenous vascular stores (Fig. 8). The demonstration of prostaglandin potentiation of potassium responses was then independent of any possible alteration in catecholamine release, uptake or inactivation. While studies were not conducted by

us using specific catecholamine uptake blockers, other researchers have addressed this question. Two groups, *Coupar* and *McLennan (1978)* and *Malik et al (1976)* reported experiments using the rat isolated perfused mesenteric vascular bed. Both groups reported that prostaglandins potentiated noradrenaline (exogenous) responses in the presence of an uptake blocker (cocaine). The concentration of cocaine used had already maximally potentiated noradrenaline responses, further increases in its concentration did not have any additional effect, prostaglandins (PGE1, PGE2, PGA1 added to the perfusate still enhanced vascular responses. In addition *Coupar* and *McLennan (1978)* ruled out any alteration of the α adrenoceptor as the prostaglandin mechanism of action. They utilized the competitive antagonist phentolamine and examined the effects of prostaglandins (PGA1, PGE2) on affinity of the antagonist for the α adrenoceptor, no effect of the prostaglandins was detected. Indeed our demonstration that angiotensin II responses werepotentiated by both PGE2 and PGF2α support their findings for lack of involvement of the α adrenoceptor and adrenaline metabolism. Thus point No. **3** appears to be untenable as an explanation.

The remaining hypothesis is that prostaglandins alter the events of excitation contraction coupling. The demonstration here that these substances exhibit differential actions on vascular responses with the same prostaglandin having opposite effects on contractions to either potassium or noradrenaline supports this view. Similar conclusions have been made by *Northover (1968)*, *Malik et al (1976)*, *Manku and Horrobin (1976)*, *Coupar and McLennan (1978)* as a result of their work in the rat vasculature. More recently *Herman et al (1978)* using canine isolated veins demonstrated that PGI2 inhibited the amplitude but not the frequency of contractions

(spontaneously contracting mesenteric veins), as well as noradrenaline contractions. In contrast no inhibition was seen when the preparation was contracted by electrical depolarization. This group also concluded that PGI2 was altering some event of excitation-contraction coupling. From the data presented here showing that PGI2 and PGE1 inhibited noradrenaline but not potassium responses it is possible to theorize that both compounds inhibited the release of intracellular calcium. Because noradrenaline mobilizes primarily loosely membrane bound and intracellular calcium (*Hinke 1965; Jhamandas and Nash 1967; Hudgins and Weiss 1968*) such an effect would prevent its action. Depolarizing stimuli on the other hand utilize extracellular calcium primarily. The muscle will contract when the plasma membrane is depolarized because it then becomes permeable to calcium, which then diffuses down its concentration gradient into the cytosol. The calcium ions can then interact with the contractile proteins resulting in tension development.

One of the perennial suggestions in smooth muscle research is that increases in intracellular cAMP levels inhibit vascular responsiveness (*Goldberg et al 1973; Lee et al 1972; Anderson 1972*), by increasing the uptake and binding of calcium by the intracellular organelles. While there exists some debate concerning this it now appears that this position is untenable. In several smooth muscle preparations (vascular and non vascular) no correlation between relaxation of, or inhibition of muscle contraction and cyclic AMP levels could be demonstrated (rat uterus, *Diamond and Hartle 1974;* rat myometrium, *Diamond and Holmes 1975;* rabbit portal vein, *Collins and Sutter 1975;* guinea pig taenia coli and canine small intestine, *Inatomi et al 1975;* dog carotid media strip, *Herlihy et al 1976;* rabbit ileum, *Ally 1976*). Similarly no effect of cyclic AMP on aortic microsome binding and uptake of calcium could be demonstrated by

either *Allen (1977)* or *Clyman et al (1976)*. In other studies examining the role of cyclic AMP in mesenteric vasodilation, *Shepherd et al (1973)* found that the dilation seen was caused by metabolites of cyclic AMP not the cyclic nucleotide itself. Recently *Schror et al (1979)* reported that PGI2 decreased cyclic AMP levels in hearts and strips of coronary vessels, even though this prostaglandin decreased coronary perfusion pressure (as a result of vasodilation) and relaxed the smooth muscle strips. They concluded that PGI2 relaxation was independent of any alterations in cyclic AMP levels.

In a recent study (*Kahn and Brachet 1979*) in which the effects of prostaglandins (PGE1, PGE2, PGF2, PGA1, and PGF1) on cyclic AMP levels were measured in the rat mesenteric artery, no changes in cyclic AMP levels were seen with concentrations up to 200 ng/ml ($> 10^{-7}$M). Higher concentrations ($> 10^{-6}$M) of all the prostaglandins tested significantly increased cyclic AMP levels (*Kahn and Brachet 1979*).

Of those PGs tested by *Kahn and Brachet (1979)* only PGE1 and PGA2 have been shown to inhibit noradrenaline pressor responses (see results) but at concentrations below the threshold for increasing cyclic AMP levels. Those prostaglandins which potentiated mesenteric vascular pressor responses also increased cyclic AMP levels. These data provide evidence against the hypothesis that increases in cyclic AMP mediated prostaglandin inhibition of mesenteric vascular responses.

In preparations perfused with calcium free buffer pressor responses to noradrenaline remain stable for a considerable period of time (see results) while superfusion of the intact rat mesenteric vasculature with calcium free solution (10 mM EDTA) failed to significantly attenuate

arteriolar constriction induced by noradrenaline or adrenaline (*Altura 1978*). These data indicate that noradrenaline contractions in the mesentery are independent of extracellular calcium.

16,16 diMePGE2 (2.6×10^{-11}M) potentiated noradrenaline responses in preparations perfused with calcium free buffer however the potentiation was not stable. This might be explained by the findings of *Deth and Van Breeman (1974)*. These researchers identified two different stores of intracellular calcium, one subserving activation and the other constriction. They also discovered that the activator calcium stores could not be replenished by the stores which functioned as uptake sinks during relaxation. It is therefore possible that 16,16 diMePGE2 facilitated the release of calcium ions from the activator stores and facilitated the resequestration and/or extrusion of calcium while simultaneously preventing the replenishment of the activator stores from the low concentration of calcium (10^{-6} M) present in the calcium free buffer; in normocalcium buffer higher concentrations of 16,16 diMePGE2 increased vascular tone suggesting an increase in cytosolic calcium, perhaps from the ECF. These concentrations potentiated potassium pressor responses while inhibiting noradrenaline contractions.

It would appear that at least three sites involved in cellular calcium homeostasis are affected by the prostaglandins and perhaps each prostaglandins affect these sites differently resulting in the observed different actions in this preparation. The definitive proof for this mechanism of action will have to await further advances and availability of sensitive techniques to follow subtle changes in calcium levels within these small smooth muscle cells.

In terms of prostaglandin actions in this preparation there is some disparity between the data presented here and that of *Coupar and McLennan (1978)*. They reported that PGE2 had no effect on noradrenaline responses whereas the present data and that of *Malik et al (1976)* and *Kondo et al (1979)* showed that it did. This is most likely the result of differences in methodology and buffer composition. *Coupar and McLennan (1978)* used a buffer much closer in composition to that originally described by *Krebs-Henseleit (1932)* whereas that used in the present work is a modified buffer made by substituting MgCl2 for the MGSO4 and lowering the glucose concentration from 10 mM to 5.5 mM while increasing the NaCl to compensate. Also all the experiments reported here were done at 30°C instead of 37°C, because at the lower temperature the responses to both exogenous noradrenaline and periarterial nerve stimulation remained stable for up to 10 hours. At the higher temperature responses to periarterial nerve stimulation decayed or became erratic within 4 hours. Perhaps the major difference which prevented this group from seeing any effect of PGE2 was their flotation of the preparation in its own effluent, with periodic washes with fresh buffer. Their preparations (from rats average weight 310 g) were as a result exposed to PGE2 levels ranging from 60-200 pg/ml (Fig. *43*, 300 g rat). It is possible that they were already working against a background of PGE2 stimulation of vascular reactivity. In addition the noradrenaline added cumulatively to the perfusate would simultaneously increase prostaglandin levels (see Table 2). Their preparations were thus also immersed in effluent containing noradrenaline and this could also increase the release of prostaglandins from the vasculature and attendant tissues (e.g. mesenteric fat) further compounding the situation. Their ability to demonstrate potentiation with PGAl can be

explained by the fact that it is the product of acid hydrolysis of PGE1 and would not normally be in the effluent.

Our preparations were suspended in a thermostatted water jacket and the effluent flowed directly into a drain, both *Malik et al (1970)* and *Kondo et al (1979)* have used similar systems. These groups have reported that PGE2 potentiated noradrenaline responses, which agrees with the present results.

While the effects of TXB2 on rat mesenteric vascular responses have not been previously reported, it has been shown to potentiate noradrenaline responses in the rat spleen (*Malik 1978*) and to constrict the canine pulmonary vascular bed (*Wasserman and Griffin 1977*). In the rat mesenteric vascular bed, TXB2 was a weak inhibitor of noradrenaline pressor responses with no effect on potassium responses.

TXA2 (from aggregating platelets), the parent compound of TXB2, constricted the mesenteric vasculature (two experiments). This data is in agreement with the findings of *Needleman et al (1977)* and *Tuvemo (1978)* that TXA2 constricts all blood vessels.

The experiments shown in Figures *9-13* indicate that vascular reactivity can be preferentially modified by either PGE1, PGE2, PGA1, PGA2, PGF2α or PGI2. The inactivity of PGD2 and the poor efficacy of TXB2 show that not all the metabolites of arachidonic acid will exert a physiological effect. Arachidonic acid itself infused into the mesenteric vasculature in concentrations above 50 ng/ml potentiated noradrenaline and potassium pressor response. No changes in baseline perfusion were seen. This suggested that the levels of PGI2 synthesized by the endothelial cells in the presence of excess arachidonic acid was insufficient to inhibit

noradrenaline responses. The effect seen is compatible with the view that the prostaglandin responsible was either PGE2, a mixture of PGE2 and PGF2, or an unidentified metabolite of arachidonic acid. The stability of the baseline was indicative of low PGF2α concentrations because as noted in results, PGF2α raised baseline perfusion pressure. The precursor of the 1 series prostaglandins, dihomogammalinolenic acid has been shown to have an effect similar to that of PGE1 (*Manku et al 1979*). Thus the precursor fatty acids yield results compatible with the view that conversion to an active metabolite was responsible for their biological actions. Indeed this has been shown to be the case for arachidonic acid in the perfused mesenteric and femoral beds of the dog (*Dusting et al 1978*). In this study sodium linoleate was inactive, indicating that the effect seen was specific for the cylooxygenase substrate.

Recently *Malik (1978)* reported that in another rat vascular bed (splenic) PGE1, PGE2 and arachidonic acid inhibited noradrenaline responses (contraction) whereas both PGF2α and TXB2 potentiated these responses. Thus within the same specie PGE1 and PGE2 have opposite effects in the mesenteric and splenic vasculature. In contrast PGF2α potentiated noradrenaline responses in the splenic vasculature as it did in the mesenteric vascular bed.

This type of data and the dose response curves presented here indicate that protaglandins differentially modulate vascular reactivity. It is apparent that either the prostaglandin receptors for PGE1 and PGE2 are different in the splenic and mesenteric vasculature or that interaction of prostaglandins with similar receptors in both types of vascular muscle resulted in the activation of different intracellular processes.

In receptor binding studies *Siegl et al* (*1979*) reported that the PGI2 receptor in human platelets did not bind other prostaglandins to any significant degree. They tested seven prostaglandins (PGI2, PGE1, PGE2, PGD2, PGF1α, 6 keto PGF1α and PGF2α) for their ability to cross react with the PGI2 receptor, and found that PGE1 exhibited the greatest cross reactivity (5%). The PGI2 metabolite 6 keto PGF1α (1-100 μM) was devoid of any affinity for the PGI2 receptor while the other prostaglandins exhibited < 0.3% cross reactivity. In another binding study using platelets *Shafer et al* (*1978*) reported that either PGE1, PGI2 or PGE2 could displace radiolabelled PGE1 from its platelet receptor. In vascular smooth muscle (splenic vasculature) *Malik* (*1979*) showed that either PGF2α or TXB2 antagonized PGE2 inhibition of noradrenaline responses. Using the perfused rat mesenteric vascular bed *Manku et al* (*1977*) found that PGE1 antagonized PGE2 facilitation of noradrenaline responses. Later *Ally et al* (*1977*) reported that low concentrations of PGE1 antagonized PGI2 actions in the rat mesentery whereas *Manku et al* (*1978*) found that higher concentrations of PGE1 potentiated PGI2 actions. Thus depending on the prostaglandin present and its concentration, the action of the prostaglandin added may be enhanced or attenuated. The primary determinant will be their relative affinities for the available receptors.

Additional support for the view that the rat mesenteric vasculature has several types of prostaglandin receptors was provided by data obtained using 16,16 diMePGE2. This analogue of PGE2 had a spectrum of activity different from that of its parent compound. To reconcile these findings, it is necessary to accept the suggestion that the two dimethyl groups altered the specificity thus allowing binding to, and activation of

PGE2, PGE1 and PGF2α receptors. This interpretation is consistent with the observed data, showing that 16,16 dimethyl PGE2 potentiated potassium pressor responses in a manner similar to PGE2 and PGF2α;inhibited noradrenaline responses like PGE1 and elevated the basal perfusion pressure, an action similar to that of PGF2. Recent data from *Gryglewski and Nicolaou (1979)* showed that a prostacyclin analogue, 6,9 thiaprostacyclin combined the properties of PGI2, TXA2 and PGE2 (or PGF2). Again the specificity for the receptors had been altered by the molecular changes which stabilized the prostaglandin. Other data indicating the existence of different types of prostaglandin receptors have been provided by *Pfaffman and Chu-Sun (1979)*. They reported that PGF1α and PGE1 potentiated phenylephrine contractions of rabbit aorta; however, if the preparation was exposed to high concentrations of phenylephrine, PGF1α caused a further contraction whereas PGE1 was inactive. Again this data is compatible with the view that the prostaglandins were acting at different sites. *Pfaffman and Chu-Sun (1979)* suggested that the lack of effect of PGE1 in the presence of high phenylephrine could be a result of desensitization of the receptor. The data presented here (Figs. *21, 22, 23, 24, 25*) show that desensitization had not developed as a result of repeated exposure to high concentrations of prostaglandins (PGE2, PGE1, TXB2 or 6-keto-PGF1α). Therefore, it is highly possible that a prostaglandin synthesized by the vessels in response to the high phenylephrine had already activated the PGE1 receptor, as a result the added exogenous PGE1 had no discernible effect.

The idea that different prostaglandin receptors exist had been suggested by *Bennet (1974)* and more recently re-emphasized by *Langs et al (1977)*. Using radiolabelled prostaglandins *Johnson et al (1974)*

demonstrated that uterine contraction was correlated with PGE1 binding while *Lefkowitz et al* (*1977*) implicated purine nucleotides in the regulation of these receptors. The importance of uterine disulphide and sulphydryl groups for prostaglandin action and binding was stressed by *Johnson et al* (*1974*) while *Rao* (*1975*) demonstrated the requirement for positively charged ions for PGF2α binding to corpus luteum sites. Utilizing subcellular fraction of the corpus luteum *Mitra and Rao* (*1978*) have demonstrated specific prostaglandin binding sites on the rough endoplasmic reticulum and Golgi fractions in addition to the plasma membrane. Similar data has been reported by *Dyer et al* (*1979*) in vascular smooth muscle (aorta, vena cava). Thus specific prostaglandin binding sites are not restricted to the plasma membrane. This suggests that endogenously synthesized prostaglandins can perhaps exert profound effects by interacting with similar intracellular receptors.

One aspect of prostaglandin action, rarely mentioned in smooth muscle studies, is their ability to cause contractions in preparations simultaneously treated with indomethacin and noradrenaline. As shown above (Fig. *17*) depolarizing stimuli were unable to cause contractions in preparations prepared as described earlier. Prostaglandins injected as a bolus, singly or in combination, did cause a contraction (Fig. *18, 19, 20*). This cannot be explained by an ionophore-like or depolarizing action of the prostaglandin(s), because neither high KCl nor CaCl2 concentrations contracted the smooth muscle. These data underscore the essential requirement for the presence of a single or several prostaglandins for the activation of the excitation-contraction coupling mechanism or the activation of the contractile proteins. This does not mean that the prostaglandin must cross the plasma membrane since activation of a plasma

membrane receptor could bring about changes intracellularly.

One possible explanation for the necessity for the prostaglandin(s) is that a prostaglandin and calcium must bind simultaneously at complimentary sites. These sites do not necessarily lie adjacent to each other, however the rapid onset of contraction (Figs. *3, 18, 19, 20*) upon the injection of prostaglandin suggests that the prostaglandin site might be on the plasma membrane, though it does not prove it.

It has been shown by us and several other researchers that low concentrations (nanogram/ml range) of prostaglandins can reverse indomethacin inhibition of mesenteric vascular reactivity (*Malik and McGiff 1976; Manku and Horrobin 1976; Ally et al 1977; Coupar and McLennan 1978; Kondo et al 1979*). When prostaglandin biosynthesis was blocked by a cyclooxygenase inhibitor, pressor agents could trigger a contraction only if exogenous prostaglandins were present in the buffer,with the amplitude of the contraction being dependent on the concentration of prostaglandin added to the perfusate (*Manku and Horrobin 1976; Coupar and McLennan 1978*). This argues in favour of some role of prostaglandins (modulatory) in vascular reactivity. Any argument that the concentrations of indomethacin used prevented calcium uptake has been elegantly nullified by the findings of *Northover* (*1968*). This researcher reported that neither indomethacin (100 µg/ml, 2.4×10^{-4} M) nor flufenamate (both cyclooxygenase inhibitors) inhibited the uptake of calcium by the rat anterior mesenteric artery; in contrast amenthocaine HCl and cinchocaine HCl (local anaesthetics, membrane stabilizers) did prevent the uptake of calcium.

In the absence of noradrenaline and indomethacin high concentrations of prostaglandins can contract the mesenteric vasculature (Figs. *21-27*).

It is unlikely that such high concentrations normally occur in the milieu surrounding the vessels. These data do show that given a pathological state or traumatic condition(perhaps with some stasis of the blood), accumulating prostaglandins including PGI2 can cause vascular spasm. Of particular interest was the demonstration that PGI2 caused a contraction of the longest duration. This finding becomes more important given the fact that PGI2 is the major prostaglandin synthesized by vascular smooth muscle. A similar contractile action of PGI2 has been shown by *Pomerantz et al (1977)* on human umbilical artery, which apparently lacks any sympathetic innervation. These researchers also reported that PGI2 increased the perfusion pressure of segments of the umbilical artery. In the rat coronary vascular bed *Karamazyn et al (1978)* showed that low concentrations of PGI2 ($< 10^{-10}$M) constricted the vasculature; similar findings have been reported by *Dusting et al (1977)* in the guinea pig heart.

Thus depending on the vascular bed either a high affinity PGI2 receptor can cause constriction (*Dusting et al 1977; Karmazyn et al 1978*) or a low affinity PGI2 receptor can cause constriction (present study and *Pomerantz et al 1977*). It should not be forgotten that all the prostaglandins tested (PGE2, PGE1, TXB2, PGI2, 6 keto PGF1α, PGF2α and PGD2) contracted the vasculature though not to the same extent (Figs. *21-27*). This might indicate that at high concentrations they all activate a common receptor. The duration and shape of contractions suggest otherwise.As a result of the limited availability of the prostaglandins only 2 experiments each were done,thus statistical analysis of the contraction duration was not possible.

Effect of Nonsteroidal Anti-inflammatory Agents on Vascular Reactivity

These experiments were designed to explore the contribution of endogenous prostaglandin biosynthesis to vascular responses to pressor agents which recruit calcium primarily from either extracellular, membrane bound or intracellular stores.

Effect of indomethacin on noradrenaline pressor responses

Infusion of indomethacin (a prostaglandin cyclooxygenase inhibitor) did not alter basal perfusion pressure, but inhibited noradrenaline pressor responses. This inhibitory action of indomethacin was concentration dependent yielding a linear concentration response line (Fig. **28**). The threshold concentration for inhibition was 1 μg/ml (2.8×10^{-6} M), the concentration producing 50% inhibition (IC50) was 8.0 μg/ml (2.2×10^{-5}M) and the maximum inhibition at 64 μg/ml (1.9×10^{-4} M) indomethacin respectively. In two experiments angiotensin II pressor responses were inhibited by indomethacin (IC50 2.2×10^{-5} M).

Effect of indomethacin on pressor responses to depolarizing stimuli

Indomethacin in three experiments inhibited responses to both potassium and calcium ions over a similar concentration range as shown in Figure *28*. The IC50 concentrations were within $\pm$ 20% of that for noradrenaline pressor responses (range 6 to 10 μg/ml; 1.7×10^{-5} to 2.8×10^{-5} M). The maximal concentration of indomethacin abolishing pressor responses did not differ (64 μg/ml, 1.9×10^{-4} M) for both pressor agents.

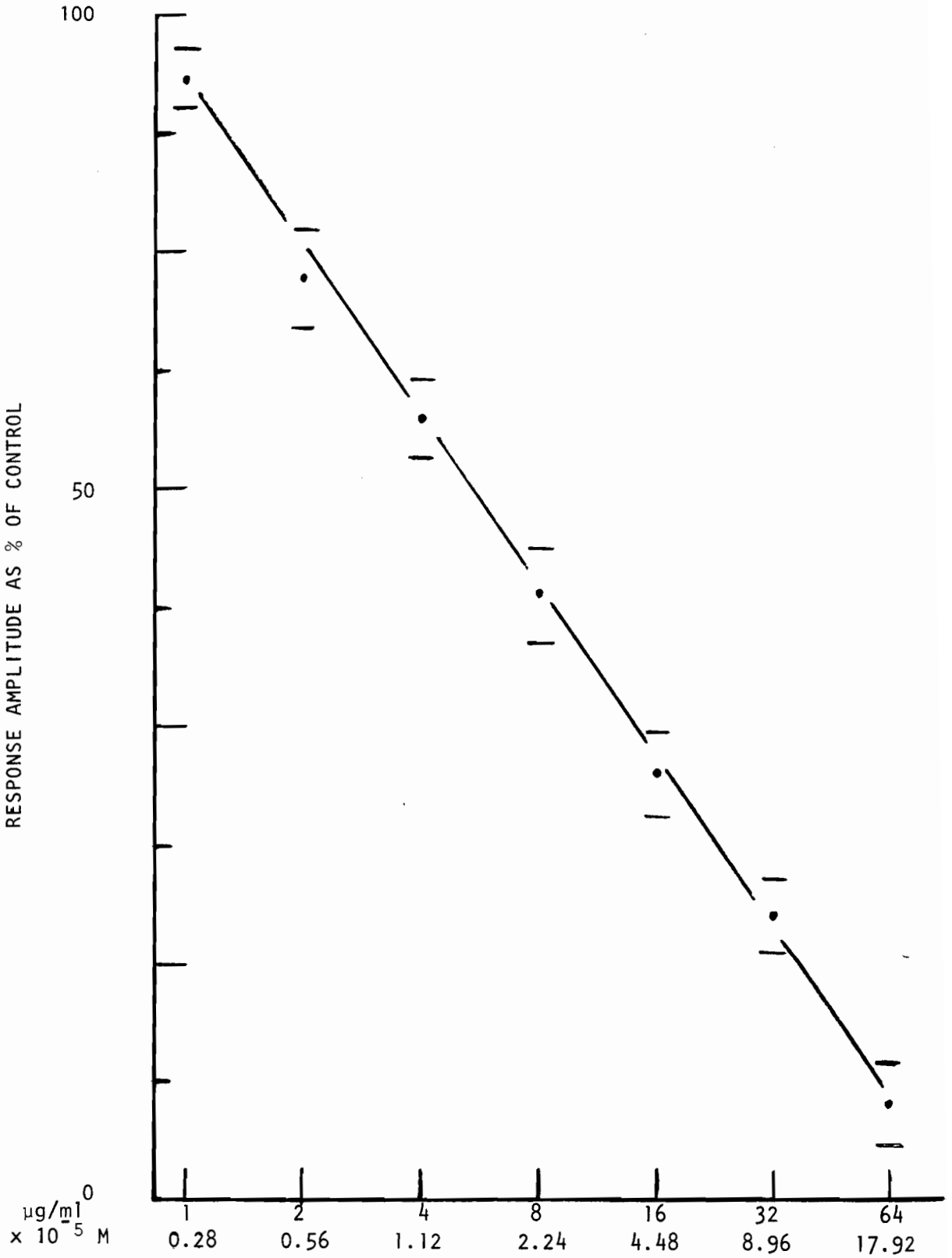

Fig. 28. Inhibition of pressor responses to noradrenaline by increasing concentrations of indomethacin in the perfusate. Before the addition of indomethacin, four injections of noradrenaline were made and the mean taken as 100%. Responses in the presence of indomethacin were expressed as percentages of this mean. Each point shown represents the mean ± SE , n = 6.

Effect of thromboxane A2 synthetase inhibitors on vascular reactivity

The thromboxane synthetase inhibitor imidazole inhibited both noradrenaline and angiotensin II pressor responses (imidazole, IC50 5.7 x 10^{-4} M). Imidazole in concentrations up to 2 x 10^{-3} M did not inhibit potassium pressor responses (two experiments).

In preparations perfused with indomethacin (50 µg/ml, 1.4 x 10^{-4} M) and with vascular reactivity restored by PGE2 (5 ng/ml, 1.4 x 10^{-8} M), imidazole did not inhibit noradrenaline pressor responses.

Imidazole analogues with substituents in positions other than the 1-position displayed lower potency as inhibitors of thromboxane A2 synthetase (*Tai and Yuan 1978*). One of the more potent analogues described was 1-benzyl imidazole. In the perfused mesenteric vascular preparation 1-benzyl imidazole was indeed more potent than imidazole and exhibited the same characteristics as imidazole (specific inhibition of noradrenaline pressor responses), whereas 2-methyl imidazole and imidazole-4 acetic acid were inactive.

Effect of 1-benzyl imidazole and nicotinic acid on vascular reactivity

1-benzyl imidazole (0.95 x 10^{-6} M to 1.5 x 10^{-5} M) inhibited noradrenaline pressor responses. Over this same concentration range potassium pressor responses were unchanged (Figs. *29*, *30*). In two preparations blocked by indomethacin (50 µg/ml, 1.4 x 10^{-4} M) and vascular reactivity restored by PGE2 (5 ng/ml, 1.4 x 10^{-8} M), 1-benzyl imidazole was not inhibitory. As can be seen in Figure *29* no significant alteration in baseline perfusion pressure was caused by 1-benzyl imidazole. Complete

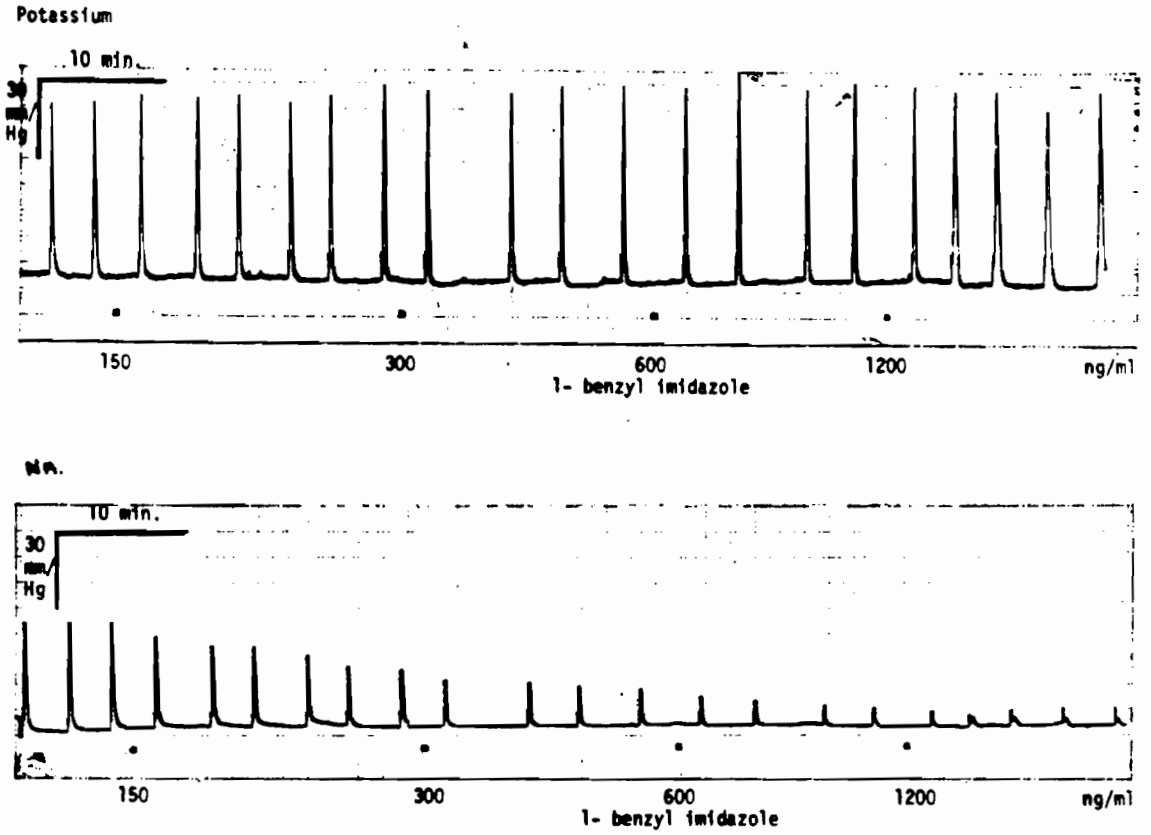

Fig. 29. Effects of 1-benzyl imidazole on pressor responses. Recording was made with the mesenteric preparation. Upper figure: pressor response to a fixed bolus injection of potassium ions, 1-benzyl imidazole did not inhibit potassium responses. Lower figure: pressor response to a fixed bolus injection of noradrenaline, 1-benzyl imidazole inhibited these responses in a concentration dependent manner. Note that no changes in basal perfusion pressure were seen (150 ng/ml = 9.5×10^{-7} M).

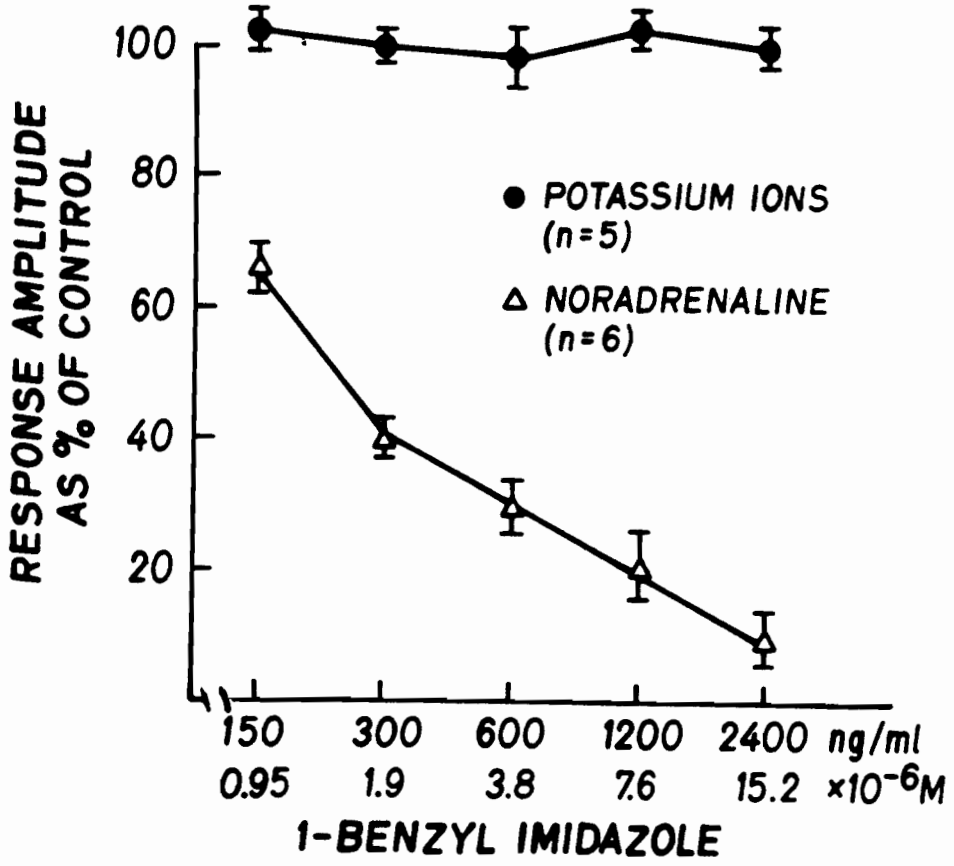

Fig. 30. The effects on the pressor responses to noradrenaline and potassium stimulation by increasing concentrations of 1-benzyl imidazole values shown are the mean ± SE, n=5 or 6. Prior to the addition of 1-benzyl imidazole, four test injections of the appropriate pressor agent were given and the mean taken as 100%. Responses in the presence of 1-benzyl imidazole were expressed as percentages of this.

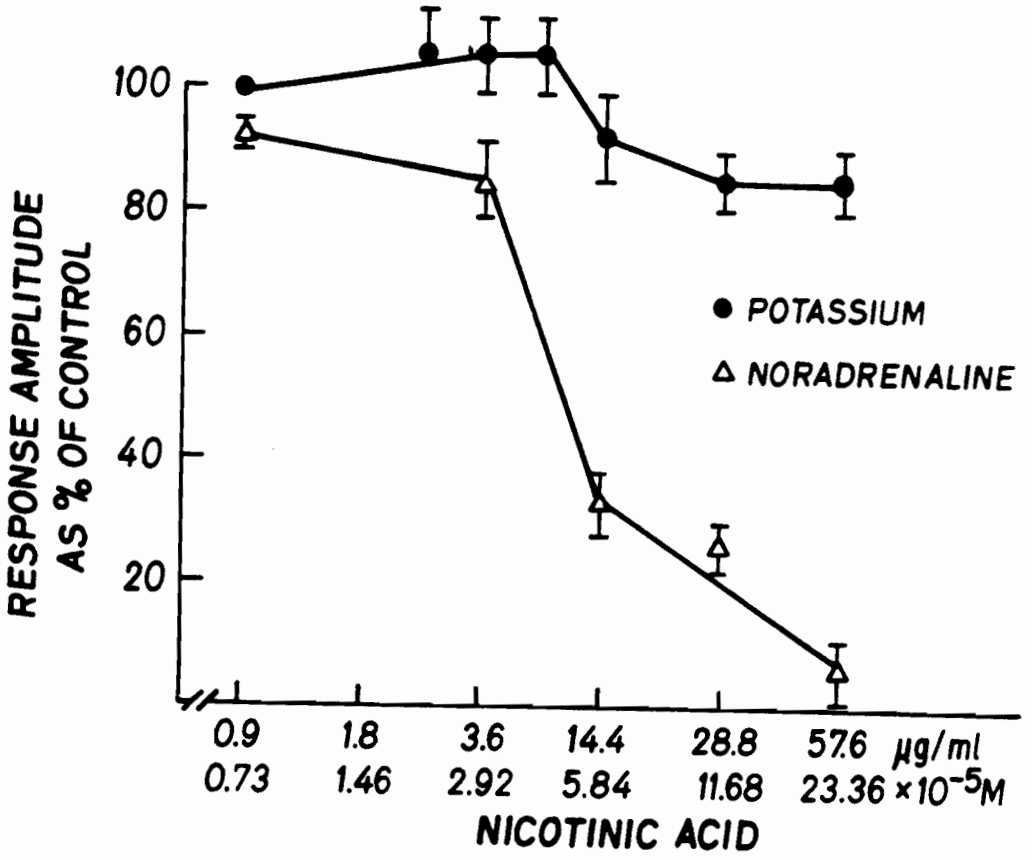

Fig. 31. Effects of nicotinic acid on noradrenaline and potassium pressor responses in the mesenteric vascular bed. Other conditions as described in Fig. *30*. Each point represents the mean ± SE , n = 6.

concentration response curves for 1-benzyl imidazole effects on pressor responses are shown in Figure *30*.

In other experiments using nicotinic acid (another thromboxane A2 synthetase inhibitor) a profile of inhibitory actions similar to that of imidazole and 1-benzyl imidazole was seen. Nicotinic acid preferentially inhibited noradrenaline pressor responses; potassium pressor responses were inhibited by approximately 20% by a concentration of nicotinic acid (2.4×10^{-4} M) which abolished noradrenaline responses (Fig. *31*). In three preparations perfused with indomethacin (1.9×10^{-4} M) plus PGE2 (1.4×10^{-8} M), nicotinic acid did not inhibit vascular pressor responses upon stimulation with noradrenaline.

Summary

These data indicate that cyclooxygenase and thromboxane A2 synthetase inhibitors can inhibit vascular responsiveness. Thus endogenous prostaglandin biosynthesis and/or action appears necessary for vascular responsiveness to noradrenaline, angiotensin II and potassium ions. Further support for this would be the ability to demonstrate a temporal relationship between changes in prostaglandin synthesis and pressor responses. These data are presented in the next section.

DISCUSSION

In 1968 *Northover* reported that indomethacin can inhibit the effects of all pressor agents tested in the isolated perfused rat mesenteric vascular bed. Subsequent papers from this laboratory (*Northover 1971, 1972*) explored the mechanism of this inhibition. *Northover* (*1968*) suggested that indomethacin directly affected calcium movements; however, in the mesenteric artery he found that high concentrations of indomethacin (100 µg/ml) did not inhibit calcium uptake. He concluded that indomethacin affected calcium interaction with or its activation of the contractile proteins.

As shown in Figure *28*, indomethacin inhibited vascular responsiveness in a log linear concentration dependent manner. *Manku and Horrobin* (*1976*) have shown that indomethacin inhibited vascular responses to injected angiotensin, vasopressin, histamine, serotonin, calcium ions and potassium ions. As found here a detectable inhibition by indomethacin was seen at 1 µg/ml (2.8×10^{-6} M) while abolition was complete at concentrations greater than 64 µg/ml (1.9×10^{-4} M).

It is possible that the effects of high concentrations of indomethacin were independent of its inhibitory effect on the cyclooxygenase. However, in preparations which had previously been inhibited by aspirin or mefenamic acid and vascular reactivity restored with PGE2, indomethacin (1.9×10^{-4} M) had no further inhibitory effect. If indomethacin action had been non specific, i.e. unrelated to prostaglandin biosynthesis, some inhibition should have been present (*Manku and Horrobin 1976*).

The effects of indomethacin can be specifically reversed by the addition of either PGE2 or PGF2α to the perfusate (*Manku and Horrobin 1976; Coupar and McLennan 1978;* and previous section this thesis).

As shown earlier the effects of various prostaglandins on vascular reactivity to different stimuli are complex. Indomethacin when perfused through the vasculature inhibits all prostaglandin biosynthesis (*Flower 1974*). It is becoming apparent that the concentrations of indomethacin and other agents normally used, while inhibiting stimulated prostaglandin biosynthesis, do not inhibit basal prostaglandin biosynthesis (*Hood and Vincent 1978; Scherer et al 1978; Pace-Asciak et al 1978).* Thus while different stimuli may require the presence of a particular prostaglandin, indomethacin at high concentrations abolished the synthesis of all prostaglandins by eliminating the availability of the endoperoxides PGG and PGH.

One other problem in interpreting data with indomethacin is that cyclooxygenases from different tissues exhibit differential susceptibility to inhibition. An example of this is that the IC50 of indomethacin in platelets was 0.17 μM(*Smith and Willis 1971*) while that in aortic microsomes was 2.77×10^3 μM (*Gryglewski et al 1976;* see Table 2 and 3 for a range of IC50's in different tissues).

Manku and Horrobin (*1976*) and *Coupar and McLennan* (*1978*) have shown that the amount of prostaglandin needed to reverse indomethacin inhibition of mesenteric vascular reactivity appears to be positively correlated with the concentration of indomethacin used, higher prostaglandin concentrations being required when higher concentrations of indomethacin were used.

Granted that in this vascular bed low concentrations of exogenous PGs (10^{-14}- 10^{-12}) have an effect on pressor responses, by how much

must endogenous prostaglandin synthesis be inhibited, to significantly alter vascular responsiveness?

Another way of testing the idea that endogenous prostaglandins play a role in vascular reactivity was to selectively alter the levels of the prostaglandin and look at its effect.

Imidazole and 1-benzyl imidazole are thromboxane synthetase inhibitors (*Needleman et al 1977; Tai and Yuan 1978*). Both of these compounds inhibited pressor responses to noradrenaline and angiotensin but not to potassium ions. In preparations perfused with indomethacin plus PGE2 neither imidazole nor its analogue, 1-benzyl imidazole, inhibited noradrenaline responses. These data indicate that the effect of these compounds was specifically related to inhibition of a stage of prostaglandin synthesis distal to indomethacin's site of action. Any possibility that imidazole or 1-benzyl imidazole were prostaglandin antagonists was simultaneously disproved.

Imidazole appears to be a selective thromboxane A2 synthetase inhibitor (*Needleman and Bryan 1977; Sun et al 1977; Nijkamp et al 1977*) and appears to compete with the endoperoxide substrate PGH2 for the active site on thromboxane synthetase (*Tai and Yuan 1978*). This then provided pharmacological evidence for a possible role of thromboxane A2 in rat mesenteric vascular reactivity.

Further pharmacological data was provided using a structurally unrelated thromboxane synthetase inhibitor, nicotinic acid (*Vincent and Zijlstra 1978*). This compound, like imidazole and 1-benzyl imidazole, inhibited noradrenaline but not potassium responses. In the experiments with indomethacin plus PGE2, nicotinic acid did not inhibit vascular

responses, supporting the view that its action was related to inhibition of thromboxane synthetase, not antagonism of prostaglandin action.

In man, nicotinic acid and its derivative (nicotinyl alcohol) produced greater vasodilation of the blood vessels of the blush areas (ears, face, neck) than of the extremeties in normal subjects. It also caused postural hypotension (*Coffman 1979*) whereas the cyclooxygenase inhibitor, indomethacin, which decreases the levels of PGE1, PGE2, PGI2, etc., caused a slight increase in blood pressure in essential hypertensives (*Ylitalo et al 1978*).

So far it has been demonstrated that prostaglandins modulate vascular reactivity in the rat mesenteric vascular bed. This is supported by the data in the previous section and that presented in this one.

In the following section, prostaglandin biosynthesis by the mesenteric vasculature and its modification by pharmacological agents is examined. In addition, some evidence is presented for TXB2 release from the perfused mesenteric vascular bed and incubates of mesenteric arterial rings.

SECTION II: PROSTAGLANDIN ANALYSIS

"The strongest evidence for a prostaglandin being a mediator of a physiological or pathological event requires the isolation, identification and quantitation of the prostaglandin produced"

Philip Needleman: Experimental criteria for evaluating prostaglandin biosynthesis and intrinsic function, Biochemical Pharmacology, 1978.

In the present study the data discussed earlier indicate that in this preparation prostaglandins may play a role as modulators of vascular reactivity. It is known that in the presence of indomethacin (1.4×10^{-4} M) responses to all stimuli are abolished. If PGE2 or PGF2$\alpha$is added to the perfusate it can reverse this inhibition (*Manku and Horrobin 1976; Coupar and McLennan 1978*). This restoration is dependent on the presence of the prostaglandin, not the stimulation of its biosynthesis.

In this vascular bed (rat mesenteric) each prostaglandin so far tested had distinct actions on vascular reactivity to potassium and noradrenaline stimulation, and on basal tone.

The quotation above should be modified to accommodate this distinction in prostaglandin effects, allowing for their modulatory or permissive actions.

Recently the isolation, identification and quantitation of prostaglandins released by the rat mesenteric vasculature was accomplished (*Wolfe et al 1979*). Utilizing the techniques of gas chromatography-mass spectrometry (LKB 900, multiple ion detector unit) the following prostaglandins were positively identified: PGE2, PGF2α, 6-keto-PGF1α and TXB2.

One limitation of this technique is that large samples are required for processing through some ten or more steps, each associated with some degree of loss of compound. It is therefore incapable of providing data following changes in prostaglandin levels over small intervals of time.

It was with this in mind that radioimmunoassay of the prostaglandins was initially undertaken. The data presented below is designed to reflect changes in prostaglandin levels, in a series of samples collected at small time intervals.

Time Course of Prostaglandin Release by the Mesenteric Vascular Bed

Prostaglandin biosynthesis was measured by assaying the release of immunoreactive PGE2. The mesenteric vascular bed was perfused continuously at a flow rate of 3 ml/minute from the moment of cannulation of the mesenteric artery. One minute samples of the perfusate were collected at 5 minute intervals and PG -like material determined by direct radioimmunoassay of the perfusate.

PGE2 release

In Figure *32*, the time course of the release of PGE2 by the isolated mesenteric vascular bed of a 160 g male rat is shown. The rate of release was initially high and fell within 1 hour to a stable rate of release (25 pg/ml or 75 pg/minute). These data also show the reproducibility of the radioimmunoassay.

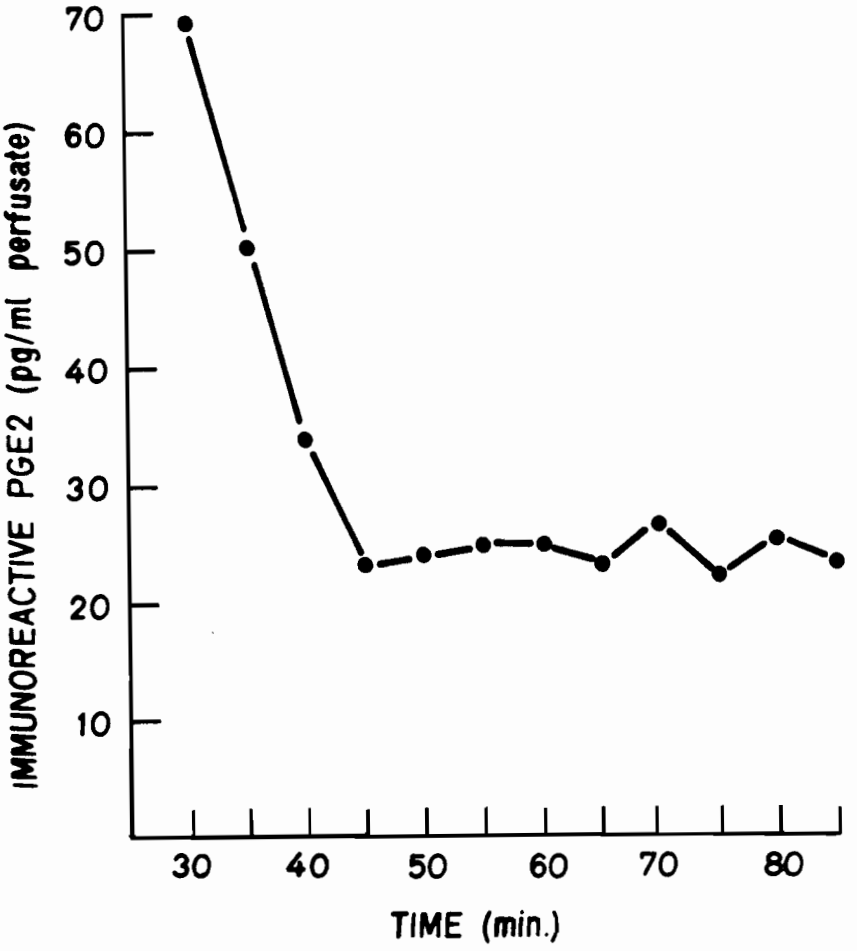

Fig. 32. Time course of the release of PGE2 by the isolated rat mesenteric vascular bed. Samples were collected every five minutes for one minute periods and analyzed by radioimmunoassay.

Ordinate: Concentration of PGE2 in the perfusate (pg/ml multiply by 3 to convert to pg/min).

Abscissa: Time (min.) from the cannulation of the mesenteric artery.

PGE2 and 6-keto-PGF1α release

The time course of PGE2 release was not unique to PGE2. In another experiment the perfusate collected at each 5 minute interval was assayed for both PGE2 and 6-keto-PGF1α (the spontaneous breakdown product of PGI2). The values obtained were plotted using semilogarithmic paper over the same time course (Fig. *33*). As can be seen the rate of release of PGE2 from this preparation taken from a 280 g male rat (Fig. *33*) was approximately twice that found for the 150 g male rat (Fig. *32*). At 1 hour (post cannulation) their respective rates of PGE2 release had stabilized at 180 pg/minute and 75 pg/minute. The rate of release of immunoreactive 6-keto-PGF1α by the larger vascular bed (280 g rat) fell from 3600 pg/ml (10,800 pg/min) to approximately a stable rate of release of 1700 pg/ml (4100 pg/min). The release of immunoreactive PGE2 fell from 195 pg/ml (385 pg/minute) to 58 pg/ml (174 pg/minute). The time course for the decrease of PGE2 and 6-keto-PGF1α release paralleled each other (Fig. *33*).

Temporal Correlation of Prostaglandin Biosynthesis with Changes in Pressor Response Amplitude

Time course of indomethacin inhibition of noradrenaline pressor responses

In 6 experiments each, the time course of the inhibition of responses by two concentrations of indomethacin was determined. The results are shown in Figure *34*. In the presence of a low concentration of indomethacin (8 μg/ml, 2.2×10^{-5} M) pressor responses declined, plateauing within 15 - 20 minutes at a new equilibrium. In a similar manner responses of preparations exposed to a high concentration of indomethacin (40 μg/ml, 1.1×10^{-4} M) fell to a new equilibrium over a similar period of time.

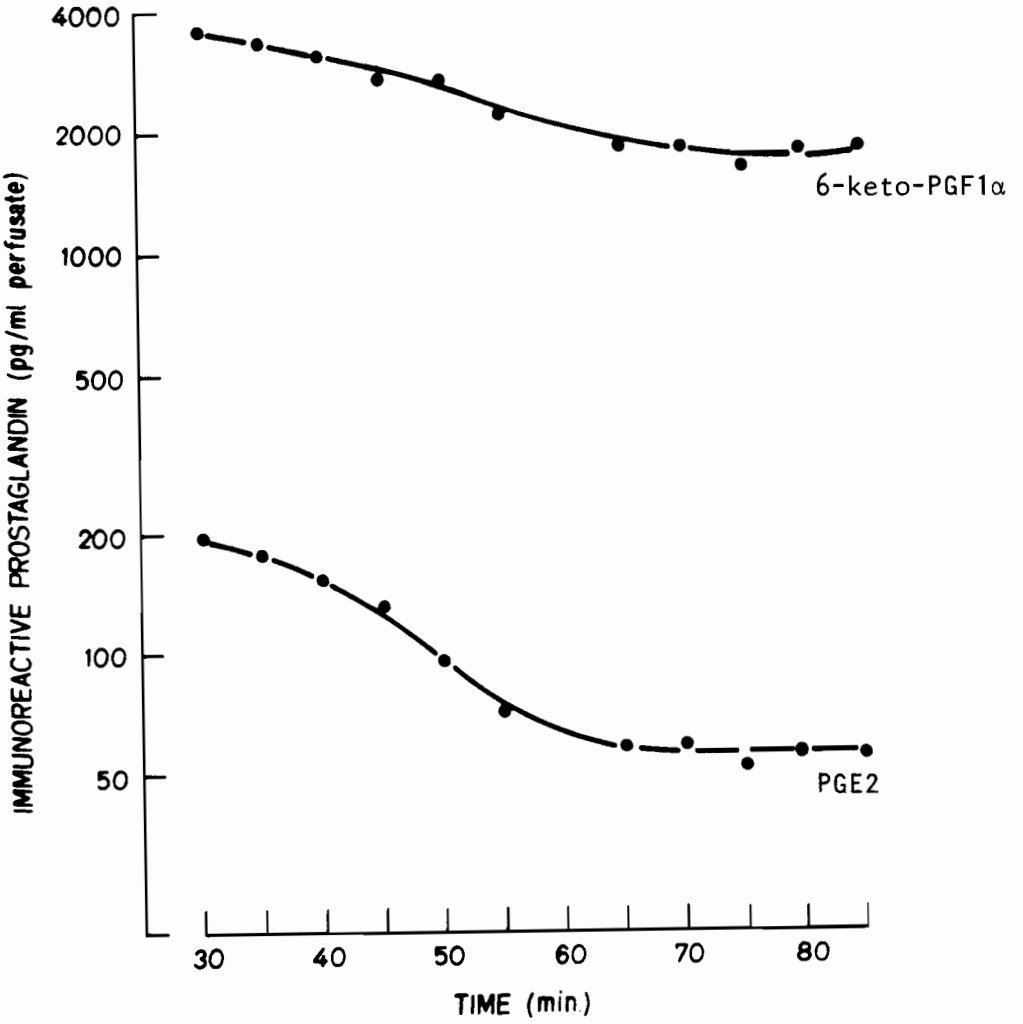

Fig. 33: Time course of the release of prostaglandins from the isolated rat mesenteric vascular bed.

Ordinate: concentration of 6-keto-PGF1α or PGE2 in the perfusate (pg/ml multiply by 3 to convert to pg/min.).

Abscissa: time(min) from cannulation of the mesenteric artery.

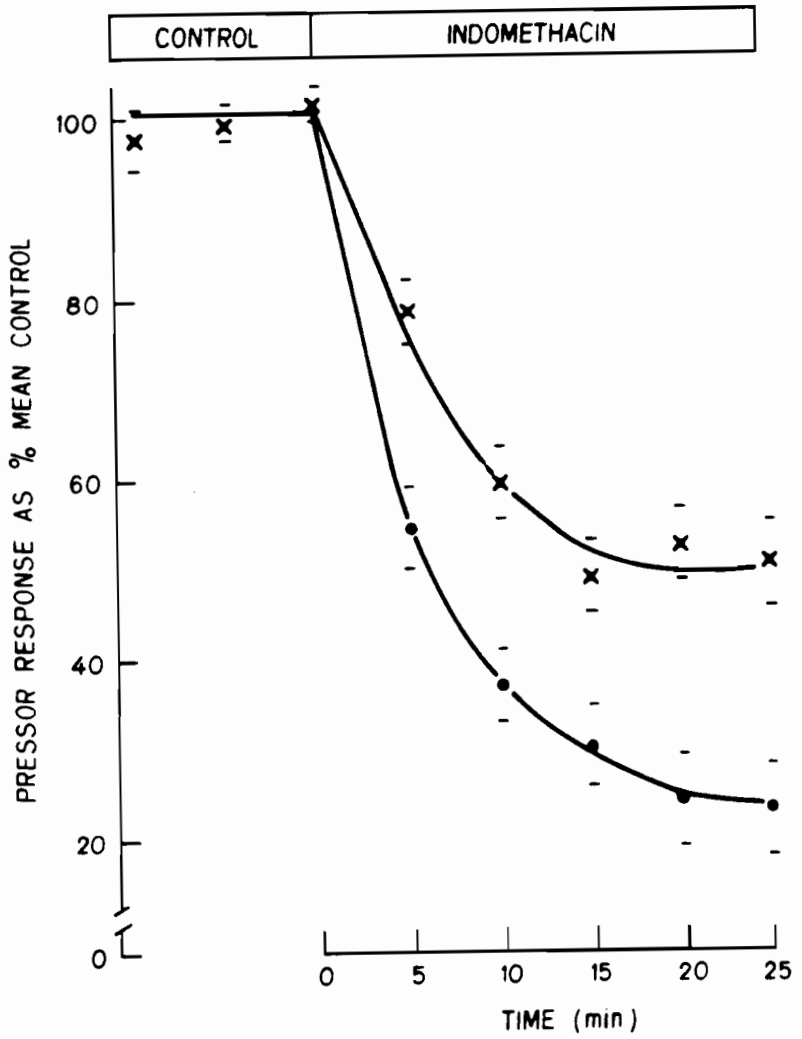

Fig. 34: Time course of indomethacin inhibition of noradrenaline pressor responses in the isolated rat mesenteric vascular bed.

Ordinate: pressor response to a fixed concentration of noradrenaline as a percentage of mean control response amplitude.

Abscissa: time (min) from the addition of indomethacin to the buffer resevoir: (X) 2.2×10^{-5} M (•) 1.1×10^{-4} M.

Each data point shown represents the mean ± SE for six experiments.

Time course of indomethacin inhibition of prostaglandin biosynthesis

Indomethacin inhibition of prostaglandin biosynthesis was monitored by assaying for immunoreactive PGE2. As before two concentrations of indomethacin were used (8 μg/ml, 2.2×10^{-5} M, and 40 μg/ml, 1.1×10^{-4} M). The preparations were equilibrated for 1 hour. After this period, 1 minute fractions of the perfusate were collected every 5 minutes over a 20 minute interval prior to the addition of indomethacin. Thereafter, sampling was continued for another 30 minutes. Within 15 minutes of adding either concentration of indomethacin the rate of release of PGE2 had fallen to a new basal level (Fig. *35*). These time courses were similar to those shown earlier (Fig. *34*) for the decline in mechanical responses to noradrenaline. The control rates of release of PGE2 in each experiment were 17 pg/ml and 30 pg/ml. After indomethacin (2.2×10^{-5} and 1.1×10^{-4} M) the new basal rates of release were 14 pg/ml and 9 pg/ml respectively. The flow rate in both cases was 3 ml/min.

Stimulation of Prostaglandin Biosynthesis by Noradrenaline (NA) and its Inhibition by Indomethacin

Preparations were equilibrated at a flow rate of 3 ml/min for 2 hours before beginning the experiment. Samples of the perfusate were collected before noradrenaline stimulation of the vascular bed (basal). The noradrenaline injections doubled the release of PGE2 (basal 67.3 ± 1.87 pg/ml, to NA stimulated 160.8 ± 16.19 pg/ml). The amount of immunoreactive 6-keto-PGF1 was also increased by about 40% from 1043.3 ± 122 pg/ml to 1447.2 ± 192 pg/ml in response to noradrenaline stimulation (Fig. *36*).

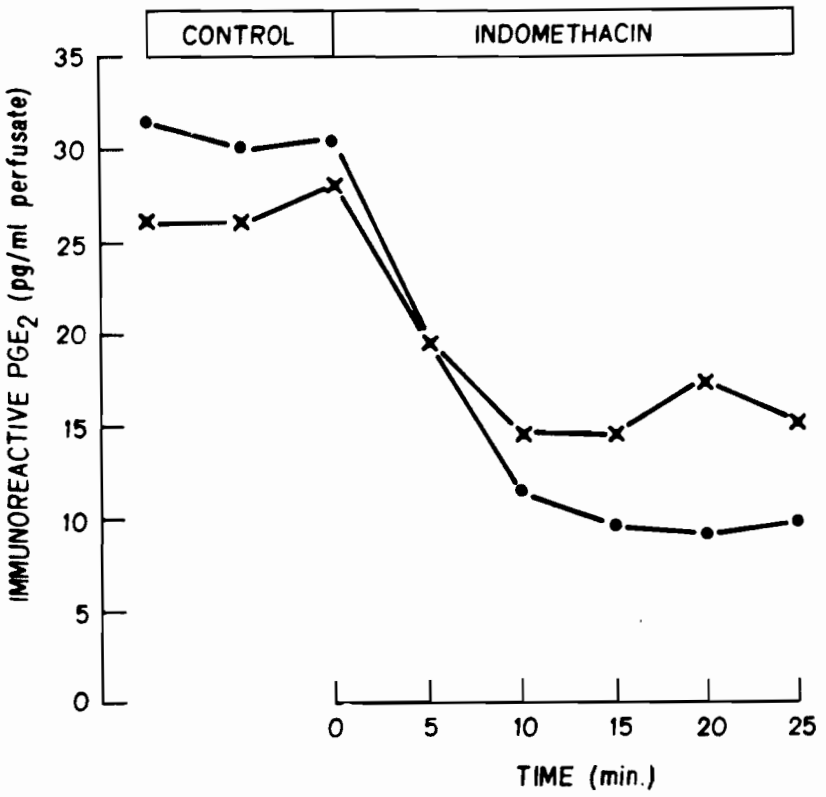

Fig. 35: Time course of indomethacin inhibition of prostaglandin biosynthesis in the isolated perfused rat mesenteric vascular bed.

Ordinate: concentration of PGE2 in the perfusate (pg/ml).

Abscissa: time (min) from the addition of indomethacin to the buffer resevoir. (X) 2.2 X 10^{-5} M; (•) 1.1 X 10^{-4} M.

Each data point shown represents the mean of duplicate determinations from a single sample in each experiment.

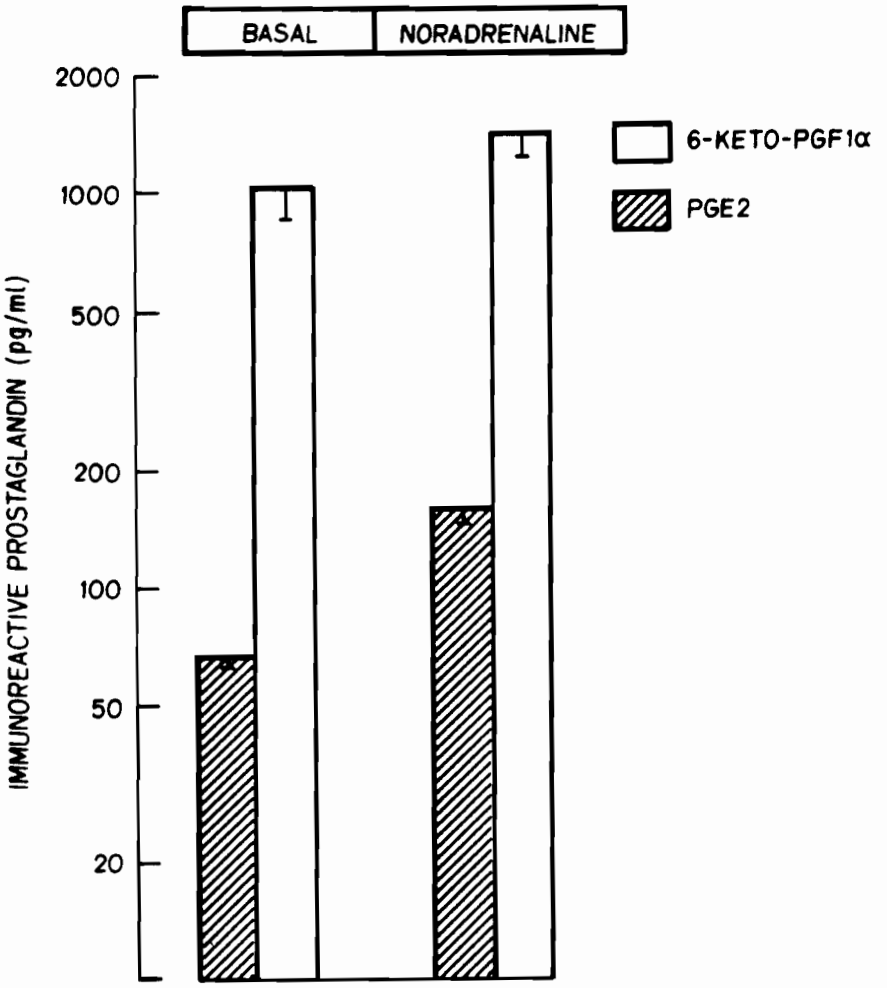

Fig. 36. Effect of noradrenaline on prostaglandin biosynthesis by the isolated perfused mesenteric vascular bed. Noradrenaline was injected intraarterially and the 1 minute fraction of the perfusate coinciding with the contraction collected and assayed by RIA for PGE2 and 6-keto-PGF1α.

Ordinate: concentration of prostaglandin in the perfusate (pg/ml; multiply by 3 to convert to pg/min).

Abscissa: PGE2 is indicated by the slashed bar and 6-keto-PGF1α by the open bar.

Values shown are the mean± SE of duplicate determinations $n \geq 5$.

Indomethacin (2.2×10^{-5} M) inhibited the basal synthesis of both PGE2 and 6 keto PGF1α. PGE2 basal release prior to indomethacin was 50.7 ± 4.06 pg/ml whereas after indomethacin the new equilibrium was at 15 pg/ml (n = 3). 6-keto-PGF1α basal pre-indomethacin release was 745.2 ± 80.6 pg/ml and after indomethacin release was reduced to 284.01 ± 30.9 pg/ml. Noradrenaline was unable to stimulate either PGE2 or 6-keto-PGF1 synthesis in the presence of indomethacin. Their respective rates of release remained at 14.35 ± 3.65 pg/ml and 284.01 ± 30.9 pg/ml (Fig. *37*).

Release of immunoreactive TXB2 by the perfused mesenteric vascular bed

The time course of TXB2 release by the mesenteric vascular bed of two preparations is shown in Figure *38*. The mesenteric artery was cannulated at t = 0 minutes and perfusion begun immediately at a flow rate of 3 ml/min. At t = 15 minutes the preparation was perfused with collagen* (12 μg/ml) for 5 minutes. At t = 20 minutes, perfusion was switched back to normal buffer and sampled for 1 minute periods at 5 minute intervals.

In a similar manner, as noted earlier for PGE2 release, the rate of TXB2 release from the individual preparations decreased with time from 75.5 pg/ml (226 pg/minute) and 65 pg/ml (195 pg/minute) at t = 30 minutes and within 20 minutes to 26 pg/ml (78 pg/min) and 35 pg/ml (105 pg/min) respectively. In this experiment the animals used were larger (350 g) than those normally used (180 g). It had been noted in preliminary experiments that with preparations from smaller animals at a fixed flow rate of

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*At a concentration used routinely by the Medical Clinic, University of Freiburg, to aggregate platelets.

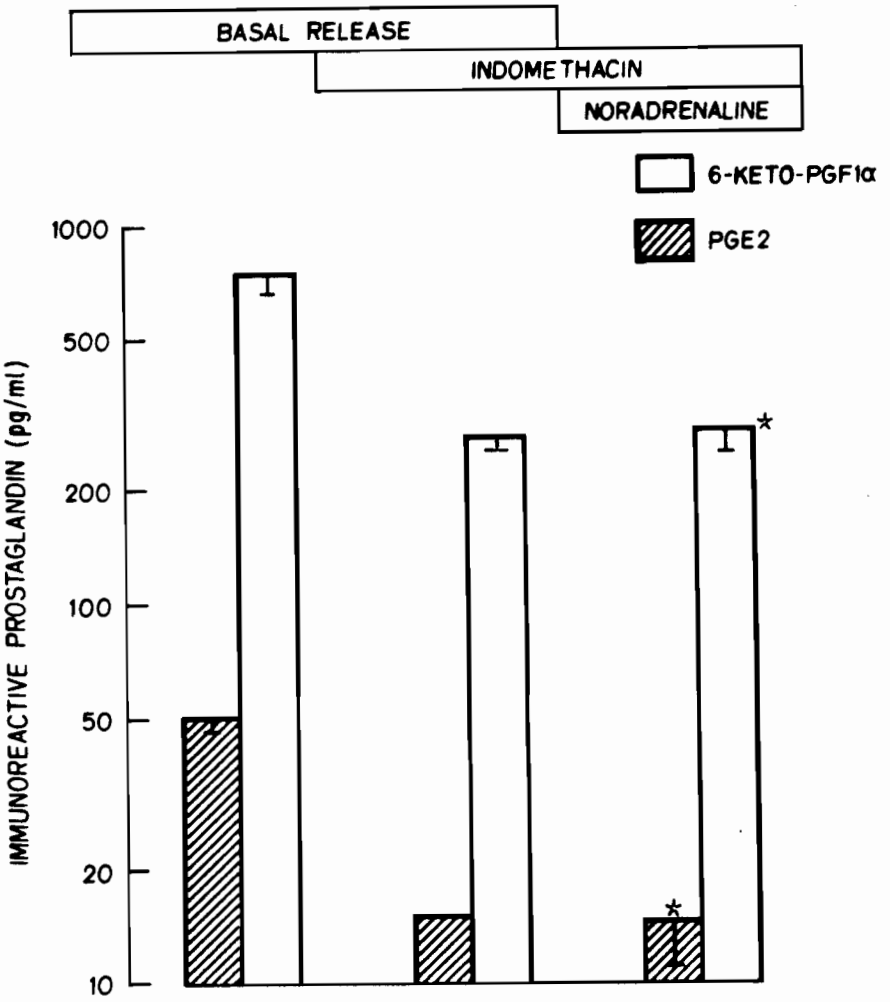

Fig. 37. Indomethacin (2.2 X 10^{-5} M) effect on basal and noradrenaline stimulated prostaglandin biosynthesis in the isolated perfused rat mesenteric vascular bed.

Ordinate: concentration of prostaglandin in the perfusate (pg/ml; multiply by 3 to convert to pg/min).

Abscissa: PGE2 is indicated by the slashed bar and 6-keto -PGF1α by the open bar.

Values shown are the mean ± SE of duplicate determinations $n \geq 5$, * $p < 0.01$ ANOVA.

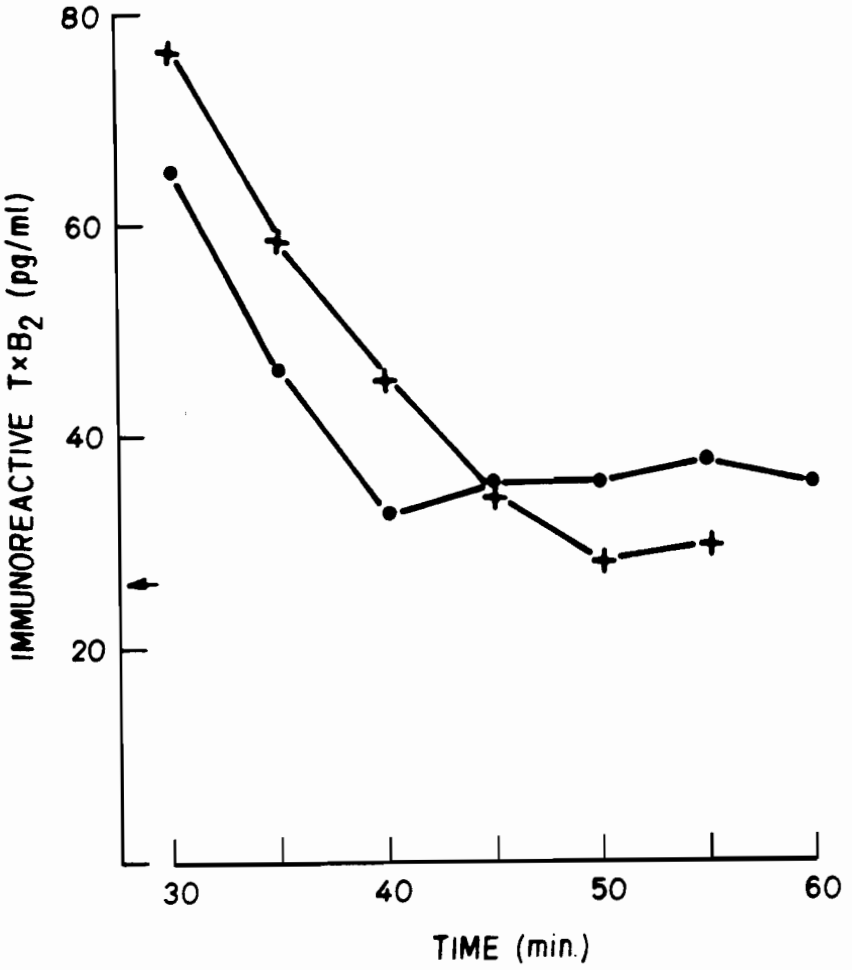

Fig. 38. Time course for the release of TxB2 from the isolated perfused rat mesenteric vascular bed of 350g animals. Aliquots of perfusate were collected every 5 mins for a 1 min period and assayed for TxB2 by RIA.

Ordinate: concentration of TxB2 in the perfusate (pg/ml; multiply by 3 to convert to pg/min).

Abscissa: time (min) from the cannulation of the mesenteric artery.

Each curve shown represents data from a single preparation. The arrow indicates the 10% confidence limit of the RIA.

3 ml/min the concentration of TxB2 in the perfusate rapidly fell below the detection limit of the radioimmunoassay. The 10% confidence limit for the TxB2 radioimmunoassay was 26 pg.

Pharmacological modification of TxB2 release by the mesenteric vascular bed

Preparations were prepared as previously described and samples collected, beginning at t = 30 minutes, every five minutes. One preparation was perfused with imidazole (100 µg/ml, 1.5×10^{-3} M) from t = 15 minutes, and the control preparation was perfused with plain buffer. Each sample collected was assayed for immunoreactive PGE2 and TxB2. The results for the control experiment are shown in Figure *39*, and that for the imidazole perfused preparation in Figure *40*.

In the control experiment both PGE2 and TxB2 levels are reasonably close, whereas in the imidazole perfused preparation PGE2 levels are approximately twice that of TxB2 for the period t = 30 min to t = 55 min. This is in agreement with the data of *Nijkamp et al (1977)* showing diversion of PG synthesis upon inhibition of thromboxane synthesis with imidazole. For this experiment preparations from rats weighing 220 - 240 g were used.

Effect of imidazole and 9,11 azo prosta 5,13 dienoic acid on TxB2 release

Both of these compounds have been shown to inhibit thromboxane synthesis in a variety of tissues. For these experiments normal weight rats were used (180 g). Preparations were perfused with drug beginning at t = 15 min and samples collected at 1 minute intervals for 5 minutes

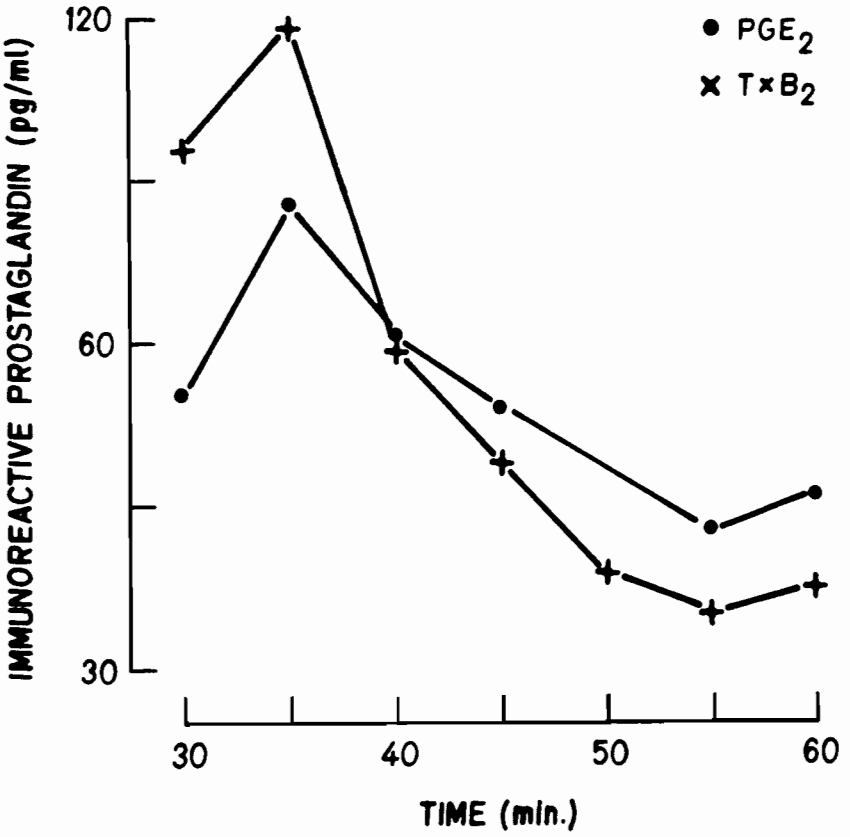

Fig. 39. Time course for the release of TxB2 and PGE2 from the isolated perfused mesenteric vascular bed of a 220 g rat. Aliquots of perfusate were collected every 5 mins and assayed by RIA for TxB2 and PGE2.

Ordinate: concentration of TxB2 and PGE2 in the perfusate (pg/ml; multiply by 3 to convert to pg/min)

Abscissa: time (min) from cannulation of the mesenteric artery.

Each point represents the mean of duplicate determinations.

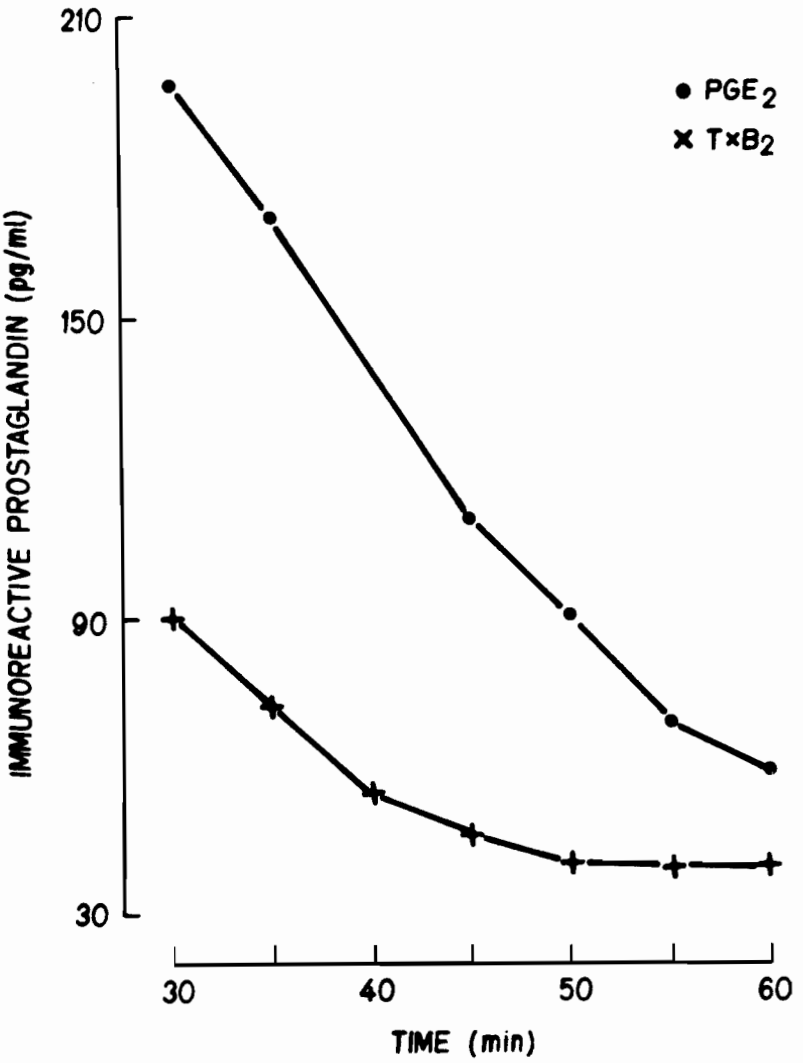

Fig. 40. Time course for the release of TxB2 and PGE2 from the isolated perfused mesenteric vascular bed of a 220 g rat. The preperation was perfused with imidazole containing buffer (1.5×10^{-3} M) beginning at t=15 min, aliquots of the perfusate were collected every 5 mins beginning at t=30 min and assayed.

Ordinate: concentration of TxB2 and PGE2 in the perfusate (pg/ml; multiply by 3 to convert to pg/min).

Abscissa: time (min) from cannulation of the mesenteric artery.

Each point represents the mean of duplicate determinations.

beginning at t = 25 min. The mean value TxB2/ml over this 5 minute period is shown in Figure *41*. Preparations were perfused with collagen containing buffer for 5 minutes from t = 5 to t = 10 min to eliminate platelets as a possible source of the TxB2.

In experiment A, control immunoreactive TxB2 was approximately 50 pg/ml, whereas in the imidazole (1.5×10^{-3} M) perfused preparation TxB2 levels were approximately 17 pg/ml (in this case extrapolated from the standard curve because the TxB2 level was below the 10% confidence level of the assay). In experiment B, control TxB2 levels were about 45 pg/ml, and in the 9,11 azo prosta 5,13 dienoic acid (3 μg/ml) perfused preparation TxB2 levels were about 24 pg/ml (this value borders on the 10% confidence limit of the assay). Thus, in both treated preparations the TxA2 synthetase inhibitors suppressed the release of immunoreactive TxB2 to levels below the 10% confidence limit of the assay.

Evidence that TxB2-like material in the perfusate was not 6-keto PGF1α cross reacting in the TxB2 RIA

In two experiments, mesenteric vascular beds from two 300 g rats were perfused for 5 hours. These preparations were perfused simultaneously with buffer from a common reservoir, all samples were similarly treated and assays performed at the same time. Samples were collected at 5 minute intervals. For sake of clarity only the values of the various PGs assayed at 1 hour intervals are shown in Figures *42* and *43*. As can be seen, prostaglandin biosynthesis increases slowly over the 4 hour period.

In Figure *42* the results of the analyses of the perfusate from animal A show that 6-keto-PGF1α, PGE2 and TxB2 increased in a parallel manner. 6-keto-PGF1α increased from 1.2 ng/ml (3.6 ng/min) to 2.2 ng/ml

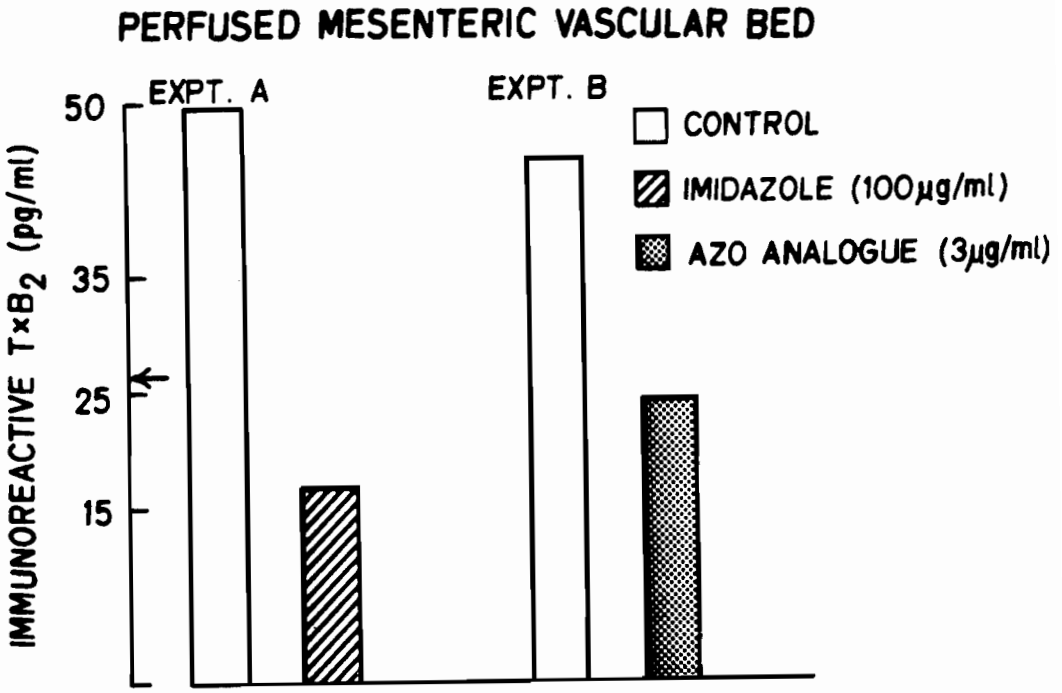

Fig. 41. Inhibition of TxB2 biosynthesis in the mesenteric vasculature by two structurally unrelated Tx synthetase inhibitors. Mesenteric vascular beds were prepared as described in methods with additional procedures as noted in results. Each preperation was perfused with buffer alone or buffer plus a Tx synthetase inhibitor. The effluent from control and treated preperations were individually collected for a 5 min period and analyzed by RIA for TxB2.

Ordinate: concentration of TxB2 in the perfusate (pg/ml; conversion factor as in Fig. 40).

Abscissa: control:open bar; imidazole (1.5×10^{-3} M) slashed bar; AZO (3 µg/ml): stippled bar.

Data shown represent the mean concentration (pg/ml) in the 15 ml of perfusate collected over the 5 mins. As can be seen the Tx synthetase inhibitors reduced the levels of TxB2 below the 10% confidence limit of the assay (indicated by the arrow).

(6.6 ng/min). PGE2 increased from 62 pg/ml (18.6 pg/min) to 60 pg/ml (180 pg/min).

In Figure *43* the results of the analyses of the perfusate from animal B show that 6-keto-PGF1α and PGE2 increase in a parallel manner whereas TxB2 fell below the 10% confidence level of the assay and rose above it at t = 5 hr. 6-keto-PGF1α increased from 1.0 ng/ml (3.0 ng/min) to 2.25 ng/ml (6.75 ng/min). PGE2 increased from 64 pg/ml (192 pg/min) to 260 pg/ml (780 pg/min). TxB2 fell from 35 pg/ml (105 pg/min) to below the 10% confidence limit of the assay and was undetectable at 3 to 5 hours. At the 5 hour mark TxB2 was again detectable at 30 pg/ml (90 pg/min) (10% confidence limit, 26.6 pg/ml or 79.8 pg/min).

Although the levels of 6-keto-PGF1α released were similar in both preparations, the TxB2 RIA did not indicate a similar rise in TxB2 levels. This indicated that the TxB2 antisera was not nonspecifically cross-reacting with 6-keto-PGF1α to give false positive results.

This was further confirmed by thin layer chromatographic separation of TxB2 from 6-keto-PGF1α, PGE2, PGF2α and PGD2.

Thin layer chromatographic separation of immunoreactive prostaglandins released by the mesenteric vascular bed

The perfusates from each of two mesenteric vascular beds (from 350 g males) were pooled, acidified to pH 3 and extracted with ethyl acetate. The ethyl acetate fraction was evaporated under reduced pressure at a temperature of 40° C. The residue was solubilized and half of the amount spotted on each of two TLC plates, together with authentic standards (Upjohn, 1 μg each) of PGF2α, 6-keto-PGF1α, PGE2, TxB2 and PGD2 in

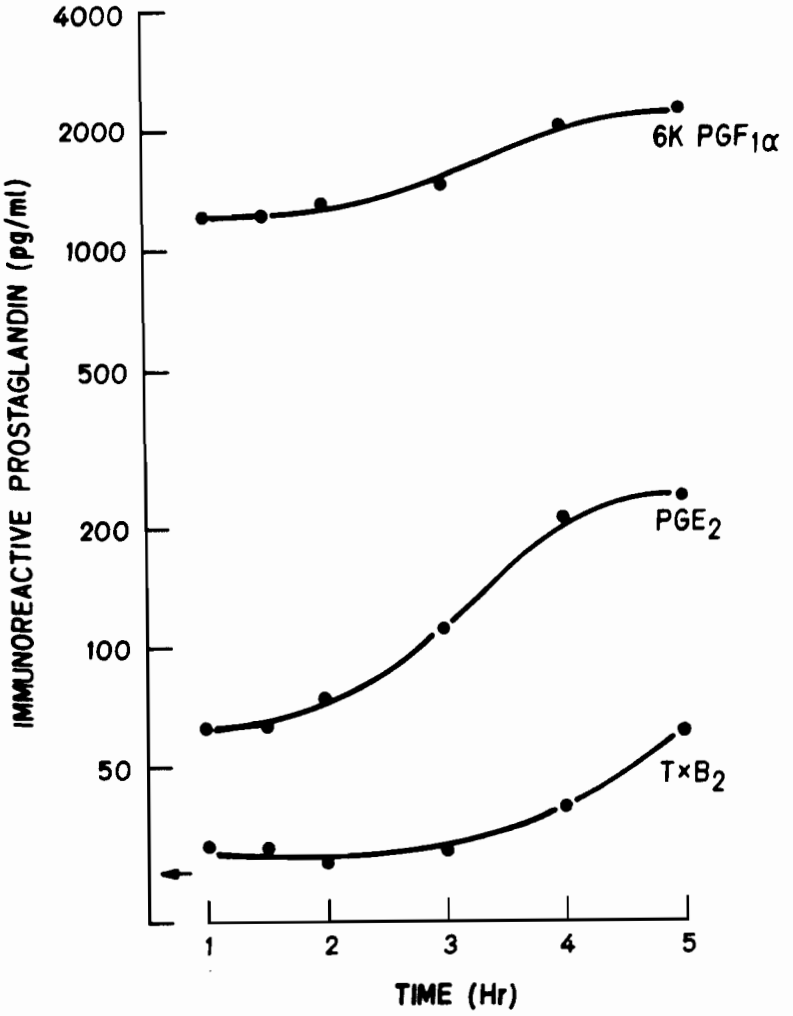

Fig. 42. Time course for the biosynthesis and release of 6-keto -PGF1α, PGE2 and TxB2 from an isolated perfused mesenteric vascular bed. Aliquots of perfusate were collected at 5 min intervals beginning 30 mins after cannulating the mesenteric artery and assayed by RIA.

Ordinate: concentration of prostaglandin in the perfusate (pg/ml; flow rate 3 ml/min).

Abscissa: time (hr) from the cannulation of the mesenteric artery (300 g rat).

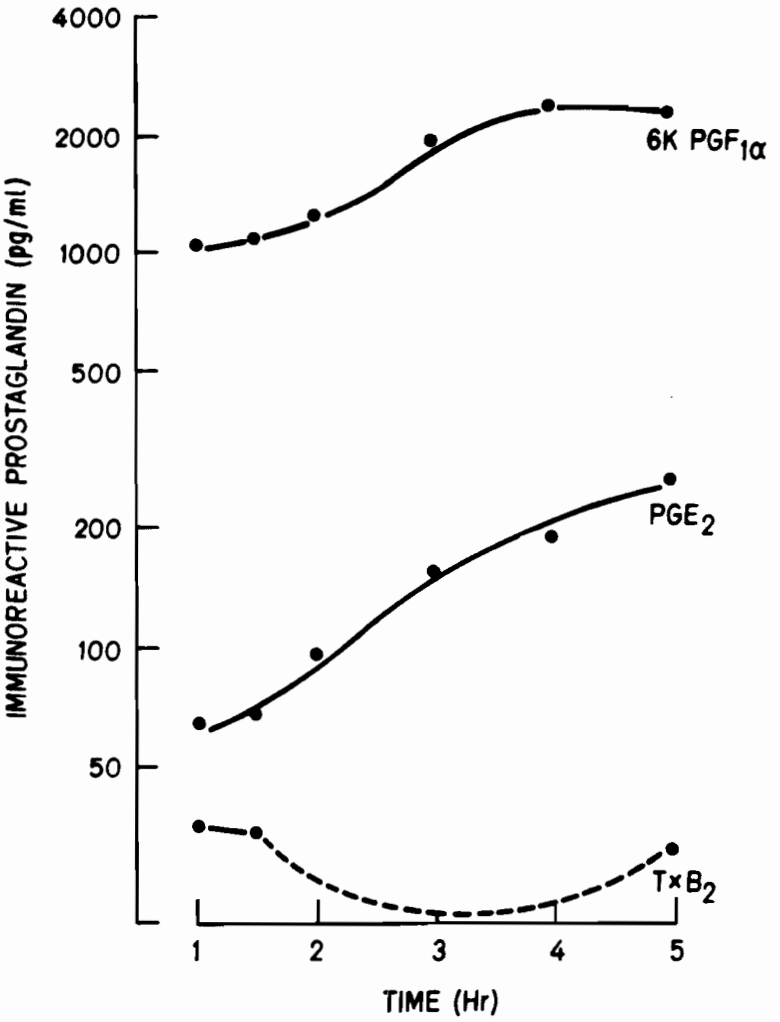

Fig. 43. Legend as in *Fig. 42.*

parallel channels. Each plate was developed in a different solvent system and the standards visualized by spraying with a 10% solution of phosphomolybdic acid and then heating at 120° C for 1 minute. The plates were divided into thirteen 1 cm zones and these sections scraped off the plates and extracted into methanol overnight. The methanol was evaporated and the residue redissolved in assay buffer and the amount of immunoreactive TxB2-like material determined.

In both solvent systems the predominant TxB2-like material comigrated with the authentic TxB2 (Figs. *44* and *45*). When the perfusate was concentrated some 600-fold, as was done for the TLC analyses, the levels of 6-keto-PGF1 and PGD2 were high enough to crossreact in the TxB2 assay. This is shown by the apparent TxB2-like material corresponding to the location of authentic 6-keto-PGF1α and PGD2 on the TLC plate.

Prostaglandin biosynthesis by arterial rings of the mesentery and its attendant smaller arteries

The mesenteric artery was cannulated and the mesenteric vascular bed perfused with physiological salt solution (Krebs). The adherent fatty tissue was removed and the mesenteric veins discarded. The arterial bed was perfused with collagen as described previously and then equilibrated in Krebs buffer at 4° C for 10 minutes. At this time the wet weight of the tissue was noted and the arteries (superior mesenteric, ileal and jejunal) were transferred to another vial containing fresh buffer (4° C) or buffer plus inhibitor (indomethacin or imidazole) for 5 minutes. At the end of this period the arteries were cut into arterial rings (at 4° C) and placed in a third vial containing 2 ml buffer alone or buffer plus inhibitor and equilibrated for a further 5 minutes. The

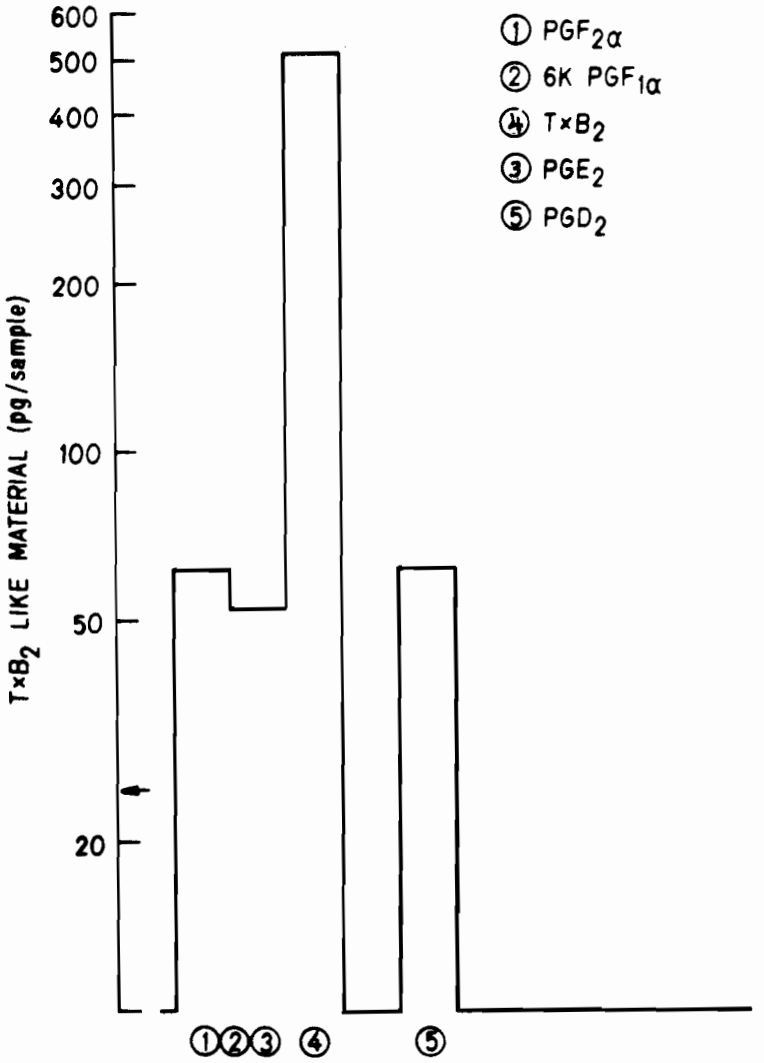

Fig. 44. Thin layer radioimmunoassay chromatogram of pooled effluent from two vascular beds. 60 ml of effluent was collected over a 10 min period (30 ml/preperation). The sample was then processed as described in methods and the chromatogram developed in diethylether:methanol: acetic acid (180:2:4 V:V). The immunoreactive TxB2 was determined by RIA and its position on the chromatogram is indicated by the heavy line. At the base of the chromatogram the positionsof authentic standards are shown: ①PGF2α; ② 6-keto-PGF1α; ③ PGE2; ④ TxB2; ⑤ PGD2. Arrow indicates the 10% confidence limit of the TxB2 RIA. Note the close correlation of the position of the maximum TxB2-like material in the effluent with the position of authentic TxB2 on the chromatogram.

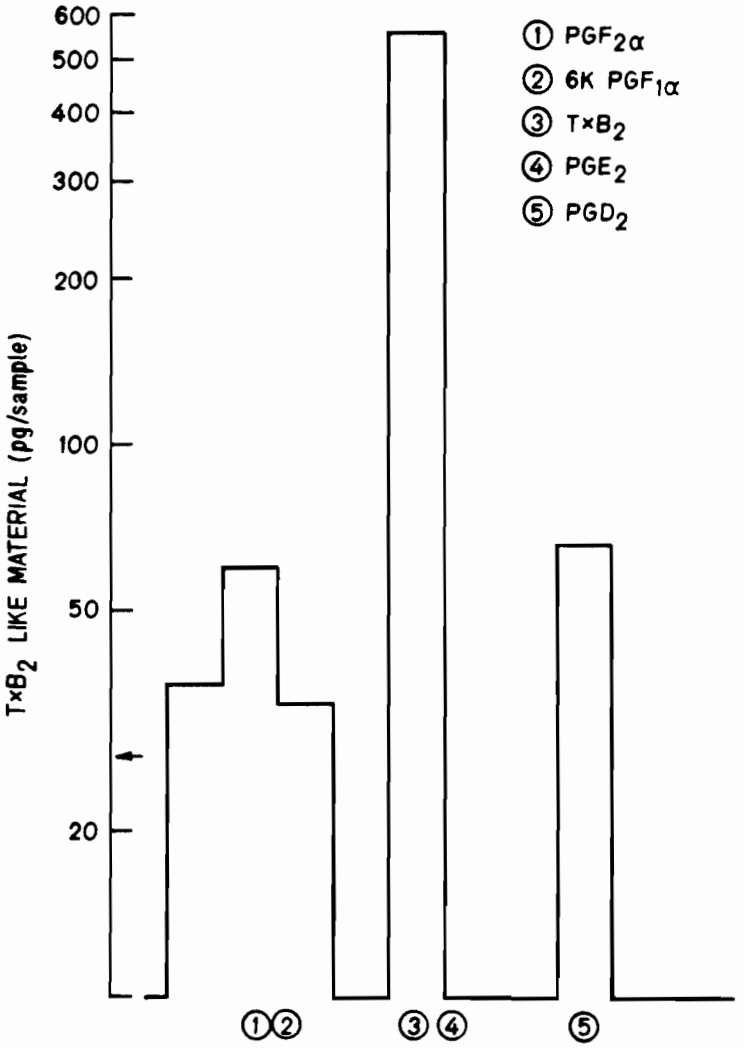

Fig. 45. Legend as in *Fig. 44* with exception that the chromatogram was developed in ethyl acetate: ethanol: acetic acid (100: 1:1 V:V). Arrow indicates the 10% confidence limit of the TxB2 RIA. Note the close correlation of the maximum TxB2 -like material in the effluent with the position of authentic TxB2 on the chromatogram.

reaction was started by transferring the vial to a waterbath (37° C), adding noradrenaline (if required), and incubated for 10 minutes. The reaction was terminated by filtering the buffer through a glass wool plug. The filtrate was stored at -60° C until assayed by RIA. Both PGE2 and TxB2 were determined for each sample and the results expressed as prostaglandin ng/gram wet weight tissue produced over 10 minutes by arterioles from a single animal. The results of these experiments are shown in Fig. *46* and *47*. In series A (Fig. *46*) the sensitivity of TxB2 and PGE2 biosynthesis to imidazole and indomethacin was determined. In Experiment No. 1 immunoreactive TxB2 and PGE2 release in response to noradrenaline stimulation are shown and appear to be approximately equal. In Experiment No. 2, the effect of imidazole (38 μg/ml, 5.5×10^{-4} M) on unstimulated PG biosynthesis was determined. This drug did not appear to have a dramatic effect on basal TxB2 release at this concentration. In Experiment No. 3, the effect of imidazole on noradrenaline stimulated PG biosynthesis was determined. In this case, PGE2 synthesis was greater than that of TxB2. In Experiment No. 4, the effect of the cyclooxygenase inhibitor indomethacin on PGE2 and TxB2 biosynthesis was determined. Indomethacin clearly reduced biosynthesis of both PGE2 and TxB2, by approximately 66%. Again, the levels of PGE2 and TxB2 did not appear to be different. In experiment No. 5, the effect of imidazole (5.5×10^{-4} M) on noradrenaline stimulated PG biosynthesis was determined as before (Experiment No. 3). Again, TxB2 production did not increase whereas the PGE2 production did increase. In Experiment No. 6, the effect of indomethacin (2.2×10^{-5} M) on unstimulated biosynthesis was determined; both PGE2 and TxB2 were comparably reduced.

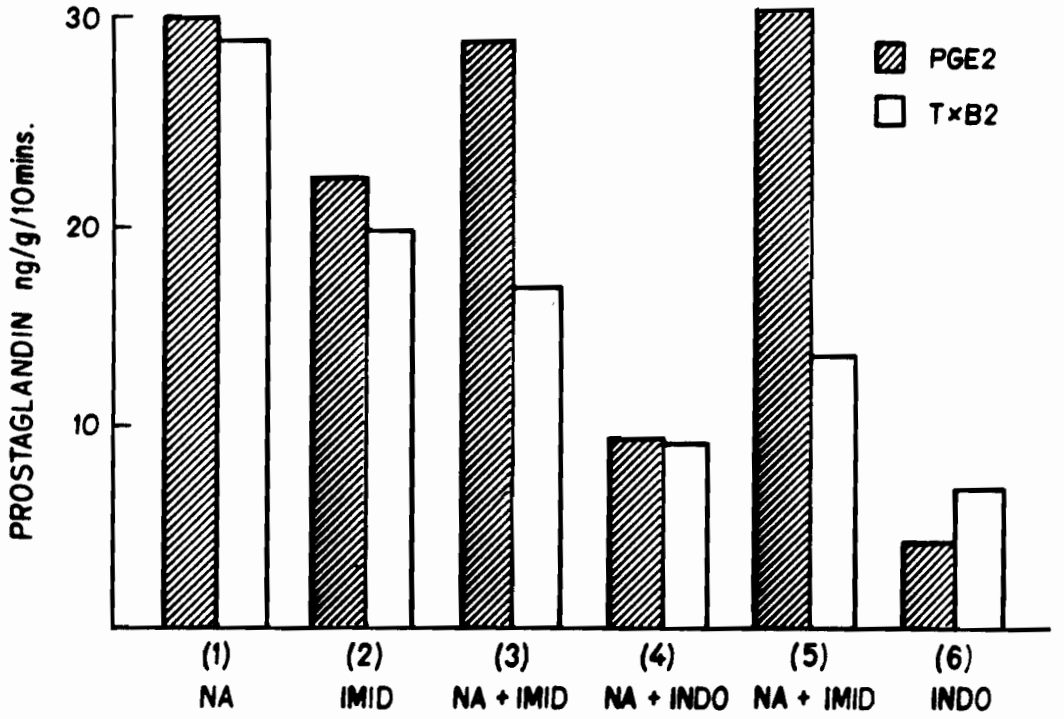

Fig. 46. Production of immunoreactive TxB2 and PGE2 by incubates of rat mesenteric artery and arteriole rings over a 10 min incubation period in Krebs buffer at 37 ^{0}C. Aliquots of the incubation buffer were analyzed by direct RIA for TxB2 and PGE2 as described in methods.

Ordinate: prostaglandin production ng/g wet weight/10 min.

Abscissa: (1) Noradrenaline stimulation; (2) Basal synthesis in the presence of 5.5 X 10^{-4} M imidazole (3) Noradrenaline stimulation in the presence of 5.5 X 10^{-4} M imidazole; (4) Noradrenaline stimulation in the presence of 2.2 X 10^{-5} M indomethacin; (5) Noradrenaline stimulation in the presence of 5.5 X 10^{-4} M imidazole; (6) Basal biosynthesis in the presence of 2.2 X 10^{-5} M indomethacin.

Note that indomethacin reduced basal biosynthesis and blocks noradrenaline stimulation of PG biosynthesis. Imidazole blocked noradrenaline stimulation of TxB2 biosynthesis, with no effect on PGE2 biosynthesis.

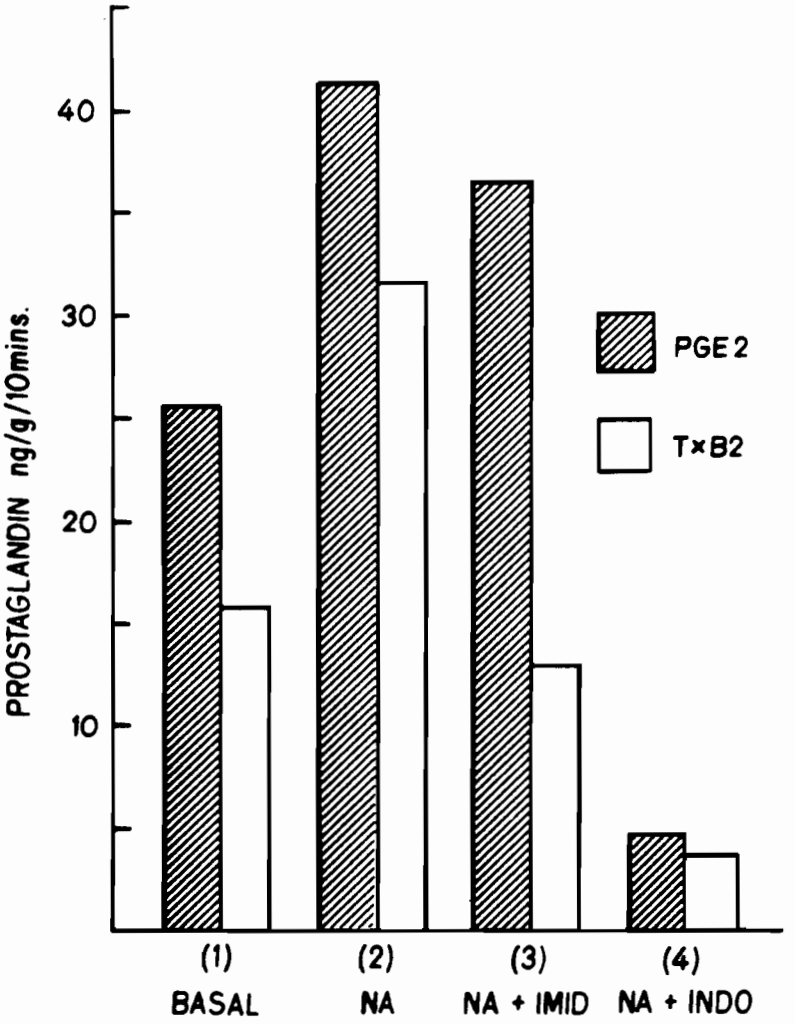

Fig. 47. Production of immunoreactive TxB2 and PGE2 by incubates of rat mesenteric artery and arteriole rings over a 10 min incubation period in Krebs buffer at 37^0C. Aliquots of the incubation buffer were analyzed by direct RIA for TxB2 and PGE2 as described in methods.

Ordinate: prostaglandin production ng/g wet weight/10 min.

Abscissa: (1) Basal biosynthesis; (2) Noradrenaline stimulation; (3) Noradrenaline stimulation in the presence of 5.5×10^{-4} M imidazole; (4) Noradrenaline stimulation in the presence of 2.2×10^{-5} M indomethacin.

Note that imidazole blocked noradrenaline stimulation of TxB2 biosynthesis with little effect on PGE2 biosynthesis. Indomethacin blocked noradrenaline stimulation of both TxB2 and PGE2 biosynthesis.

In series B (Fig. *47*), this study was repeated using a different batch of animals. As before, imidazole (5.5×10^{-4} M) inhibited NA stimulated production of immunoreactive TxB2 whereas PGE2 biosynthesis was not affected and the cyclooxygenase inhibitor indomethacin (2.2×10^{-5} M) inhibited the synthesis of both TxB2 and PGE2.

Thin layer chromatographic separation of immunoreactive prostaglandins in arterial ring incubates

Approximately 2.4 ml of incubates from arterial rings (8 rats) were pooled and then acidified (pH 3.2) and extracted with ethyl acetate. The organic fraction was then evaporated to dryness with a rotary evaporator, the prostaglandins were redissolved in 50 µl ethanol and spotted on a TLC plate, together with PGE2, PGF2α, 6 keto PGF1α and TxB2 standards in parallel channels. The plate was developed and the standards visualized by spraying with 10% solution of phosphomolybdic acid and then heating for 1 minute in an oven at 120° C. The plate was divided into thirteen 1 cm zones and these were eluted with methanol overnight. The methanol was evaporated and the residue redissolved in phosphate-buffered saline. The apparent immunoreactive TxB2-like material in each zone was determined by TxB2 RIA (the results of this are shown in Figure *48*). As can be seen, the main immunoreactive zone corresponded to the position of authentic TxB2 on the TLC plate.

These data clearly show the release of TxB2-like material from both the perfused mesenteric vascular bed and incubates of arterial rings. With this knowledge it was now possible to test the hypothesis that endogenous TxA2 opposed the actions of exogenous TxB2 infused through the vascular bed (Fig. *13*) on noradrenaline pressor responses. If this

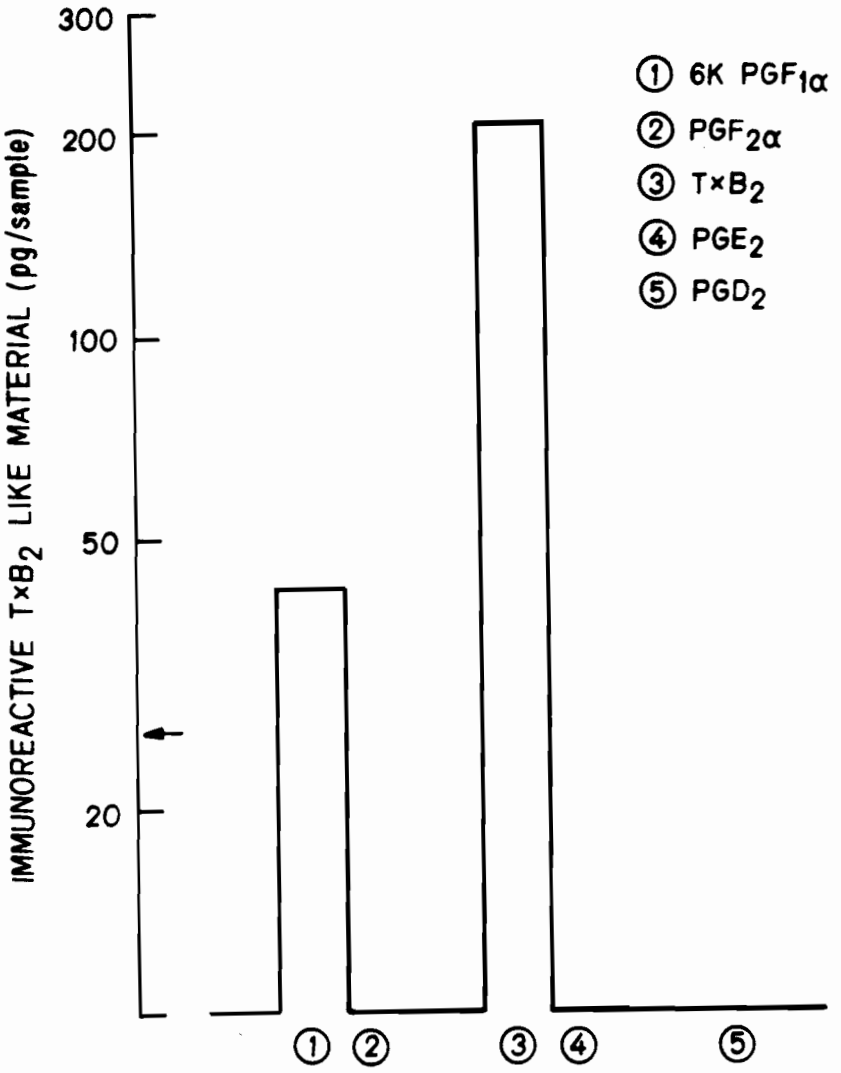

Fig. 48. Thin layer radioimmunoassay ch omatogram of pooled incubation media of mesenteric artery and arteriole rings (n=8). The media was processed as described in methods and the TLC plate was developed in ethyl acetate:ethanol: acetic acid (100:1:1 V:V). The immunoreactive TxB2-like material in each 1 cm zone of the plate was determined by TxB2 RIA. At the base of the chromatogram the positions of the authentic standards are shown: ① 6K PGF1α; ② PGF2α; ③ TxB2; ④ PGE2; ⑤ PGD2. Note the close correlation of the maximum TxB2-like material in the incubation media with the position of authentic TxB2 on the chromatogram.

hypothesis was correct then inhibition of endogenous TxA2 synthesis should be accompanied by enhanced effectiveness of exogenous TxB2 added to the buffer.

Effect of TxB2 on noradrenaline pressor responses in preparations perfused simultaneously with imidazole

Perfusion of the mesenteric vascular bed with imidazole (7.4×10^{-5} , 2.5×10^{-4} and 5.9×10^{-4} M) unmasked a potent and effective inhibitory action of TxB2 on noradrenaline responses (Fig. *49*). In the presence of imidazole the maximum inhibition by TxB2 was increased ($p < 0.001$ ANOVA) when compared to responses of preparations perfused with TxB2 alone. Increasing the concentration of imidazole up to 5.9×10^{-4} M shifted the TxB2 concentration response curve progressively leftwards (Fig. *49*).

Effect of TxB2 on potassium pressor responses in preparations perfused simultaneously with imidazole

Imidazole increased both the effectiveness and potency of TxB2 inhibition of noradrenaline pressore responses;in contrast the combination of this TxA2 synthetase inhibitor and TxB2 resulted in a potentiation of potassium pressor responses (Fig. *49*). This potentiation of potassium responses was not seen in preparations perfused with TxB2 alone (Fig. *13*).

Effect of TxB2 on baseline perfusion pressure in preparations perfused simultaneously with imidazole

In preparations perfused with TxB2 (10^{-12} to 10^{-8} M) alone, no significant effect was seen on baseline perfusion pressure (Fig. *13*). When the preparations were perfused with imidazole at either of three

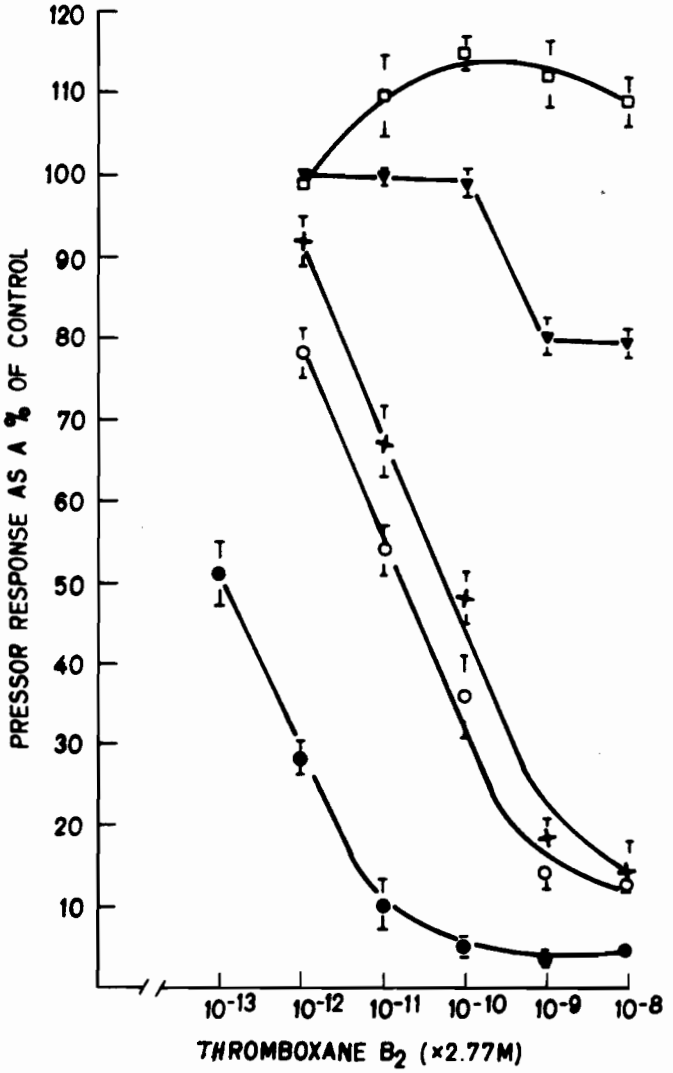

Fig. 49. Inhibition of noradrenaline contractions of the isolated perfused mesenteric vascular bed by TxB2 (▼) and the potentiation of its actions ($p < 0.01$, ANOVA) by imidazole 7.4×10^{-5} M (+), 2.5×10^{-4} M (o), 5.9×10^{-4} M (o). Also shown is the effect of TxB2 on potassium contractions in the presence of 5.9×10^{-4} M imidazole (●).

Ordinate: pressor response as % of mean control response prior to the addition of TxB2.

Abscissa: concentration of TxB2 (M).

Each data point shown represents the mean ± SE for six experiments.

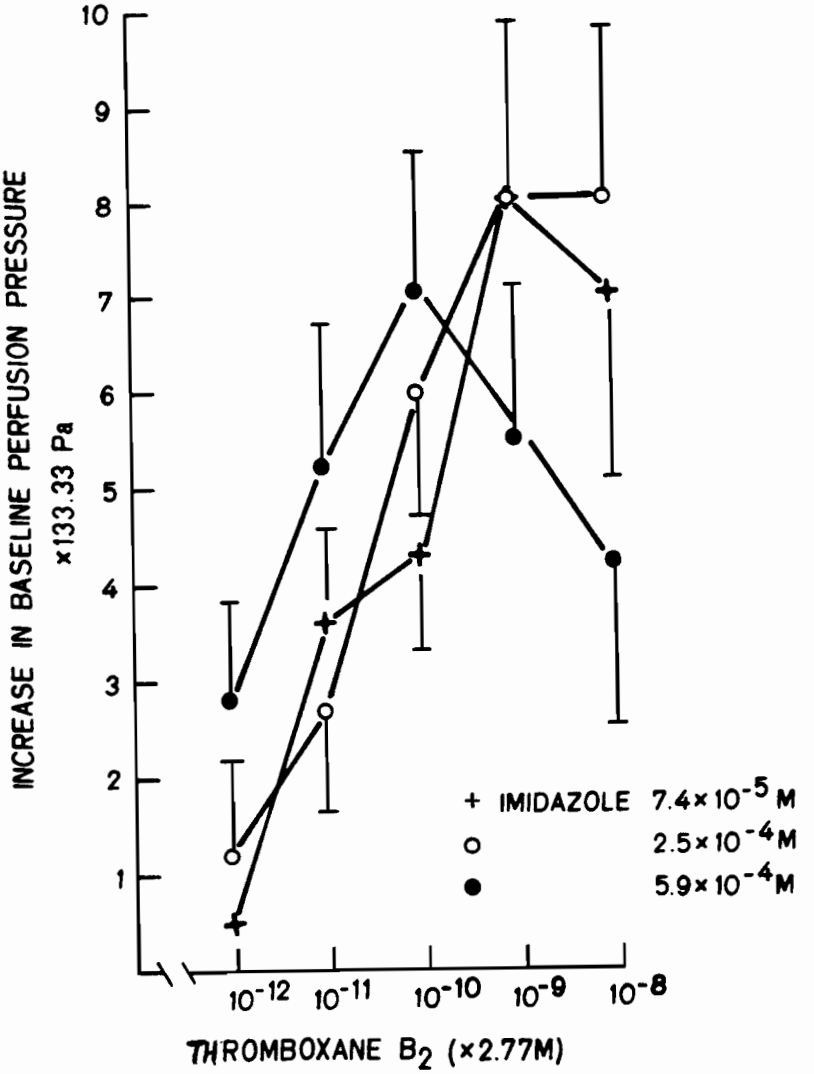

Fig. 50. Increase in base line perfusion pressure by increasing concentrations of TxB2 in preparations simultaneously perfused with imidazole 7.4×10^{-5} M (+), 2.5×10^{-4} M (o), 5.9×10^{-4} M (●). In the absence of imidazole, TxB2 did not influence base line perfusion pressure (see inset Fig. *13*).

Ordinate: increase in base line perfusion pressure (mm Hg; 1 mm Hg = 133.33 Pascals).

Abscissa: concentration of TxB2 (M).

Each data point shown represents the mean ± SE for six experiments.

concentrations (7.4×10^{-5} , 2.5×10^{-4} and 5.9×10^{-4} M), TxB2 significantly increased baseline perfusion pressure in a concentration related manner ($p < 0.01$ ANOVA, Fig. *50*).

DISCUSSION

The rat mesenteric vasculature synthesizes and releases prostaglandins continuously even in the absence of stimuli (*Wolfe et al 1979;* present data). The amount of prostaglandins released appears to be correlated with the size of the vascular bed (comparison made at identical flow rates, Figs. *32* and *33*), i.e. dependent on the muscle mass. A similar observation was made by *Wolfe et al (1979)*.

The time course of PGE2 and 6-keto PGF1α release from a preparation showed a temporal parallelism. This suggested that the source(s) were the same. The initial high rate of release seen was probably related to the trauma associated with the preparation of the vascular bed. Within one hour the rate of release for both PGE2 and 6-keto PGF1α had plateaued. The preparations from smaller animals (Fig. *32*) appeared to stabilize much earlier than those from large animals (Fig. *33*). However, as shown in Figure *38* this observation was not necessarily correct. It is more likely that the time required for stabilization was directly determined by the severity of the trauma the preparations were subjected to. The observation which remained constant was that the larger preparations released a greater amount of prostaglandins in comparison with the smaller preparations (Fig. *33* and *42* vs Fig. *32* and *39*).

In an unrelated system (perfused rat kidney) *Scherer et al (1978)* found that the rates of release of PGE2 and PGF2 were 46.2 ± 9.3 pg PGE2/minute/kidney and 27 ± 3.4 pg PGF2α/minute/kidney. These values are of the same order of magnitude as PGE2 and PGF2α release by the perfused mesenteric vascular bed.

In experiments designed to explore the role of, and possibly implicate, a substance in the physiology of a system, a classical approach has been ablation procedures. In this system there is a superimposability of the time curves for indomethacin (ablation/inhibition) effect on mechanical and prostaglandin biosynthetic activity (Figs. *34* and *35*). These data clearly indicate that the onset and maximum inhibitory action of indomethacin was definitely correlated with its inhibition of the prostaglandin cyclooxygenase. The effect of indomethacin on both mechanical and biosynthetic activity was concentration dependent and thus satisfies the criterion of *Needleman et al* (*1978*) quoted above. The superimposable nature of these time-effect curves (Figs. *34* and *35*) are persuasive evidence indeed.

While it is now generally accepted that noradrenaline stimulates prostaglandin biosynthesis, it was of interest to determine if it increased all prostaglandins by the same factor. As can be seen in Figure *36*, noradrenaline stimulated PGE2 biosynthesis by 100% whereas 6-keto PGF1α biosynthesis was increased by 40%. Thus there is a differential stimulation of prostaglandin synthesis in the mesenteric vascular bed.

In the rabbit kidney *Schwartzman et al* (*1979*) found that bradykinin or antiogensin II injected intraarterially, caused a 400% increase in PGE2 release while arachidonic acid infusion resulted in a 10,000% increase in PGE2 release (basal release of PGE2, no stimulation: 47.5 - 65.5 ng/min). Using endotoxin as a stimulus *Harper et al* (*1978*) showed that PGF and PGE levels in the rabbit carotid arterial plasma were increased by 400% and 30% respectively (pre-endotoxin levels: PGF 270 $\pm$ 30 pg/ml; PGE 280 $\pm$ 0.00 pg/ml). In nonvascular smooth muscle, noradrenaline increased PGF release

from rat uterus by 300% (pre-noradrenaline release 225 ± 37.6 pg/mg wet weight/60 min, *Ishikawa and Fuchs 1978*). These data demonstrated that in a variety of preparations, a number of dissimilar stimuli all alter prostaglandin biosynthesis, resulting in the release of different amounts of the prostaglandins monitored.

In the mesenteric vascular bed, the nonsteroidal antiinflammatory drug, indomethacin (2.2×10^{-5} M) abolished noradrenaline stimulation of prostaglandin biosynthesis (Fig. *37*) but did not abolish basal prostaglandin biosynthesis. Basal prostacyclin release appeared resistant to inhibition by indomethacin in this preparation (see Tables 2 and 3 for a comparison of indomethacin potency as an inhibitor of the cyclooxygenase from various tissues and species). It is possible that in this vascular bed the free arachidonic acid levels were high enough to oppose indomethacin's action, as has been shown to occur by *Flower (1974)*. Other researchers have reported data showing that moderate concentrations of indomethacin did not inhibit basal prostaglandin biosynthesis but effectively inhibited synthesis in response to stimulation (*Hood and Vincent 1978; Scherer et al 1978; Pace-Asciak et al 1978*). In the mesenteric vascular bed, the time course data (Fig. *35*) for indomethacin inhibition of basal biosynthesis showed that a high concentration of indomethacin (by literature values, see Tables 2 and 3) did not abolish PGE2 biosynthesis, indicating the resistance of the cyclooxygenase.

It is generally accepted that little thromboxane A2 is synthesized by vascular smooth muscle (*Needleman et al 1977; Tuvemo 1978*), although this prostaglandin is one of the most potent constrictors of vascular smooth muscle (*Tuvemo 1978; Needleman et al 1979*).

Thromboxane A2 is normally undetectable in the effluent from the heart (*Anhut et al 1977*) and kidney (*Morrison et al 1977*); however, both groups have shown that under special conditions both preparations synthesize and release TxB2 (the metabolite of TxA2). *Anhut et al* (*1977*) have reported that the anaphylactic guinea pig heart releases TxB2 while *Morrison et al* (*1977*) have shown that the ureter obstructed rabbit kidney released TxB2. It has now been shown that an endogenous inhibitor of prostaglandin biosynthesis is present in the kidney cortex (*Terragno et al 1978*). It is therefore possible that a similar endogenous inhibitor of thromboxane synthetase exists and that it is either inactivated or its synthesis abolished during anaphylaxis (*Anhut et al 1977*) or in the uremic kidney (*Morrison et al 1977*). Recently *Ogino et al* (*1979*) reported the isolation and identification of an activator of prostaglandin hydroperoxidase. This activator, "uric acid", is a metabolite of adenosine, a ubiquitous substance found in all cells. Thus both endogenous activators and inhibitors of different enzymes in the prostaglandin biosynthetic pathway exist within the cells.

The release of immunoreactive TxB2 appeared to follow a similar time course to that found for PGE2 and 6-keto-PGF1α. In the smaller preparations at the fixed flow rate of 3 ml/min the levels of TxB2 often fell below the reliability of the assay within 30 minutes. This did not occur in preparations from larger animals. The release of TxB2 was sensitive to pharmacological modification (Figs. *39* and *49*). Imidazole (a thromboxane synthetase inhibitor) altered the ratio of PGE2 to TxB2 detected in the perfusate. The amount of PGE2 was increased while the levels of TxB2 were decreased (Fig. *40*). In the control preparation the levels of PGE2

and TxB2 in the effluent were similar (Fig. *39*). This effect of imidazole wasconsistent with the data of *Nijkamp et al* (*1977*) and *Wolfe et al* (*1979*) showing diversion of prostaglandin endoperoxides upon thromboxane synthetase inhibition. A structurally unrelated thromboxane synthetase inhibitor, 9, 11 azo prosta-5,13 dienoic acid reduced the release of TxB2 from the mesenteric vasculature, to a concentration below the 10% confidence level of the assay (26 pg, Fig. *41*).

Because there are many factors which alter antibody-antigen interaction, the possibility of a nonspecific substance crossreacting in the assay had to be considered. The sensitivity to imidazole and 9,11 azo-prosta 5,13 dienoic acid inhibition identified the active substance as being a product of thromboxane synthetase.

Further support is provided in Figures *42* and *43* showing that in two separate preparations, prostacyclin levels were similar in the effluent as were the PGE2 levels, in contrast to the TxB2 levels. PGI2 is the major arachidonic acid metabolite produced; thus, it is possible that it could have been crossreacting in the TxB2 assay. The data in Figure *43* clearly shows that TxB2 did not parallel the increase in PGE2 and 6 keto PGF1α. Additional verification was provided by TLC separation of the mixture of prostaglandins released by these blood vessels. In two solvent systems the substance having the greatest affinity for the TxB2 antisera comigrated with authentic TxB2. The apparent TxB2 present in the PGD2 zone indicated its biosynthesis. PGD2 was not identified by *Wolfe et al* (*1979*) in their studies using GC-MS analysis.

In the experiments using arterial rings, TxB2 synthesis was again confirmed using the radioimmunoassay. Its bioassay was sensitive to both

indomethacin and imidazole inhibition. These data provided positive pharmacological and biochemical data for the endogenous biosynthesis of TxA2 by the mesenteric artery and its associated arterioles. Previously vascular smooth muscle synthesis of TxA2 had only been shown in the human umbilical artery (*Tuvemo 1978*). Combined TLC separation and radioimmunoassay again identified the antigenic substance as being TxB2. This data is in agreement with the findings of *Wolfe et al (1979)* using GM-MS analysis, which categorically identified TxB2 in the incubates of rat mesenteric arteries. Because prostaglandins are not stored, this then represented *de novo* synthesis from endogenous arachidonic acid stores.

Recently, two groups reported the identification of TxB2 as a product of arachidonic acid metabolism in incubates of bovine cerebral arterioles. *Maurer et al (1979)* identified TxB2, PGE2, PGF2α and 6-keto PGF1α by combined high performance liquid chromatography and radioimmunoassay in incubates of cerebral microvessels. In the second study, *Hagen et al (1979)* using radiochromatographic and gaschromatographic-mass-spectrometric procedures positively identified TxB2, PGE2, PGF2α, PGD2 and 6-keto PGF1α produced from added 1-^{14}C-arachidonic acid as well as from endogenous arachidonic acid. These data as well as that presented here indicate that TxA2 (detected as TxB2) was produced by the vessel wall in amounts significantly lower than that of either PGE2 or 6-keto PGF1α (PGI2). Since TxA2 was much more potent than the other prostaglandins (*Tuvemo 1978; Needleman et al 1979*), then low concentrations would be sufficient for its biological actions. The data presented above and earlier, using thromboxane synthetase inhibitors support this view.

The human umbilical artery synthesized 20 - 52 ng TxB2/gram tissue/

3 hours (*Samuelsson et al 1978*) while we found that the rat mesenteric artery synthesizes 12.8 ± 1.3 ng TxB2/gram tissue/10 minutes. These values can be compared, because it has now been shown that incubates of smooth muscle release 80% of the prostaglandins in the first 5 minutes, thereafter little further accumulation takes place (*Pace-Asciak et al 1978; Ishikawa and Fuchs 1978*). In contrast, *Wolfe et al (1979)* found that rat aortic and mesenteric rings produced 4.7 ng TxB2/gram tissue/ 10 minutes. The lack of agreement in the absolute concentrations of TxB2 found by ourselves and *Wolfe et al (1979)* may be a result of their use of primarily the abdominal aorta - mesenteric artery junction and the use of phosphate buffer in their incubates. In the present study only the mesenteric, ileal and jejunal arteries and small arterioles were used in Krebs-Henseleit buffer (the abdominal aorta, superior mesenteric vein and other small veins were discarded). Furthermore, our preparations were prequilibrated for 20 minutes with constant perfusion at 30° C and were then transferred to cold buffer (4° C) whereas *Wolfe et al (1979)* transferred their arteries directly to cold buffer (phosphate). The amount of 6-keto PGF1α synthesized by the rings prepared from aortic and mesenteric arteries was reported as 3427 ± 325.8 ng/gram tissue/10 minutes by *Wolfe et al (1978)* whereas we found that the mesenteric arterial rings synthesized 674.4 ± 28.3 ng/gram/10 minutes. The reasons for this difference are probably those noted above. Some data for buffer related effects, is the reported PGI2 synthesis of 6400 ng/gram tissue/10 minutes by rat aortic rings in borate buffer (*Okuma et al 1979*, using platelet bioassay for PGI2). It is of interest to note that *Herman et al (1978)* found as much as five-fold difference in prostacyclin (PGI2) production

between tissues incubated in plasma and Tris buffer. Thus, the incubation media may be one key factor responsible for the lack of agreement of these sets of data.

In their study *Wolfe et al (1979)* were unable to detect TxB2 in their perfusates of the vascular bed. This was most likely the result of not having enough TxB2 in the collected perfusate to yield a final minimal amount of 2 ng (after work-up) for GC-MS analysis. The procedure required six extraction steps followed by derivatization. If one assumes that in the effluent from four preparations collected for 1 hour, the total amount of TxB2 was 20 ng, then in the work-up with a recovery of 60% at each extraction, after five extractions, only 1.2 ng of TxB2 would be left. This quantity is below the sensitivity of the GC-MS method which required 2 ng of TxB2 for processing (*Wolfe et al 1979*).

The limit of sensitivity of the TxB2 radioimmunoassay was 26 pg. In many cases (150 - 200 g rats) after 35 minutes (from time of cannulation) the levels of TxB2 had fallen below the sensitivity of the methods. As a result the weight of the animals used was increased to approximately 300 grams. This kept the perfusate levels of TxB2 within the detection range as shown in Figures *38* and *42* and the TLC separation of the prostaglandin mixtures from the perfusates as shown in Figures *44* and *45*.

Having identified TxB2 in the perfusate it was now possible to generate TxB2 dose response curves in the presence of fixed concentrations of a TxA2 synthetase inhibitor. This would provide further indirect evidence about the role of TxA2 in these vessels.

This procedure unmasked a potent inhibitory effect of TxB2 on noradrenaline contractions, in addition to a direct smooth muscle stimulation

seen as an increase in basal perfusion pressure. Potassium pressor responses were potentiated by TxB2 in the presence of the TxA2 inhibition. Imidazole did not affect potassium responses by itself.

The increase in basal tone in the presence of TxB2 plus imidazole suggests that TxB2 inhibited the lowering of cytosolic calcium levels even though it simultaneously blocked the mobilization of activator calcium. Its inability to block or inhibit potassium responses indicated that the contractile proteins and other intracellular machinery needed for their activation were still functional, thus ruling out a toxic effect.

SECTION III: PROSTAGLANDINS AND PURINES

INTRODUCTION:

Physiological role of purines

The existence of a "non-adrenergic non-cholinergic" branch of the autonomic nervous system has been demonstrated in a number of systems. Some examples are: stomach and small intestine of a variety of mammallian species (*Furness 1969; Lock 1971; Weston 1971; Hirst and McKirdy 1974; Abrahamsson 1975*), rabbit portal vein (*Hughes and Vane 1970*), dog retractor penis (*Luduena and Grigas 1966*) and guinea pig seminal vesicles (*Nakanishi and Takeda 1972, 1973*).

Recently *Burnstock* (*1972*) has proposed a model suggesting that the transmitter substance released by activation of "non-adrenergic, non-cholinergic" nerve is a purine (probably adenosine triphosphate (ATP) or adenosine. This model is based on a number of lines of evidence which fulfill to varying degrees the criteria which must be met before any substance can be seriously considered as a neurotransmitter (*Werman 1966*). These criteria are listed below and will be discussed considering either adenosine or ATP as likely candidates.

1. The substance and the enzymes necessary for its synthesis must be present.
2. The substance must be released by nerve activation.
3. Application of the substance must mimic nerve activation.
4. A system for termination of responses must exist.
5. Drug effects whether potentiation or antagonism should be similar when the nerve is activated or the substance is applied.

Synthesis

This criterion states that the substance must be found in the site from which it is presumed to be released and that the precursors and mechanisms for its synthesis be available.

Preformed purines are not required in the diet of mammals because all cells are able to carry out "*de novo*" synthesis of adenosine and ATP (*Friedkin and Roberts 1954; Bohinsky 1973; Zimmer et al 1973*). Hence the transmitter (ATP or adenosine) is potentially available from all cell types. As an alternative to *de novo* synthesis, cells can reutilize the degradation products of adenosine and the adenine nucleotides to resynthesize ATP and adenosine via the salvage pathways (*Roux 1973; Namm 1973; Bohinsky 1973*), thus maintaining intracellular levels. In addition, it has been found that ^{3}H adenosine is taken up by organs and tissues innervated by "non-adrenergic, non-cholinergic" nerves (*Su et al 1971; Kuchi et all 1973; Su 1975; Burnstock 1975b*). In one case the site in which the ^{3}H-adenosine was stored following uptake has been identified. *Burnstock (1972)* reported that liquid scintillation spectrometry of serial sections of the guinea pig taenia coli indicated that most of the ^{3}H label was stored in nerves.

It seems clear that if either ATP or adenosine are considered as a putative transmitter there are mechanisms for fulfilling the criterion of availability for release. The first mechanism, synthesis, finds an analogy in the parasympathetic branch in which acetylcholine is synthesized from acetate and choline. The second mechanism, uptake, has its counterpart in the sympathetic branch in which norepinephrine is taken up by the postganglionic nerves.

Although this first criterion is adequately fulfilled with respect to ATP or adenosine, this by itself is not as convincing as with other substances such as acetylcholine. Because these substances are ubiquitous, the demonstration that nerves contain either adenosine or ATP is not by itself convincing evidence that these substances are neurotransmitter agents.

Release

In order to satisfy this criterion it must be verified that during activation of "non-adrenergic, non-cholinergic" nerves the putative transmitter is released into the extracellular fluid (ECF).

It has been shown that purines (adenosine and ATP) are released by a number of preparations. These include brain, neuromuscular junction, electroplaque, heart, stomach and blood vessels.

A number of studies have been undertaken which demonstrate that adenosine or its phosphorylated derivatives are released during field stimulation of brain tissue. *Heller and McIlwain* (*1973*) preloaded superfused slices of the lateral olfactory and optic tracts with 8 ^{14}C adenine; upon electrical stimulation increased amounts of labelled material were released. This material was identified as adenine nucleotides and their degradation products (ATP, AMP, adenosine and inosine), confirming the earlier findings of *Pull and McIlwain* (*1972a,b; 1973*) who used the superfused guinea pig neocortex preparations. Similar data were reported by *Heller and McIlwain* (*1973*) from experiments using isolated preparations of the superior colliculus and lateral geniculate body of the brain, stimulated via an incoming optic tract. Later *Kuroda and McIlwain* (*1974*)

verified the release of ^{14}C adenine nucleotides from neural tissue using electrically stimulated isolated synaptosome beds, although this output was not as large as that obtained with the neocortex preparation (*Pull and McIlwain (1972a,b; 1973)*. In addition to these *in vitro* experiments *Sulakhe and Phillis (1975)* have demonstrated the *in situ* release of ^{3}H-adenosine and its derivatives from the pericruciate sensorimotor cortex of cats upon electrical stimulation.

Utilizing a peripheral neural preparation, *Silinsky and Hubbard (1973)* and *Silinsky (1975)* measured the release of ATP from the rat phrenic nerve hemidiaphragm. In a similar experiment using torpedo electroplaque, *Meunier et al (1975)* also demonstrated the release of ATP during nerve stimulation. The release of ATP in the former study was prevented by hemicholinium (blocks acetylcholine synthesis) suggesting a direct link between the release of ATP and acetylcholine. On the other hand, in the latter study *Meunier et al (1975)* provided evidence for a dissociation between acetylcholine and ATP release. They found that d-tubocurare (which blocks neuromuscular junction nicotinic receptors) decreased ATP release while that of acetylcholine was unaffected. Other isolated organs (heart and stomach) have been shown to release adenosine and/or ATP. *Berne* and coworkers (*Berne 1964; Berne and Rubio 1974; Rubio et al 1974*) have found that following myocardial ischemia or hypoxia, adenosine was released into the extracellular fluid. *Burnstock et al (1970)* presented evidence that the guinea pig and toad stomachs released adenosine or ATP into the perfusate in response to stimulation of the vagus nerves. These perfusates were tested on isolated atropinized (atropine blocks the muscarinic receptors) guinea pig taenia coli, and were found to be inhibitory. Chromatographic analysis revealed that adenosine was the major

purine present in the perfusate. Because this preparation has ATP catabolizing enzymes, it was suggested that in actuality ATP was released by the "non-adrenergic, non-cholinergic" nerves and that the adenosine found was derived from the rapid degradation of ATP. A similar conclusion was made in a later paper by *Satchell and Burnstock* (*1971*) reporting the results of experiments quantitating the amounts of adenosine release by the stomachs of the guinea pig and toad.

More recently, *Paddle and Burnstock* (*1975*) measured the levels of ATP in the superfusate of guinea pig taenia coli. They found that in response to electrical stimulation of the intramural nerves (in the presence of guanethidine, which prevents norepinephrine release) there was an eight to twenty-fold increase in the superfusate levels of ATP; this is consistent with the hypothesis that ATP is released by the "non-adrenergic, non-cholinergic" nerve. Utilizing a different approach to this problem *Su* (*1975*) prelabelled rabbit vasculature (thoracic artery, ear artery, portal vein) with tritiated adenosine. When these preparations were electrically stimulated in the presence of either guanethidine or tetrodoxin (blocks the sodium channels essential in nerves for the generation of action potentials) the release of ATP normally seen in response to the stimulus was abolished. These data suggested that the source of ATP in the rabbit vasculature was the adrenergic nerves in contrast to that found in the guinea pig taenia coli described above.

To summarize, in a variety of preparations either adenosine or ATP is released upon stimulation. Available evidence supports the contention that these substances are neural in origin, probably "non-adrenergic, non-cholinergic" nerves. This criterion of release is fulfilled by either adenosine or ATP, supporting the view that either

of these substances could be a "putative" neurotransmitter.

Mimicry

It is generally accepted that the addition of a pure preparation of a neurotransmitter (synthetic or natural in origin) to a biological system can mimic the effects of activating those nerves known to release this particular substance. The corollary of this is: if a known drug can mimic the effects of nerve activation, this drug becomes a candidate for nomination as the unknown transmitter substance. This corollary is one of the criteria a putative neurotransmitter must satisfy, if it is to be accepted as a true neurotransmitter.

In *1929, Drury and Szent-Gyorgyi* found that the effects of stimulating the vagus nerve caused bradycardia in dogs and that administration of adenosine produced a similar effect. This was the first reported evidence that a purine could mimic the effects produced by nerve activation. Analogous results were obtained in studies in the lung (*Bennett and Drury 1931; Bianchi et al 1963*); here dilation was produced by a low concentration of ATP, a response which could be elicited by stimulating the vagus nerve (*Widdicome 1963*). Later *Robinson et al* (*1971*) demonstrated that the relaxation produced by activation of the vagus nerve was unaffected by antiadrenergic and anticholinergic drugs, thus identifying the effect as being due to "non-adrenergic, non-cholinergic" nerve activation.

In many other smooth muscle preparations (blood vessels, urinary bladder, gut) the actions of ATP and adenosine mimic the effects of stimulating "non-adrenergic, non-cholinergic" nerves.

The rabbit portal vein relaxes to both ATP and intramural nerve

stimulation following adrenergic blockade (*Hughes and Vane 1967*). In the urinary bladder atropine resistant contraction occurs in response to pelvic nerve stimulation and exogenous ATP (adrenergic response is relaxation) (*Ambache and Zar 1970; Burnstock et al 1972*). The close resemblance in the response to exogenous ATP and adenosine can be best seen in the gut. The response to activation of the intramural nerves of the small intestine and colon of a variety of species is inhibition of gut motility. In all of these preparations adenosine and ATP cause relaxation (see review, *Burnstock 1972*). This action of adenosine and ATP on the smooth muscle was not blocked by antagonists of acetylcholine or norepinephrine; anti-adrenergic agents such as reserpine, guanethidine or 6-hydroxydopamine; finally tetrodoxin had no effect on these responses. In these preparations adenosine and ATP (in micromolar concentrations) were highly effective inhibitors of the longitudinal muscle mechanical activity. Similar observations in other smooth muscle preparations (*Ambache and Zar 1970; Burnstock et al 1970; 1972a; Satchell et al 1973; Spedding et al 1975*) confirm the presence of these "non-adrenergic, non-cholinergic" inhibitory nerves, and mimicry of the nerve stimulated responses by adenosine and ATP.

The electrophysiological responses of the guinea pig taenia coli muscle cells to "non-adrenergic, non-cholinergic" nerve stimulation and exogenous ATP is hyperpolarization, similar to that produced by sympathetic nerve stimulation and exogenous norepinephrine. The cause of the hyperpolarization induced by the first two modes of stimulation is an increase in the potassium ion conductance whereas stimulation via adrenergic nerves and exogenous norepinephrine causes hyperpolarization by increases in the conductance of both the potassium and chloride ions (*Tomita 1972; Tomita and Watanabe 1973*).

A comprehensive catalogue of species and their organs from which these types of observations have been made is available in reviews by *Burnstock (1972; 1975a)*.

In summary, adenosine and ATP mimic the effects of "non-adrenergic, non-cholinergic" nerve activation at either the mechanical or the electrophysiological level and the criterion of mimicry has been adequately fulfilled by these two purines.

Inactivation

Termination of the effects of neurotransmitters appears to depend on two mechanisms: (a) catabolism to inactive metabolites, and (b) uptake of the neurotransmitter, lowering the concentration of the neurotransmitter in the vicinity of the receptors.

The uptake mechanism has been well documented as being the major one inactivating norepinephrine released by sympathetic nerve endings (*Iversen and Callingham 1970*), while in parasympathetic branch catabolism by acetyl cholinesterase is the major inactivating mechanism (*Michelson and Danilov 1970*). Although extracellular catabolism does occur in the sympathetic branch, this is a minor pathway for termination of responses. In the case of adenosine and ATP, uptake alone or metabolism followed by uptake, respectively, can provide a mechanism for inactivating these putative transmitters.

If ATP were released into the extracellular fluid it could be degraded to adenosine by enzymes such as ATPase and 5' nucleotidase. Both of these enzymes have been shown to be partially extracellular on guinea pig leukocytes (*De Pierre and Karnovsky 1974a,b*) and frog skeletal muscle

(*Woo and Manery 1975*). If adenosine were released or ATP converted to adenosine, this nucleoside could be removed from the extracellular fluid by uptake into cells. Once inside the cell adenosine could be degraded by adenosine deaminase (*Shenoy and Clifford 1975*) or reutilized to synthesize ATP via the salvage pathways (*Roux 1973; Maguire et al 1973; Agarwal et al 1975*). These systems could effectively lower the concentration of adenosine or ATP in the vicinity of its receptor site, thus terminating their effects.

In summary two mechanisms have been identified which can rapidly terminate the effects of adenosine and ATP; therefore, this criterion has been satisfied.

It is debatable whether this criterion is useful in deciding whether adenosine or ATP is the "non-adrenergic, non-cholinergic" neurotransmitter because these mechanisms are located in all cells (*Bohinsky 1973*). This contrasts with the situation seen in the adrenergic and cholinergic nervous systems where their major inactivating systems are discretely located in the vicinity of their respective receptors.

Drug effects

The specific modification of the biological effects of a drug (agonist) by another drug (potentiation or antagonism) may be capitalized upon by investigators in attempting to identify the release of this agonist from endogenous sources. Such pharmacological manipulations have been used in attempts to identify the putative neurotransmitter released by "non-adrenergic, non-cholinergic" nerves.

Potentiation

Dipyridamole, a drug which blocks the uptake of adenosine in a variety of tissues (red blood cells, *Bunang et al 1964;* guinea pig heart, *Hopkins 1973*), has been documented as being able to potentiate the actions of exogenously added adenosine and ATP in a number of systems (*Stafford 1966; Huang and Daly 1974b; Satchell and Burnstock 1975; Kalsner 1975*). Another drug which blocks adenosine uptake is hexobendine (*Kolassa et al 1971; Huang and Daly 1974b*); this drug has also been shown to potentiate the action (dilation of coronary arteries) of exogenously added adenosine (*McInnes and Parrat 1969*). Although these two drugs have been documented as being potentiators of the actions of exogenous adenosine and ATP, few researchers have examined the effects of dipyridamole and hexobendine on responses to activation of "non-adrenergic, non-cholinergic" nerves and to exogenously added adenosine and ATP in the same preparation.

One such study was conducted by *Satchell et al (1972)*, who found that dipyridamole ($0.4 - 5 \times 10^{-7}$ g/ml) potentiated the inhibitory responses of the guinea pig taenia coli to transmural stimulation and to either exogenous adenosine or ATP. This potentiation did not occur with the responses to perivascular stimulation (which activates adrenergic nerves) or exogenously applied norepinephrine.

These workers obtained similar results with hexobendine (10^{-6} g/ml). However, the potentiation of "non-adrenergic, non-cholinergic" nerve stimulated responses and that to exogenous ATP were only half that obtained using dipyridamole. On the other hand, *Huang and Daly (1974b)* have shown that in the guinea pig brain these two drugs were equipotent inhibitors of adenosine uptake. This raises the possibility that the potentiation seen with hexobendine might be due in part to mechanisms other than

inhibition of adenosine uptake. Indeed, support of this was presented in the study of *Satchell et al* (*1972*), where the concentration of hexobendine used to block the actions of exogenous adenosine was ten times in excess of that which blocked α adrenergic receptors in this system. In addition, the potentiation caused by hexobendine was not reversed by washing while that caused by dipyridamole was reversible.

Recently *Nakatsu and Bartlett* (*1979*) demonstrated that dipyridamole antagonized the effect of adenosine (relaxation) on the rat small intestine. This was opposite to the predicted potentiation. In the same system a specific blocker of adenosine uptake, 6-[2-hydroxy-5-nitrobenzyl] thioinosine potentiated adenosine actions (*Bartlett 1978*). Thus the action of dipyridamole must have been as a result of some other mechanism of action.

Antagonism

Attempts have been made to obtain support for the "purinergic" hypothesis utilizing a number of drugs which antagonize the actions of exogenous adenyl compounds. Researchers have made use of drugs such as quinidine, phentolamine, tolazoline, yohimbine, imidazole, 2-2'-pyridylisatogen and metoclopramide in a variety of experimental models. Those studies which utilized the above listed drugs will be discussed with emphasis on whether the data contribute to the fulfillment by adenosine or ATP of the fifth criterion -- identical drug effects on responses to both "putative" and endogenous transmitter substances.

Adenosine and ATP produce a high grade heart block in the guinea pig (*Stafford 1966*) and it was later found that the antimalarial drugs, quinine and quinidine antagonized this action of the adenyl compounds

(*Burnstock 1972*). Attempts have been made to utilize these drugs as antagonists of "non-adrenergic, non-cholinergic" nerve mediated responses and those due to either exogenous adenosine or ATP. Quinine (1×10^{-5} g/ml) had no effect on either exogenous ATP or transmural stimulation of the guinea pig taenia coli (*Burnstock et al 1970*). However, quinidine, the dextrorotary isomer of quinine, at a concentration of 2×10^{-4} g/ml antagonized the individual inhibitory actions of adenosine, ATP and "non-adrenergic, non-cholinergic" nerve activation (*Burnstock et al 1970; Burnstock et al 1972*). This drug at a much lower concentration, 5×10^{-5} g/ml, blocked the relaxation of the taenia coli induced by norepinephrine or perivascular adrenergic nerve stimulation. This evidence that quinidine was a nonspecific antagonist was supported by the work of *Spedding et al (1975)* who found that quinidine (140 μM) antagonized the relaxation of the guinea pig taenia caeci induced by either norepinephrine or isoproterenol yet was ineffective against ATP.

Recently *Manku and Horrobin (1976)* showed that chloroquine, quinine and quinidine were prostaglandin antagonists in the mesenteric vascular bed.

Other agents (phentolamine, tolazoline and yohimbine) had been shown by *Satchell et al (1973)* to block the inhibitory effects of ATP and transmural stimulation of guinea pig taenia coli. These agents at the concentrations used (phentolamine, 0.18 mM; tolazoline, 6.2 mM, yohimbine, 0.14 mM) have other blocking actions, for example at α adrenoreceptors (*Nickerson and Collier 1975*).

Earlier *Rikimaru et al (1971)* reported that imidazole in heroic concentrations blocked ATP induced relaxation of the guinea pig taenia coli, while being ineffectual against relaxation caused by activation of the "non-adrenergic, non-cholinergic" transmitter substance. These data

could not be reproduced by either *Bowman and Hall (1970)* or *Satchell et al (1973)*. The latter investigators suggested that the results of *Rikimaru et al (1971)* were due to improper control of the pH. They found that these large concentrations of imidazole (50 mM), raised the pH of the physiological solution to 8.5. When the pH was readjusted to 7, the same concentration of imidazole was ineffective in antagonizing relaxation induced by ATP or transmural stimulation of the "non-adrenergic, non-cholinergic" nerves.

In summary, quinidine, phentolamine, tolazoline and yohimbine are all capable of blocking the actions of exogenous adenosine and ATP while also blocking the responses to activation of the "non-adrenergic, non-cholinergic" nerves. However, these drugs have been shown to have other actions, hence any data provided in studies using these drugs are not by themselves compelling evidence in favour of the view that either adenosine or ATP is the "non-adrenergic, non-cholinergic" transmitter.

It has been reported that a new compound 2-2'-pyridylisatogen produced a specific blockade of the action of ATP on guinea pig taenia coli (*Hooper et al 1974*) and that this drug also tended to block the effects of activation of "non-adrenergic, non-cholinergic" nerves. *Spedding et al (1975)* have provided evidence that ATP antogonism by 2-2'-pyridylisatogen becomes irreversible after 30 min and reuqired concentrations in excess of 12.5 μM. Furthermore, this compound (40 μM) did not antagonize the relaxation of the taenia coli following field stimulation of the "non-adrenergic, non-cholinergic" nerves. Recently, *Spedding and Weetman (1976)* reported that in the guinea pig caecum, 2-2'-pyridylisatogen was a much more potent antagonist against ATP-induced than against adenosine-induced relaxation. The significance of this finding remains unknown at this

time but suggests an ability to differentially block the action of these two purines.

The specificity of this compound as an antagonist is questionable, because the dose ratio for adenosine in the presence of 2-2'-pyridylisatogen is similar to that for norepinephrine under identical conditions (*Spedding and Weetman 1976*). *Dean and Downie* (*personal communication*) have found that this drug is also effective in depressing the cholinergic responses in the bladder. Recently *Sakai et al* (*1979*) reported that in the guinea pig vas deferens 2-2'-pyridylisatogen was a specific blocker of ATP but not adenosine action.

Another compound metoclopramide, a derivative of procainamide (a local anesthetic with quinidine-like actions) has been shown to oppose the actions of adenyl compounds. *Okwuasaba and Hamilton* (*1975*) reported that metoclopramide (0.1 and 1.0 μM) antagonized the inhibitory actions of ATP, ADP and adenosine on intestinal smooth muscles of a number of species (guinea pig, rabbit and rat). In all of these smooth muscle preparations metoclopramide exhibited greater potency against ATP rather than ADP, AMP or adenosine-induced relaxation. In these studies this drug (metoclopramide) potentiated the effect (relaxation) of norepinephrine whereas *Bury and Mashford* (*1976*) demonstrated metoclopramide in similar concentrations also potentiated the effect (contraction) produced by exposure of the guinea pig ileum to substance P, acetylcholine and histamine. when the concentration of metoclopramide was increased (range 10^{-6} - 10^{-4} M) these contractions were dramatically reduced. This evidence suggests that the antagonism of adenosine and ATP might also be due to another action of metoclopramide other than that suggested. In addition, it is possible that the effects described by *Bury and Mashford* (*1976*) occur with the

adenyl compounds when lower concentrations of metoclopramide (< 0.1 μM) are used.

The studies discussed so far using drugs as pharmacological tools have all emphasized, paraphrasing *Burnstock (1972)*, that the most outstanding gap in our knowledge of the "non-adrenergic, non-cholinergic" nervous system is the discovery of drugs which can selectively block or augment the effects of addition of the "putative" transmitter (e.g. adenosine or ATP) and the responses to activation of these nerves. A drug which might help to fill this void is theophylline (a methylxanthine). Over the past decade it has become apparent that theophylline selectively and reversibly antagonizes the actions of exogenous adenosine and ATP.

The earliest report of antagonism between the methylxanthines and the adenyl compounds was by *Nichols and Walaszek (1963)*; in their studies they found that intravenous caffeine blocked the fall in blood pressure caused by adenosine and ATP in a number of species (chickens, rabbit, cat and dog). Later *De Gubareff and Sleator (1965)* demonstrated that adenosine inhibited the electrical activity of the isolated guinea pig atrium and this effect was blocked or reversed by caffeine, depending on the order in which the two drugs were added.

In studies undertaken with brain tissue, *Sattin and Rall (1970)* reported that adenosine and ATP (50 μM) each induced a 20 - 30-fold increase in the adenosine 3' 5' cyclic monophosphate (cyclic AMP) content of guinea pig cerebral cortical slices. Adenosine-induced increases in cyclic AMP content were dose related with maximum stimulation at 100 μM, at drug concentrations in excess of 500 μM adenosine became auto-inhibitory, producing a bell shaped dose response curve. When the dose response curve was generated in the presence of theophylline (0.5 mM), the rising portion of the curve was shifted to the right by a factor of ten, while the declining protion

was unaffected. Similar results were obtained using isolated neuroblastoma cells (*Penit et al 1976*). Here too, theophylline (0.001 - 1.0 mM) caused a rightward shift in the rising portion of the dose response curve suggesting that this antagonism seen with theophylline might be of a competitive nature.

In the rabbit ileum, *Ally and Nakatsu (1976)* have shown that theophylline but not its 7 substituted analogues,was a competitive adenosine antagonist. Neither adenosine nor theophylline compounds increased the tissue levels of cAMP (*Ally 1976*). In similar studies using vascular smooth muscle (dog and guinea pig), *Herlihy et al (1976)* reported complementary data showing that neither adenosine nor theophylline, in biologically effective concentrations, had any effect on tissue cyclic AMP levels. In a study in which changes in tissue cyclic AMP levels and adenylate cyclase activity was monitored (*McKenzie et al 1977*), it was concluded that neither adenylate cyclase inhibition nor changes in tissue cylic AMP levels were part of the mechanism of the smooth muscle relaxant action of adenosine or ATP.

In a more recent report, the potencies of 1-methyl-3-isobutylxanthine and isoamyl-3-isobutylxanthine were compared in relation to their smooth muscle relaxing and cyclic AMP elevating abilities. In pig coronary arteries isoamyl-3-isobutylxanthine was 2 - 3-fold more potent in causing relaxation although it was one-tenth as potent as a phosphodiesterase inhibitor (*Kramer and Willis 1977*).

The pool of data on brain slices has been expanded by *Daly and coworkers (Huang et al 1972; Schultz and Daly 1973; Huang and Daly 1974; Mah and Daly 1976)* verifying that theophylline antagonized adenosine-induced formation of cyclic AMP. *In situ, Sattin (1971)* had demonstrated

that theophylline and caffeine (0.2 μmol/g) effectively antagonized cyclic AMP increases in mouse forebrain normally seen during seizures. Examining another parameter within the brain, (the firing rate of cortical neurons), *Phyllis and Kostopoulos (1975)* have shown that intravenous or iontophoretic administration of theophylline or caffeine blocked the depressant action of adenosine in a reversible manner.

Similar data gathered from a variety of animal models have all documented the effective use of theophylline as an adenosine or ATP antagonist. Some examples and the parameters monitored are: guinea pig and rat sinus rhythm (*Shaumann et al 1970*); coronary blood flow in dogs (*Afonso 1970; Paolini and Wicken 1975*); blood flow, in renal artery and kidney in dogs (*Osswald 1975*); and fat cell lipolysis (*Schwabe and Ebert 1974*).

Site of adenosine action

In studies on coronary myocytes and atrial muscle cells with an adenosine derivate of approximate MW (> 30,000 daltons) *Schrader et al (1977)* demonstrated that AMP-protein conjugate caused effects similar to those of free adenosine and AMP. Using ^{14}C-radiolabelled cyclic AMP-protein conjugates they demonstrated that no radioactivity was taken up by the tissues. In control experiments using cyclic AMP and ^{14}C-adenosine significant incorporation was seen. They concluded that the data provided direct evidence for a cell surface receptor for adenosine as well as AMP action on coronary myocytes and atrial muscle cells. A similar conclusion had been made by *Ally and Nakatsu (1976)* for the actions of adenosine and its phosphorylated derivatives in the rabbit ileum. These researchers also demonstrated that an intact adenosine moiety was essential for

receptor activation.

In a recent study *Verhaeghe et al (1977)* showed adenosine and the adenine nucleotides relaxed strips of canine saphenous vein and tibial artery to a greater degree when they had been contracted by nerve stimulation than by exogenous noradrenaline. The greater effect seen was a result of inhibition of noradrenaline release from the sympathetic nerve endings. Adenosine had no effect on either basal or tyramine stimulated neurotransmitter release, although it did reduce potassium (30 mM) induced efflux. The adenosine antagonist theophylline antagonized the inhibitory effect of adenosine (low concentration). High concentrations of adenosine ($> 4 \times 10^{-4}$ M) increased the intraneuronal leakage of noradrenaline out of the storage vesicles. In *in vivo* experiments they also showed that adenosine inhibited saphenous venoconstriction induced by lumbar chain stimulation or infused noradrenaline.

In similar experiments in the rabbit kidney (*in vitro* and *in situ*) in the canine subcutaneous adipose tissue (*in situ*) and guinea pig vas deferens (*in vitro*), *Hedqvist and Fredholm (1976)* found that adenosine significantly and reversibly depressed noradrenaline action and its release from the nerve endings. These researchers concluded that adenosine modified catecholamine responses by a mechanism independent of its direct effects on the smooth muscle.

Adenosine and its related compounds are released from several tissues upon nerve stimulation (*Burnstock 1972; Su et al 1971; Pull and McIlwain 1972*). The amount of adenosine released appears to be of the same order of magnitude (10^{-7} - 10^{-5} M) as that found by *Hedqvist and Fredholm (1976)* and *Verhaeghe et al (1977)* to inhibit transmitter release. In the guinea pig heart *Schrader et al (1977)* found that isoproterenol

increased the adenosine concentration in the effluent within 45 secs from 10^{-8} M to about 10^{-6} M. It thus appears that in the ventricular as well as in the vascular muscle adenosine serves two functions: the alteration of vascular reactivity which then limits the inotropic and metabolic effects of the catecholamines, as well as a negative feedback inhibition of neurotransmitter release.

RESULTS

Adenosine inhibition of pressor responses

Adenosine (2×10^{-6} to 4×10^{-3} M) was added to the buffer reservoir in a cumulative fashion and pressor responses to either injected noradrenaline or potassium ions were determined. As can be seen in Figure 51 adenosine had a very weak inhibitory effect on responses to injected potassium with a maximum inhibition of 20% at an adenosine concentration of 4×10^{-3} M. In contrast, the dose response curve against noradrenaline was distinctly broken into two parts. The slope of the section between 2×10^{-6} M to 1×10^{-4} M was significantly greater ($p < 0.01$, ANOVA) when compared to the curve obtained between 1×10^{-4} M to 4×10^{-3} M adenosine. The concentration of adenosine causing 50% inhibition was approximately 10^{-4} M (determined from Fig. 51). Because of the differential dependency of these two pressor substances on extracellular and intracellular calcium stores, it appeared that adenosine might be acting only against that component of noradrenaline action dependent on intracellular calcium release. Adenosine did not alter baseline perfusion pressures in these experiments as shown in the representative trace, Figure 52.

Adenosine inhibition of noradrenaline responses in preparations perfused with low calcium buffer

The calcium chloride was omitted from the normal buffer and the experiments were repeated; the free calcium in a buffer prepared in this manner is about 10^{-6} M. In this situation, adenosine abolished noradrenaline responses with a linear log response curve over the entire

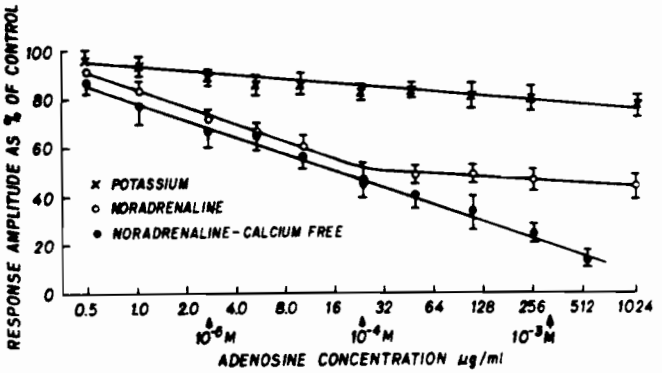

Fig.51: Inhibition of pressor responses in the rat mesenteric vascular bed by increasing concentrations of adenosine in the buffer. Potassium responses were studied in ordinary buffer and noradrenaline responses in both ordinary and calcium-free buffer. Six preparations were studied in each case. Prior to adding adenosine three test injections of the pressor agent were given and the mean pressor response taken as 100%. Pressor responses in the presence of adenosine are expressed as percentages of control ±SEM.

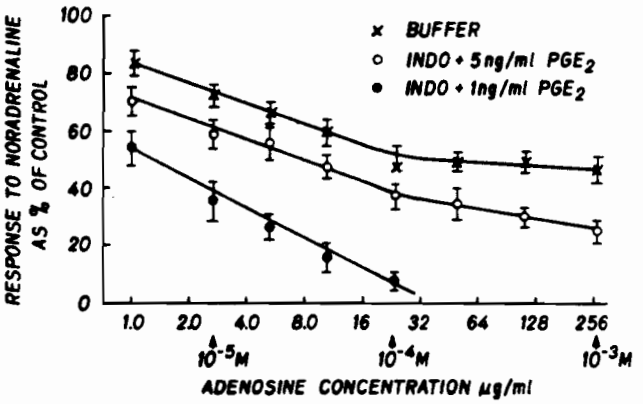

Fig.53: Inhibition of pressor responses to noradrenaline by adenosine when the preparations were perfused with buffer containing 64 µg/ml indomethacin and either 1 or 5 ng/ml PGE2. The indomethacin abolished pressor responses which were then partly restored by the PG. The experiments (six in each case) were then performed as described in fig.51.

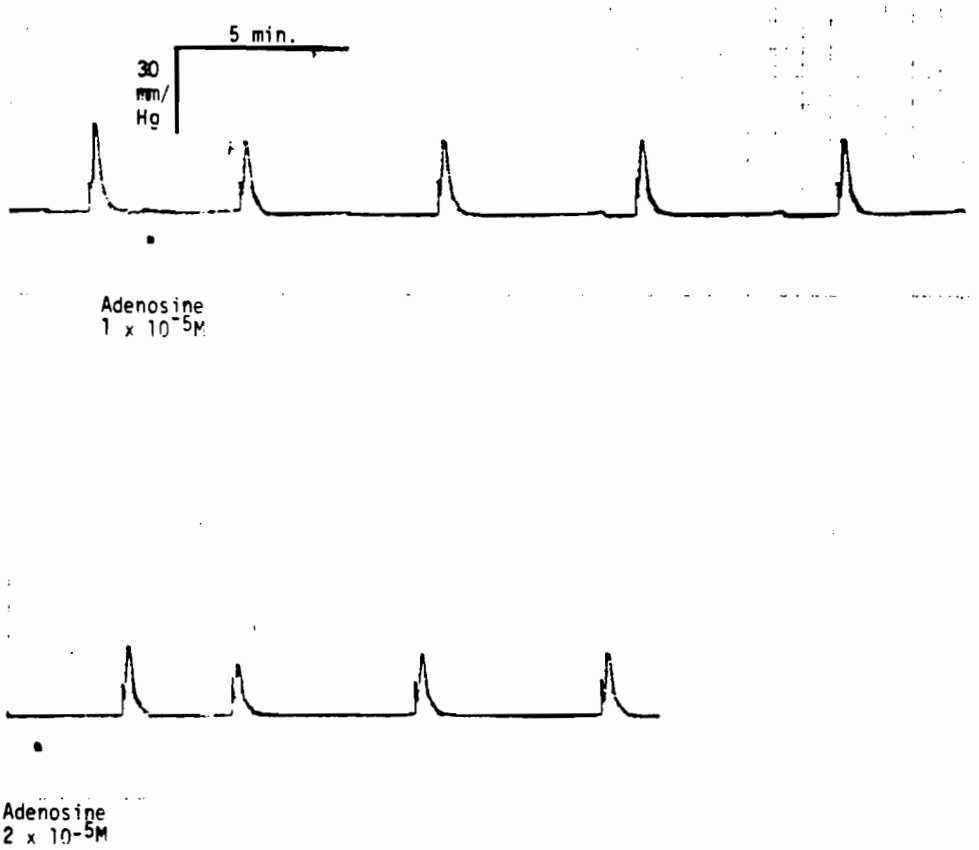

Fig. 52. Inhibition of noradrenaline pressor responses by adenosine added to the perfusate. Adenosine did not alter basal perfusion pressure or the duration of each contraction.

concentration range (2×10^{-6} M to 4×10^{-3} M, Fig. 51). The possibility that the adenosine effects might be solely due to alpha adrenoceptor blockade was eliminated by showing, in two experiments, that adenosine blocked angiotensin II responses in a similar manner. In two experiments in which the calcium concentration was lowered to 2.5×10^{-4} M, the inhibition of noradrenaline pressor responses by adenosine was similarly potentiated (see sample trace, Fig. 54).

Prostaglandin-adenosine interactions

The possibility of an interaction between adenosine and prostaglandins was first tested by repeating the study of adenosine inhibition of noradrenaline responses in preparations partially inhibited with indomethacin (8 µg/ml, 2.2×10^{-5} M). This treatment moved the dose response curve to the left in an apparently parallel manner (data not shown). The action of adenosine was then studied in preparations in which prostaglandin biosynthesis had been abolished with indomethacin (64 µg/ml, 1.8×10^{-4} M) and vascular reactivity partially restored by either 1 or 5 ng/ml PGE2 (2.8×10^{-9} or 1.4×10^{-8} M). Once the indomethacin and PGE2 had been added to the buffer and pressor responses had stabilized, adenosine was then added to the buffer and pressor responses determined at five minute intervals until the responses plateaued.

In the presence of the lower concentration of PGE2 (1 ng/ml, 2.8×10^{-9} M), adenosine abolished noradrenaline pressor responses. Adenosine was not able to abolish noradrenaline pressor responses in preparations perfused with a higher concentration of PGE2 (5 ng/ml, 1.4×10^{-8} M). In the latter experiment a dose response curve similar to that obtained in control experiments was derived. This may be indicative of a restoration of the participation of extracellular calcium in noradrenaline

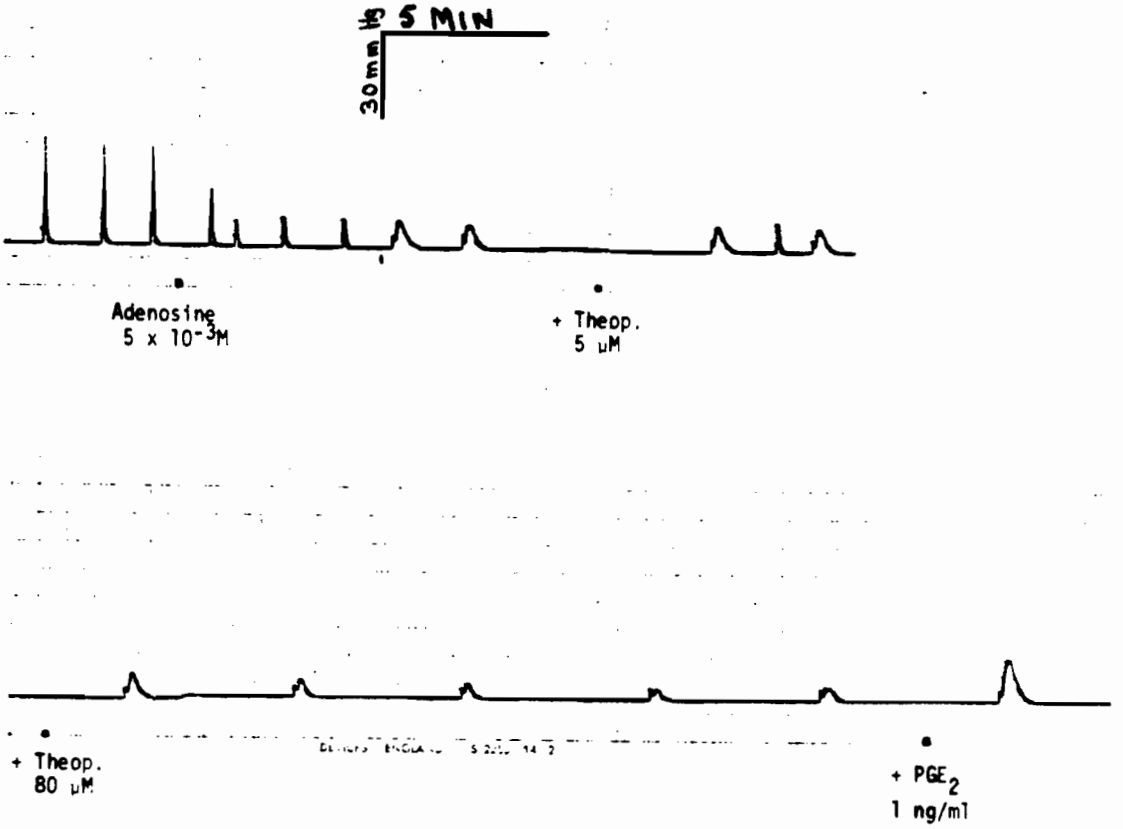

Fig. 54. The effects of adenosine on noradrenaline pressor responses in a preparation perfused with 2.5×10^{-4} M calcium containing buffer. Theophylline (5×10^{-6} and 80×10^{-6} M) added to the perfusate did not reverse or oppose adenosine action. PGE2 (1 ng/ml, 2.8×10^{-9} M) added to the perfusate with both adenosine and theophylline present partially reversed the inhibition.

contractions at the higher PGE2 concentration (Fig. 53). Adenosine therefore appeared to antagonize that component of prostaglandin action associated with the mobilization of intracellular calcium stores. The addition of PGE2 to the buffer after adenosine and theophylline (a purine an logue) can partially reverse the inhibitory effects of these substances (Fig. 54).

Potentiation of the inhibitory action of adenosine by a thromboxane synthetase inhibitor

As noted above partial inhibition of prostaglandin biosynthesis with indomethacin (8 μg/ml, 2.2×10^{-5} M) potentiated the inhibitory actions of adenosine on noradrenaline contractions whereas potassium contractions were unchanged. Using a more potent analogue of imidazole (1-benzylimidazole) to partially inhibit thromboxane A2 biosynthesis, the effects of adenosine were again examined. 1-Benzylimidazole potentiated the actions of adenosine, shifting the dose response curve to the left. In Figure 55 the effects of adenosine at three concentrations (1×10^{-5}, 1×10^{-4}, 1×10^{-3} M) in the presence and absence of 1-benzylimidazole (300 ng/ml, 1.9×10^{-6} M) are shown. As can be seen the inhibitory potency of adenosine was significantly enhanced at each concentration ($p < 0.05$ ANOVA).

PGE2 antagonism by adenosine in lymphocytes

In an unrelated experimental system (human lymphocytes transformed by Epstein Barr virus) PGE2 at low concentrations (2.8×10^{-12} M) significantly potentiated cell proliferation ($p < 0.05$ ANOVA). Increasing the concentration of PGE2 ($> 2.8 \times 10^{-12}$ M) caused a progressively increasing

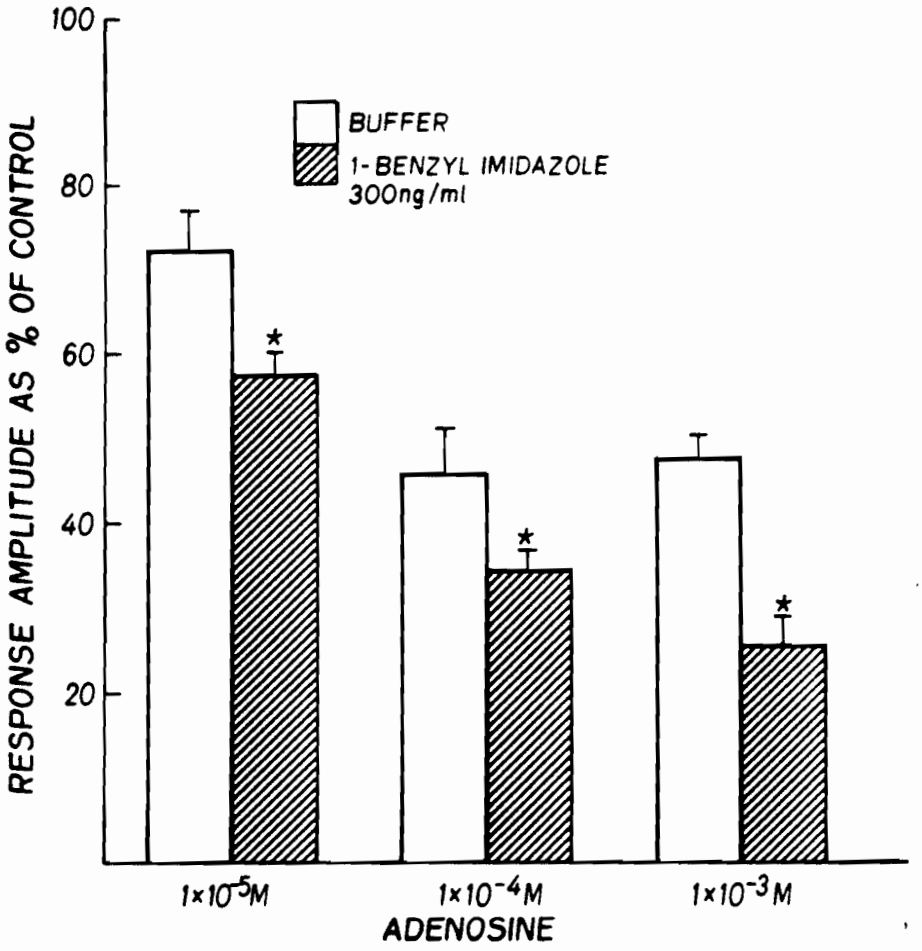

Fig. 55. Inhibition of pressor responses to noradrenaline by adenosine when the preparations were perfused with buffer alone or buffer containing a thromboxane synthetase inhibitor, 1-benzyl imidazole (300 ng/ml, 1.9×10^{-6} M). *$p < 0.05$, ANOVA.

inhibitory effect on cell multiplication. Adenosine at the two concentrations tested (1×10^{-4} M and 2×10^{-4} M) inhibited the PGE2 stimulated proliferation, shifting the concentration responses progressively rightwards (Fig. 56). Similar results were obtained with phythohemagglutinin stimulated human lymphocytes.

Structural requirements of purine analogues for activity in the mesenteric vascular bed

Having established that adenosine was a potent and effective inhibitor of noradrenaline responses, it was of interest to examine the actions of several adenosine analogues in an attempt to exclude or implicate activity at the classical "adenosine" receptor (*Burnstock 1970; 1975; Ally and Nakatsu 1976*).

The adenosine nucleotides, adenosine 5'monophosphate (5'AMP) and adenosine 5'diphosphate (5'ADP) were both effective inhibitory agents in this preparation, though less potent than adenosine itself (Fig. 58, see Fig. 57 for structure). Other purine ribosides tested (2'deoxyadenosine, inosine, 1-benzylinosine and guanosine) were much less potent than adenosine.

Adenine, which differs from adenosine by the absence of the ribose moiety at position 9 and from inosine by the absence of the ribose at position 9 and the presence of an amino group at position 6, was about half as potent as adenosine and twice as potent as inosine (Fig. 58).

Guanosine, which can be regarded as an inosine analogue made by the addition of an amino group at position 2, was equipotent with inosine. The substitution of a benzyl group at position 1 (1-benzyl inosine), increased its potency to equal that of adenosine (Fig. 58).

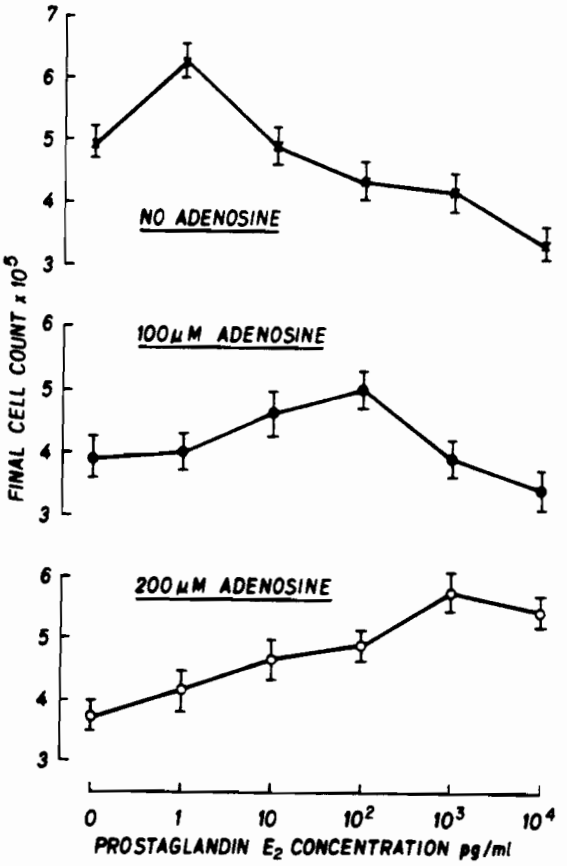

Fig. 56. The effects of adenosine and PGE2 on lymphocyte cell proliferation. Cell counts are expressed as numbers/ml of medium at the end of the three day incubation. The starting cell count was 2×10^5 cells/ml in each case. 1 pg/ml PGE2 is approximately 2.8×10^{-12} M. Each point represents mean ± SE, n = 10.

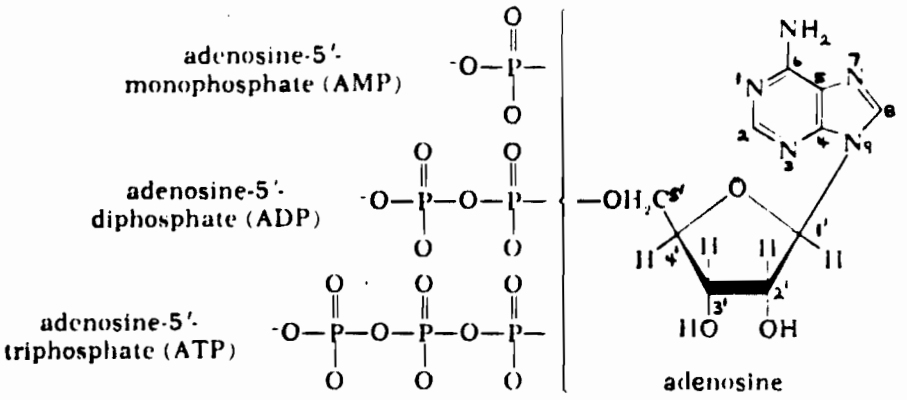

Fig. 57. Structure of adenosine and its phosphorylated derivatives. The numbering system for each ring of the purine rings and the ribose moiety is indicated.

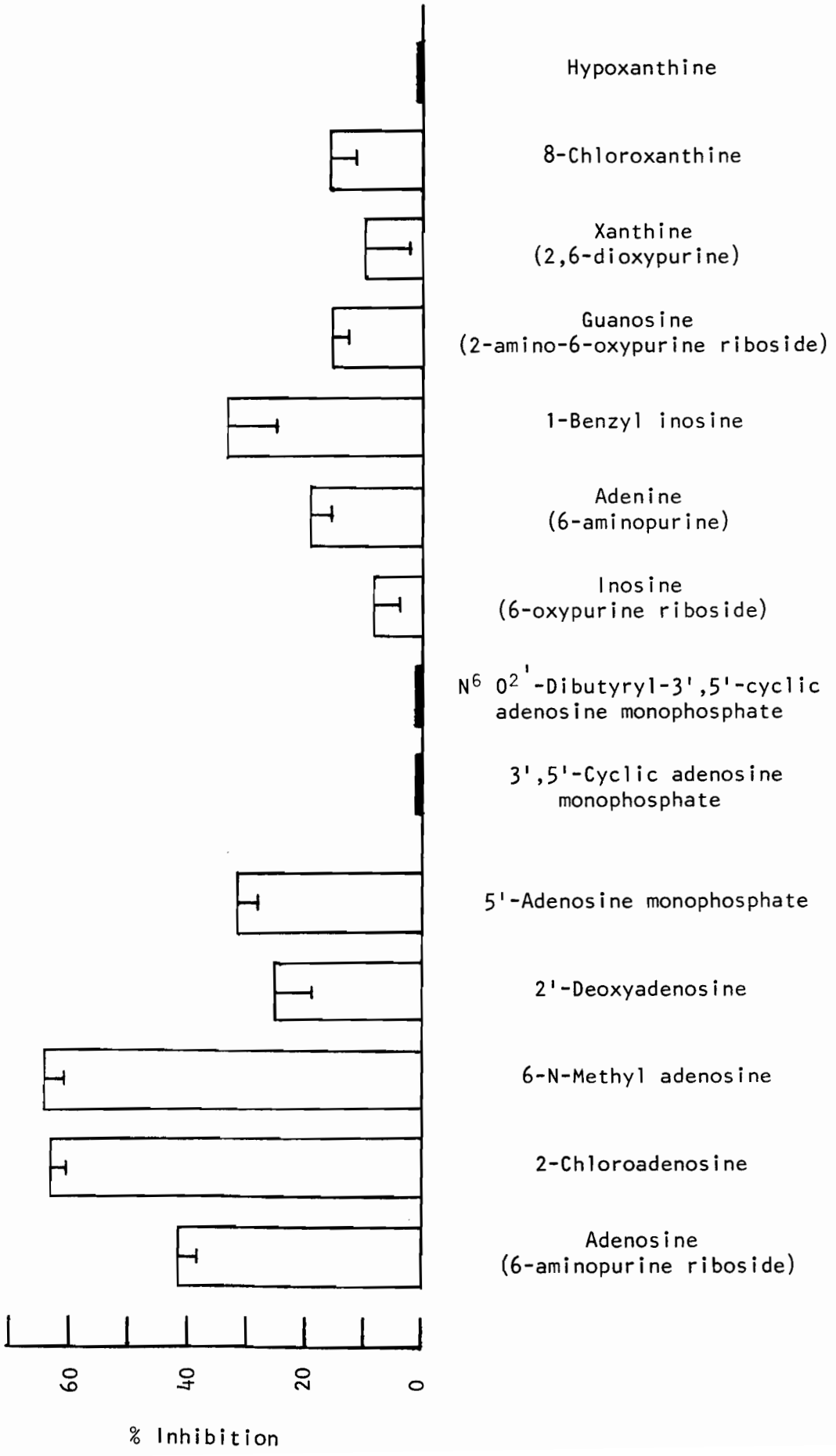

Fig. 58. Inhibition of noradrenaline pressor responses in the mesenteric vascular bed by adenosine and adenine analogues (1×10^{-4} M). The effect of each compound is expressed as % inhibition of naradrenaline control responses (mean ± SE, n = 6).

From these data it appeared that maximal activity depended upon a structure similar to the adenine moiety of adenosine. With this in mind, a number of compounds structurally similar to adenosine were tested.

The addition of a methyl group to the 6-nitrogen (6-N-methyl purine riboside) resulted in a significant increase in inhibitory potency ($p < 0.01$, ANOVA), whereas purine riboside itself was relatively inactive.

Xanthine (2,6-dioxypurine) was equipotent with purine riboside, indicating that the ribose moiety was not essential for activity. This was further supported by the inhibitory activity of adenine (6-amino-purine). This latter compound differs from adenosine by the absence of the ribose moiety and was about half as potent as adenosine.

Increasing the basicity of the purine nucleus of adenosine by the introduction of a chloro group (2-chloroadenosine) significantly increased its potency (Fig. 58, $p < 0.01$, ANOVA) to equal that of 6-N-methyl purine riboside.

As shown above, the ribose moiety was not essential for activity, however both 2'-deoxy- and 3'-deoxy-adenosine were less potent than adenosine (activity relative to adenosine $\simeq 0.5$, $p < 0.001$, ANOVA). In contrast, 3',5'-cyclic adenosine monophosphate (cyclic AMP) and $N^6,O^{2'}$-dibutyryl 3',5'-cyclic adenosine monophosphate (dibutyryl cyclic AMP) were inactive in concentrations up to 1×10^{-2} M. Increasing the lypophilicity of cyclic AMP with the butyryl groups did not result in any discernable inhibitory action. This is in agreement with the results obtained with 1-benzyl inosine showing that this lipophilic analogue was not any more potent than adenosine itself.

These data indicate that the inhibitory effect of adenosine was not mediated at the "adenosine" receptor, because compounds inactive at

this receptor in brain (*Huang et al 1972; Huang and Drummond 1976*) and smooth muscle (*Ally and Nakatsu 1976; Herlihy et al 1976*) retain inhibitory activity in the mesenteric vasculature.

2,6 dioxypurine analogues as potent prostaglandin antagonists

It appeared that suitable substituents on the 2,6 dioxypurine moiety (xanthine) could possibly result in potent prostaglandin antagonist compounds. One such analogue, 2,6-dioxy-8-chloropurine, appeared slightly more potent than 2,6 dioxypurine. In preliminary experiments 1,3,7-trimethyl-2,6-dioxypurine (caffeine) appeared to be a fairly potent prostaglandin antagonist; however, this compound was relatively insoluble.

A more suitable analogue was 1,3-dimethyl-2,6-dioxypurine (theophylline). This compound, unlike adenosine, effectively inhibited both noradrenaline and potassium responses (Fig. 59); the concentrations of theophylline inhibiting pressor responses by 50% were: noradrenaline, 1.1×10^{-5} M, and potassium, 3.5×10^{-4} M.

In two experiments, it was shown that theophylline inhibited noradrenaline responses in preparations perfused with indomethacin (64 µg/ml, 1.8×10^{-4} M) plus PGE2 (5 ng/ml, 1.4×10^{-8} M). The effective inhibitory concentration range was 5.0×10^{-6} M to 5.0 to 10^{-5} M. Similar inhibition by theophylline of potassium pressor responses was senn in preparations perfused with indomethacin (1.8×10^{-4} M) plus either 1 ng/ml PGE2 (2.8×10^{-9} M) or 5 ng/ml (1.4×10^{-8} M) PGE2 (Fig. *60*). In this case the effective theophylline concentration range was 4.4×10^{-5} M to 1.4×10^{-3} M.

The possibility existed that the effects of theophylline were the result of activation of the "adenosine" receptor, since in a number of

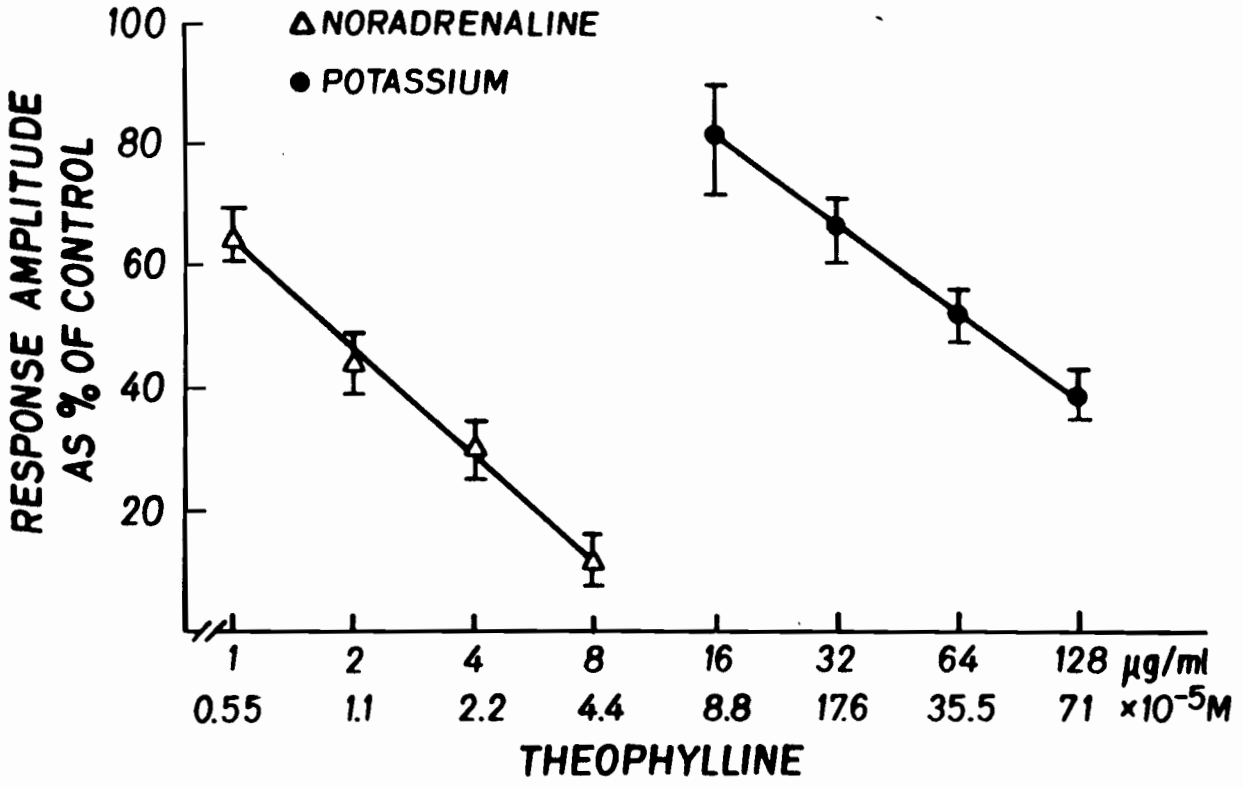

Fig. 59. Inhibition of pressor responses in the rat mesenteric vascular bed by increasing concentrations of theophylline added to the buffer. Prior to adding theophylline to the buffer four test injections of the pressor agent were given and the mean pressor response taken as 100%. Pressor responses in the presence of theophylline are expressed as percentages of control. Each point represents data from 6 preparations, mean ± SE.

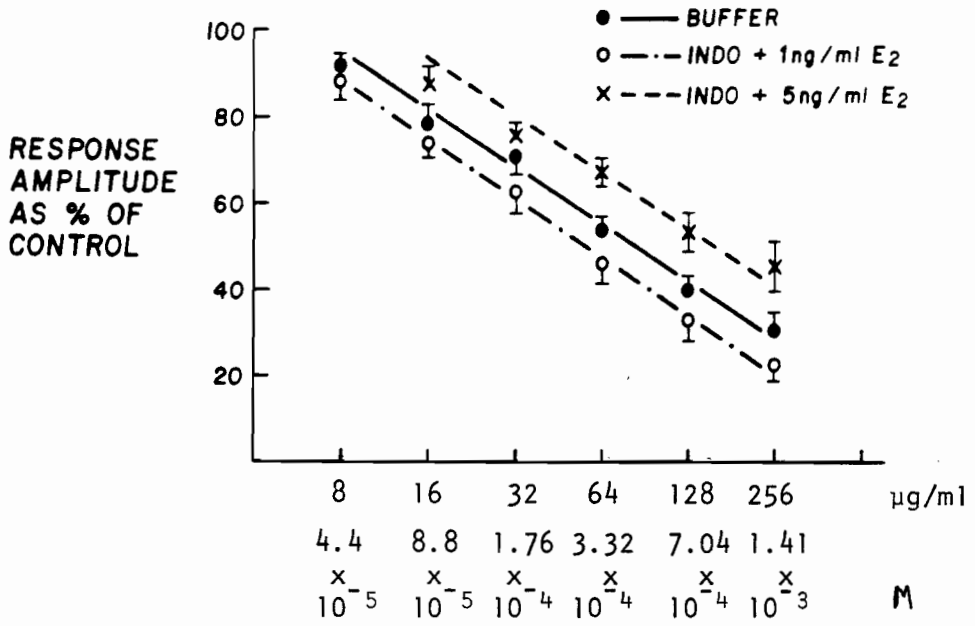

Fig. 60. Inhibition of pressor responses to potassium by theophylline when the preparations were perfused with buffer alone or buffer containing indomethacin (1.8×10^{-4} M) and either 1 ng/ml (2.8×10^{-9} M) or 5 ng/ml (1.4×10^{-8} M) PGE2. The indomethacin abolished pressor responses which were then partially restored by the PG. The experiments were then perfused as described in Fig. 59. Each point represents the response mean ± SE n = 6. The responses in the presence of either 1 or 5 ng/ml PGE2 were significantly different from each other ($p < 0.05$, ANOVA) but not from those obtained in buffer only perfused preparations.

systems theophylline has been shown to have agonist/antagonist interactions with adenosine (*Sattin and Rall 1970; Burnstock 1975; Ally and Nakatsu 1976*). It was therefore necessary to test a xanthine analogue which lacked these properties. Such an analogue, 7,(2,3) dihydroxyprophyl 1,3 dimethyl 2,6-dioxypurine (diprophylline), was inactive as an adenosine antagonist in the rabbit ileum. This is in agreement with published data that 7-substituted methylxanthines lack adenosine antagonist action (*Ally and Nakatsu 1976*).

In a manner similar to theophylline, diprophylline antagonized both noradrenaline and potassium responses (Fig. *60*). In contrast to theophylline, the concentrations of diprophylline inhibiting noradrenaline and potassium responses were similar (1.2×10^{-7} M to 7.7×10^{-6} M); however both compounds appeared to exhibit greater potency against noradrenaline responses (Figs. *59*, *61*).

In three preparations perfused with indomethacin (64 µg/ml, 1.8×10^{-4} M) plus PGE2 (5 ng/ml, 1.4×10^{-8} M), diprophylline inhibited noradrenaline responses over the same concentration range. In this situation endogenous prostaglandin biosynthesis was blocked and pressor responses were dependent upon the exogenous PGE2.

In preparations in which thromboxane synthesis was inhibited with imidazole (thus preferentially inhibiting noradrenaline responses) and vascular responsiveness restored with PGE2 (5 ng/ml, 1.4×10^{-8} M),diprophylline inhibited noradrenaline responses (Fig. *62*).

In two experiments using 1,3-dimethyl-2,6-dioxypurine-7-acetic acid (theophylline-7-acetic acid), results similar to those described above for diprophylline were obtained. This analogue was also inactive as an adenosine antagonist in the rabbit ileum (*Ally and Nakatsu 1976*).

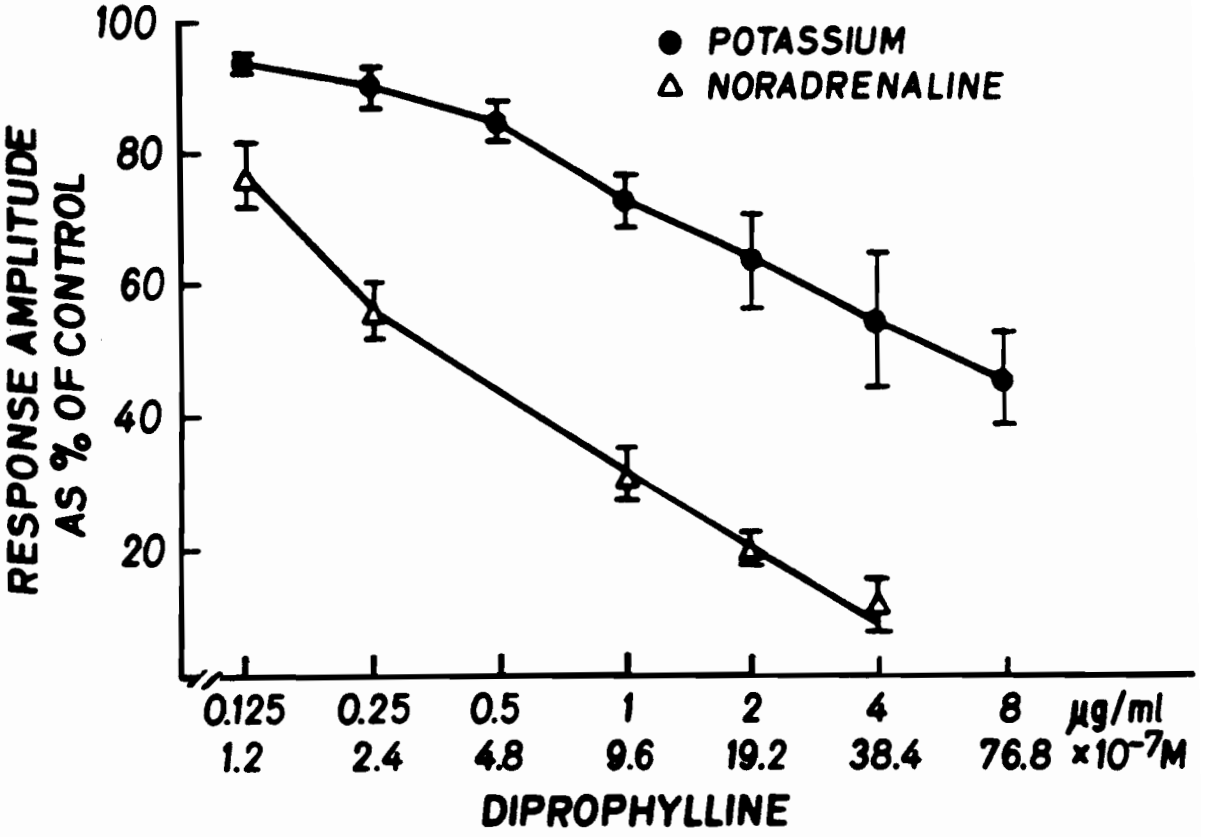

Fig. 61. Inhibition of pressor responses in the rat mesenteric vascular bed by increasing concentrations of 7-(2,3)-dihydroxyl-propyl 1,3-dimethyl,2,6-dioxypurine (diprophylline) in the buffer. Prior to adding diprophylline, four test injections of the pressor agent were given and the mean pressor response taken as 100%. Pressor responses in the presence of diprophylline are expressed as percentages of control. Each point shown represents M ± SE , n = 6.

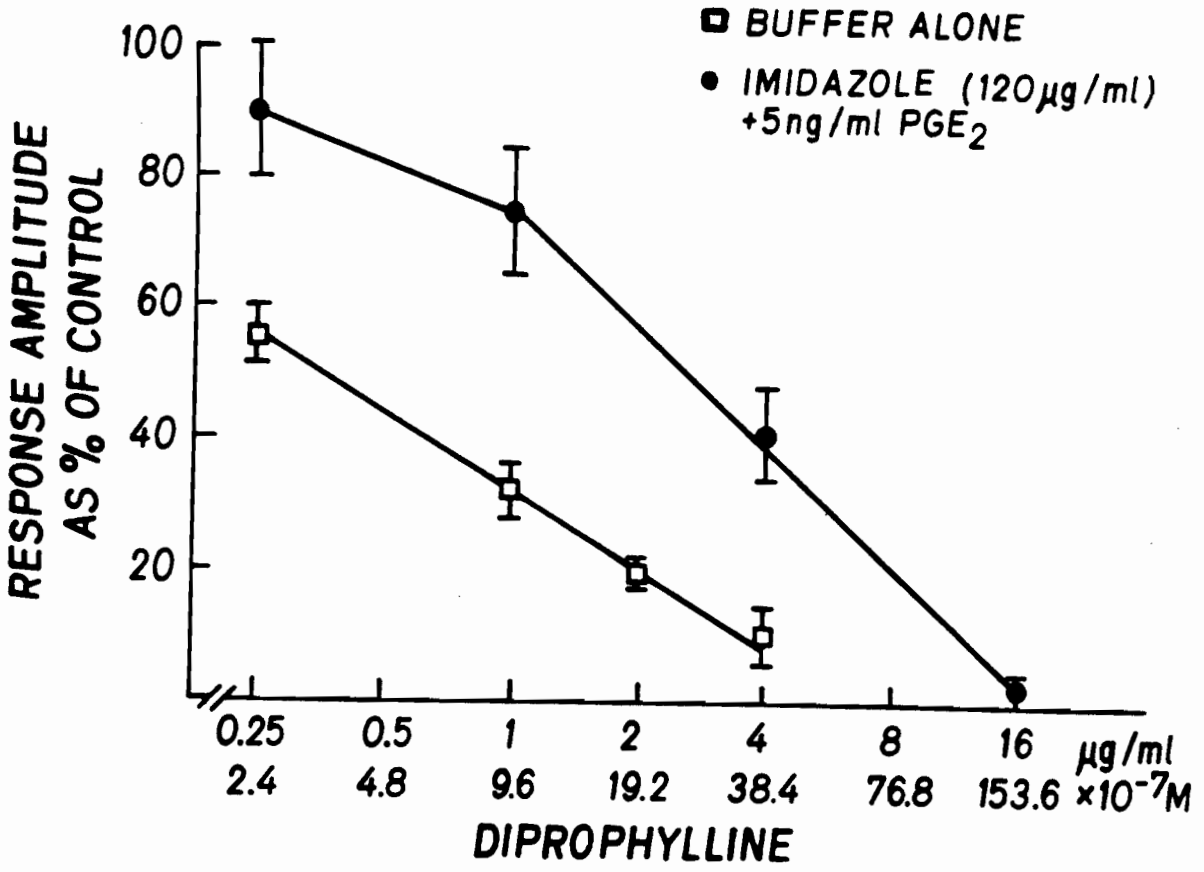

Fig. 62. Inhibition of noradrenaline pressor responses by diprophylline when the preparations were perfused with buffer alone or buffer containing imidazole (120 µg/ml 2.2×10^{-4} M) and PGE2 (5 ng/ml 1.4×10^{-8} M). The imidazole abolished noradrenaline responses which were then partly restored by the PG. Before adding diprophylline to the perfusate four test injections of noradrenaline were given and the mean response taken as 100%. Pressor responses in the presence of diprophylline are expressed as percentages of control ± SE , n = 5. Imidazole did not inhibit potassium responses whereas diprophylline did.

Neither theophylline, diprophylline nor theophylline-7-acetic acid antagonized the actions of adenosine in the mesenteric vascular bed. An example of such an experiment with adenosine and theophylline is shown in Figure 54.

Effect of dipyridamole on vascular responses

This compound has a distict chemical structure differing from that of the purines. It is an effective vasodilator and appears to act predominantly on small resistance vessels in the coronary bed. The vasodilator properties of dipyridamole have been ascribed to inhibition of adenosine deamination (*Bunabg et al 1964; Deutick and Gerlach 1966*) and to inhibition of adenosine uptake into erythrocytes or other cells (*Koss et al 1962; Gerlach et al 1964; Kubler et al 1970; Stafford 1966; Herlihy et al 1976*). If this was correct, then both adenosine and dipyridamole should have similar actions in the mesenteric vascular bed.

Dipyridamole (4.9×10^{-8} M to 1.6×10^{-6} M) was added to the perfusion buffer in a cumulative manner. This compound inhibited noradrenaline (Fig. 63) and angiotensin (2 experiments, data not shown) pressor responses at concentrations which had no effect on potassium responses (Fig. 63) or basal perfusion pressure. Dipyridamole inhibited both noradrenaline and angiotensin pressor responses by approximately 50% at a concentration of 4×10^{-7} M. In two experiments, PGE2 (10 ng/ml, 2.8×10^{-8} M) added to the perfusate after dipyridamole inhibition of noradrenaline responses reversed the inhibition. This suggested the possibility that dipyridamole was perhaps a prostaglandin antagonist.

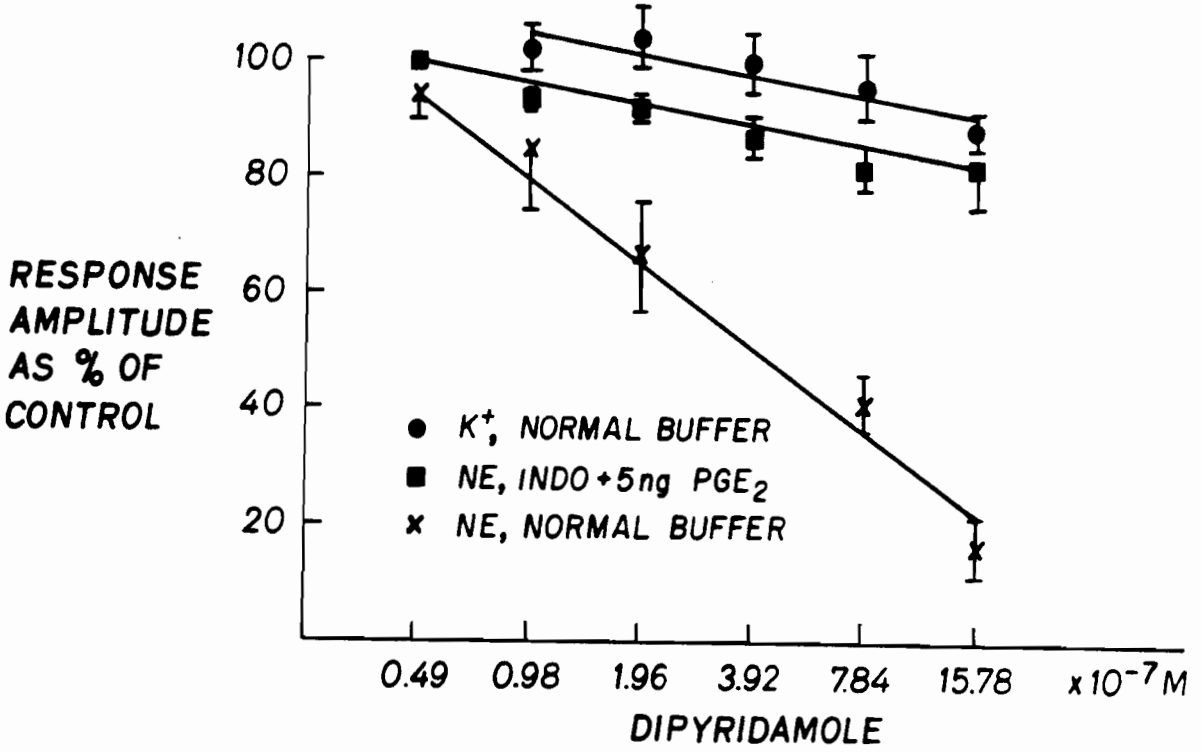

Fig. 63. The effects of dipyridamole in the perfusing buffer on responses to fixed doses of pressor agents. Results are expressed as percentages of the mean responses to either 10 ng norepinephrine or 43 millimoles potassium ions obtained prior to adding drug to the buffer. Each point represents the mean and SE for 6 experiments. Dipyridamole inhibited norepinephrine pressor responses in preparations perfused with buffer but failed to cause any significant inhibition in preparations perfused with indomethacin (to inhibit PG biosynthesis) plus PGE2 (to restore pressor responsiveness). Dipyridamole did not inhibit potassium responses in either buffer perfused or indomethacin plus PGE2 perfused preparations.

Dipyridamole-calcium interaction

Two preparations were equilibrated with buffer from which the calcium was omitted. The effect of a concentration of dipyridamole which normally inhibited noradrenaline responses by 50% was then determined. This concentration of dipyridamole (3.9×10^{-7} M) abolished noradrenaline responses. These data indicated that the action of dipyridamole was not similar to that of adenosine (see Fig. 51).

Dipyridamole: failure to inhibit noradrenaline pressor responses in preparations perfused with indomethacin plus PGE2

Preparations were perfused with indomethacin (50 µg/ml, 1.4×10^{-4} M) which abolished vascular responsiveness and presumably prostaglandin biosynthesis. Pressor responses were then partially restored with exogenous PGE2 (5 ng/ml, 1.4×10^{-8} M). Responses to three bolus injections of noradrenaline were obtained at 5 minute intervals and dipyridamole was then added to the buffer reservoir in a cumulative fashion and its effects on pressor responses determined. Dipyridamole (Fig. 63), unlike adenosine (Fig. 53) did not inhibit noradrenaline responses.

These data indicated that the action of dipyridamole was dissimilar to that of adenosine and was probably unrelated to its known inhibition of adenosine uptake.

Effect of hexobendine and lidoflazine on vascular responses

Hexobendine, which is equipotent to dipyridamole as an inhibitor of adenosine uptake and phosphodiesterase enzyme activity (*Huang and Drummond 1976*), was ineffective in the mesenteric vascular bed. Neither noradrenaline nor potassium responses were inhibited at concentrations ranging from

1×10^{-8} M to 5×10^{-6} M.

Lidoflazine, a weak adenosine uptake inhibitor (*Huang and Drummond 1976*) structurally unrelated to either dipyridamole or hexobendine but possessing quinidine like properties, inhibited both potassium and noradrenaline pressor responses at high concentrations (2×10^{-5} M, 3 experiments). Perfusion of the preparations with indomethacin (20 µg/ml, 5.6×10^{-5} M) increased the inhibitory effects of lidoflazine on noradrenaline responses. Inhibition was now seen with much lower concentrations of lidoflazine (2×10^{-6} M). These data dissociated blockade of adenosine uptake from the biological activity seen with dipyridamole.

Adenosine-dipyridamole interaction

In two experiments, noradrenaline pressor responses were partially inhibited with 1×10^{-4} M adenosine and dipyridamole (1×10^{-5} M) was then added to the perfusate. This concentration of dipyridamole inhibited adenosine uptake by approximately 80% (*Huang and Drummond 1976*). Dipyridamole abolished noradrenaline responses within 1 minute, whereas adenosine required 15 to 20 minutes to exert its maximal effect. The inhibition caused by dipyridamole was readily reversed (within 8 minutes) upon switching to buffer plus adenosine (Fig. *64*). As shown in the sample trace, neither compound (adenosine nor dipyridamole) caused any changes in baseline perfusion pressure.

Adenosine-hexobendine interaction

In 1 experiment, hexobendine (1×10^{-5} M) was added to the perfusate after adenosine (1×10^{-4} M), had no effect on pressor responses comparable to that noted above (Fig. *64*).

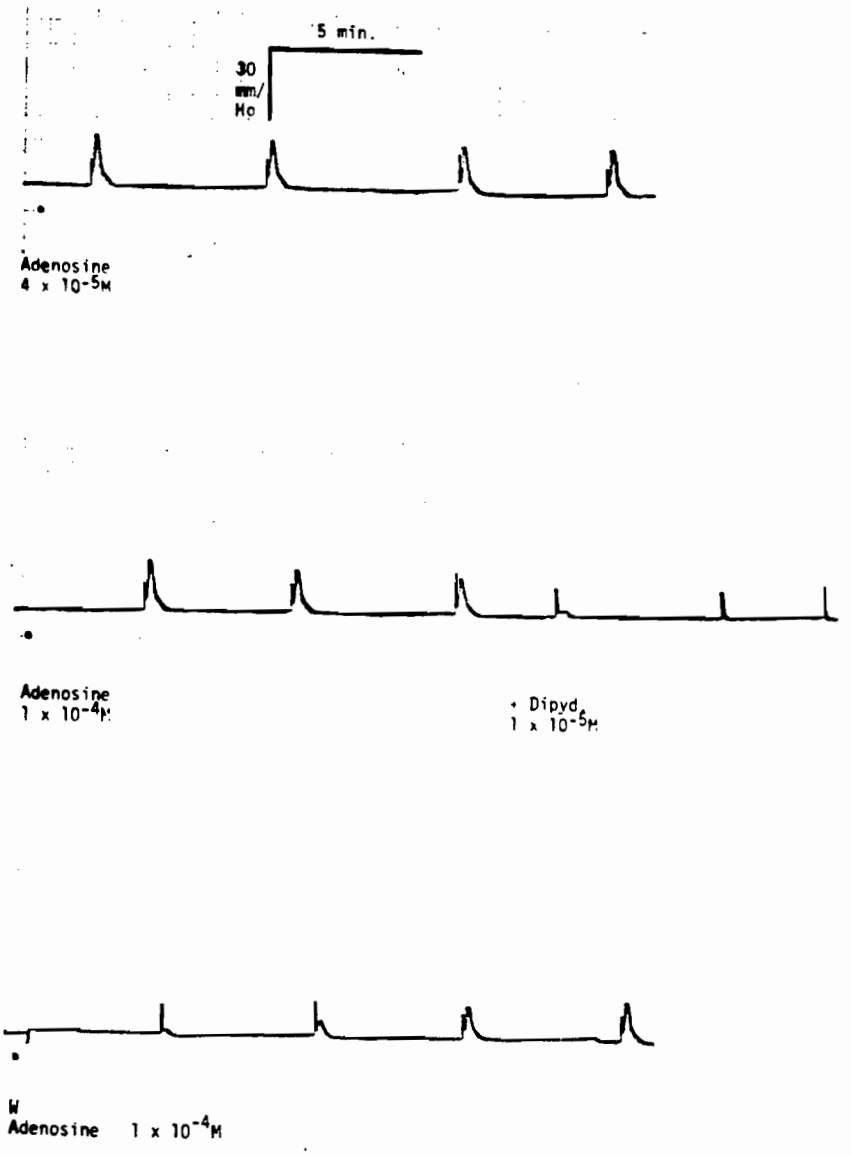

Fig. 64. Inhibition of pressor responses to noradrenaline by adenosine. After partial inhibition by 1×10^{-4} M adenosine, dipyridamole (1×10^{-5} M) added to the perfusate abolished pressor responses. Upon switching to buffer containing adenosine (1×10^{-4} M) alone, responses returned to pre-dipyridamole amplitude.

DISCUSSION

The observations made here exemplify the inhibitory effect of adenosine on vascular responsiveness to noradrenaline and angiotensin but not to potassium. The former stimuli appear to mobilize primarily intracellular or loosely bound calcium whereas the latter stimulus is depenent on the presence of calcium in the extracellular fluid (*Northover 1968*, this study).

As demonstrated in the earlier sections, vascular reactivity in this preparation appeared to require the presence of a number of prostaglandins or a particular prostaglandin.

The interpretation of the data , is that adenosine either antagonizes noradrenaline at its receptor or alternatively prevented the release of or prevented the mobilization of the activator calcium stores.

The former can be dispensed with because adenosine also antagonized angiotensin actions, this latter stimulus acts independent of the α receptor. The maintenance of vascular responsiveness to potassium depolarization in the presence of high concentrations of adenosine negates any arguement that the contractile proteins were inactivated or somehow damaged.

In experiments in which the calcium was omitted from the buffer no change in basal perfusion pressure was seen; this was in agreement with the much earlier report by *Uchida and Bohr (1969)*. In the experiments presented here adenosine attenuated noradrenaline responses to a significant degree if a high enough concentration was used, though it did not abolish pressor responses. It thus appeared that adenosine acted by blocking that component of noradenaline contractions that utilized intracellular calcium.

It has been shown that calcium can stimulate smooth muscle prostaglandin

biosynthesis (*Coburn et al 1977*) acting at a site on the plasma membrane (*Diegel and Coburn*) *1979*). Increasing the extracellular calcium concentration was accompanied by a 200 to 300% increase in PGE2 levels. Thus part of the increased inhibitory activity of adenosine in low calcium buffer was probably related to decreased levels of endogenous prostaglandins.

It has been suggested that in the guinea pig atria adenosine antagonized the movement of calcium across the plasma membrane (*Schrader et al 1975*). Had the potassium responses been inhibited by adenosine, this would have provided data in favour of this suggestion.

In an electrophysiological study using large and small coronary arteries *Harder et al (1979)* demonstrated that there are striking differences in adenosine effects in these two sizes of vessels. Despite similarities in resting potential (-55 mV) and input resistance (9.5 MΩ), adenosine inhibited the calcium dependent current in only the small coronary arteries and had no effect in the large coronary arteries. In contrast, nitroglycerine had the opposite action in each vessel, whereas verapamil blocked the calcium dependent current in both the large and small coronary arteries. In the mesenteric vascular bed, verapamil blocked pressor responses to both noradrenaline and potassium (Fig. 72).

These researchers (*Harder et al 1979*) attempted to reconcile their data by suggesting that the adenosine receptor was present in the small but absent in the large coronary arteries. They argued that the action of verapamil demonstrated that the mechanism of the genesis of the calcium dependent current was not different between the two sizes of vessels. It is possible that activation of the adenosine receptor in the large artery does not prevent the passage of calcium across the plasma membrane, but

instead decreased the availability of intracellular calcium, as shown for the mesenteric vasculature.

The inhibitory actions of adenosine on noradrenaline responsiveness could be potentiated by reducing the endogenous prostaglandin levels with indomethacin or 1-benzyl imidazole. In preparations perfused with sufficient indomethacin to abolish endogenous prostaglandin biosynthesis plus either 1 ng/ml or 5 ng/ml (2.8×10^{-9} M or 1.4×10^{-8} M) PGE2, adenosine inhibited pressor responses in a manner which suggested competitive interaction with PGE2. This interaction subsequently altered the events of excitation-contraction coupling.

These experiments did not give a definitive answer to the question of whether adenosine had to permeate the cell membrane. Calcium stores appear to be associated with the inner surface of the plasma membrane (*Hinke 1965*) and adenosine could possibly modify noradrenaline effects on these stores without entering the cell. Some support for this is the demonstration that ATP, ADP and AMP were all effective inhibitors of responses (Fig. 58). These phophorylated derivatives do not easily cross membranes (*Stilwall and Winter 1974*) possibly because of their negative charge. Whatever the case, it appeared that adenosine interacted with prostaglandins, most likely TxA2 and PGE2, to inhibit vascular responses.

Further support for an antagonism between adenosine and prostaglandins was provided by the results of experiments with lymphocytes. It appeared that adenosine inhibition of lymphocyte division was antagonized by exogenous PGE2 added to the incubation media (Fig. 56). Adenosine inhibition of lymphocyte proliferation had previously been shown by *Snyder et al (1976)* and *Hovi et al (1976)* and had been shown to be the result of activation of a membrane bound receptor (*Schwartz et al 1978*).

This inhibition is of clinical importance since in about half the patients with severe combined immunodeficiency disease there is a congenital deficiency of the enzyme adenosine deaminase, leading to elevated levels of adenosine (*Agarwal et al 1976; Polmar et al 1976*). This immunodeficiency appears to be dependent on the adenosine accumulation since *in vitro* the affected lymphocytes can be made responsive to mitogens by exposure to adenosine deaminase, and *in vivo* considerable improvements can be acheived by transfusions of adenosine deaminase containing red blood cells (*Polmar et al 1976*). In these lymphocytes, as in smooth muscle, activation is critically dependent on calcium (*Whitney and Sutherland 1972*). It may be possible to enhance lymphocyte fuction in patients with adenosine deaminase deficiency by feeding arachidonic acid or by the use of prostaglandin analogues, which may be less hazardous than repeated red blood cell transfusions.

Previous studies by *Kovatsis et al (1976)* have demonstrated physiological antagonism between PGE2, PGA1, PGF1, NADP and βNAD on isolated rabbit jejunum. Earlier *Kovatsis et al (1974)* had demonstrated a similar antagonism between PGA1/PGA2 and AMP/ATP in the same preparation while *Bouillin et al (1972)* have shown that PGE1 was a competitive antagonist of ADP binding to platelets. In the guinea pig myometrium *Dozi-Vassiliades et al (1976)* also demonstrated antagonism between PGE2, PGF2 and adenine nucleotides. It therefore appears that adenosine, in addition to its other properties, can possess prostaglandin antagonist properties. The potentiation of the actions of adenosine by indomethacin and the thromboxane synthesis inhibitor, 1-benzyl imidazole, provide further pharmacological support, which was further strengthened in the experiments with the lymphocytes. It remained to be determined whether a more potent purine

analogue could be identified using the mesenteric vasculature as a test system.

The phosphorylated derivatives of adenosine (adenosine mono-, di-, tri-phosphates) were effective but less potent inhibitors. Other purines (2'-deoxyadenosine, inosine and 1-benzyl inosine) and guanosine were also less potent than adenosine. It is interesting that the more lipid soluble 1-benzyl inosine was less potent, indicating that the loss of the amino group in the 6 position was the primary cause of the lower potency. The activity of 6-N-methyl purine riboside in contrast to the inactivity of purine riboside, again highlighted the necessity of the 6-amino group. Introducing a chloro group onto the adenosine molecule, producing 6-chloro adenosine, significantly increased the inhibitory potency.

In contrast to the requirements for the activation of the adenosine receptor in the ileum (*Ally 1976*), vasculature (*Angus at al 1971*), taenia coli (*Satchell and McGuire 1975*) and coronary vasculature (*Cobbin et al 1974*), 2'- and 3'-deoxyadenosine retained biological activity as did adenine (2,6-dioxypurine). If adenosine had been acting at a receptor similar to that found in the above tissues, it would be expected that removal of, or alteration in, the ribose moiety would result in total inacitvity. Thus it appears that the site of action of adenosine differs from the classical "adenosine" receptor.

The antagonist of adenosine at this classical receptor is theophylline (1,3-dimethyl-2,6-dioxypurine). This compound did not antagonize the inhibitory actions of adenosine, supporting the above conclusion. Theophylline was itself a potent PGE2 antagonist (Fig. *60*).

The posibility that adenosine was acting by increasing intracellular cyclic AMP was tested using cyclic AMP and its more lipid soluble

analogue $N^6,O^{2'}$-dibutyryl-3',5'-cyclic AMP. Neither compound inhibited noradrenaline pressor responses. It had been shown in the vasculature and gut that neither adenosine nor theophylline produced their effects by increasing cellular cyclic AMP (*Herlihy et al 1976; Ally 1976*). It had been shown that 2'-deoxyadenosine did not activate adenylate cyclase (*Zimmerman et al 1976*), yet was able to inhibit noradrenaline responses in the vasculature.

Because it is known that theophylline possesses agonist/antagonist interactions with adenosine (*Sattin and Rall 1970; Ally et al 1976; Wahl and Kuschinsky 1976*) it was necessary to rule out such an action in the mesentery.

The compound diprophylline was not an adenosine antagonist yet was a potent inhibitor of noradrenaline responses, and an antagonist of PGE2 (Fig. 62). Compared to theophylline, diprophylline was a hundred fold more potent as a PGE2 antagonist, yet these compounds were eqipotent as phosphodiesterase inhibitors (*Amer and Kreighbaum 1975*). These data separate the action of theophylline from any action on the adenosine receptor since its analogue diprophylline, which is inactive at this receptor, retained and was an even more potent prostaglandin antagonist.

Comparing the potency of methyl isobutylmethylxanthine, theophylline and diprophylline as phosphodiesterase inhibitors, the rank order in potency is methyl isobutylmethylxanthine > theophylline ≃ diprophylline. As antagonists in the mesenteric vasculature the order was: diprophylline ≥ methyl isobutylxanthine > theophylline. This dissociates their well known activity on the phophodiesterase enzyme from their actions described here.

With regard to adenosine and its derivatives, *McKenzie et al (1977)*

have dissociated any action on the adenylate cyclase enzyme and tissue cyclic AMP levels, from their biological actions in inhibiting contractility of smooth muscle. They showed that two compounds (adenosine-N'-oxide and 6-isopentenyl-aminopurine riboside) which effectively relaxed smooth muscle did not cause any cyclase stimulation at concentrations up to 1 mM. In contrast, three compounds (including 2-amino-6-mercaptopurine ribonucleoside and 2'-deoxyadenosine) caused greater than 30% stimulation of the enzyme without possessing any relaxant activity.

In the mesenteric vasculature both 2'- and 3'-deoxyadenosine were about half as potent as adenosine as antagonists of noradrenaline responses. These adenosine analogues did not activate the "adenosine" receptor (*Ally and Nakatsu 1976; McKenzie et al 1977; Cobbin et al 1974*).

These data suggest that the effects of adenosine, its analogues, theophylline and diprophylline were independent of the "adenosine" receptor as defined in other tissues and appeared to be divorced from actions on either the adenylate cyclase or phosphodiesterase enzymes.

Recently *Schror et al (1979)* demonstrated that PGI2, which had actions similar to adenosine in the mesenteric and coronary vasculature, decreased the level of cyclic AMP in the coronary arteries and inhibited the release of adenosine while, at the same time, causing vasodilation.

In platelets, adenosine inhibited aggregation while the activity of adenylate cyclase was stimulated and inhibited by adenosine (*Haslam and Lynham 1972*). The activation of the adenylate cyclase was prevented by aminophylline (theophylline$_2$ ethylenediamine) but not by papaverine or cyclic AMP. They interpreted their data as indicating that the activation and inhibition of the adenylate cyclase were the result of two independent processes.

In the rat uterus the prostaglandin antagonist 7-oxa-13-prostynoic acid inhibited PGE1 induced contraction, however this compound did not inhibit PGE1 stimulation of adenylate cyclase. This then dissociates prostaglandin actions on contractility from their effects on the adenylate cyclase enzyme.

Dipyridamole is a potent coronary vasodilator, thought to produce its effects by inhibiting adenosine uptake (*Stafford 1966*). This compound was shown to be a pulmonary vasodilator in the intact dog when hypoxic vasoconstriction was present, as well as being a potent systemic vasodilator (*Mlczoch et al 1977*). Dipyridamole was also a phosphodiesterase inhibitor (IC50, 6.2×10^{-5} M) whereas adenosine was inactive (*Fredholm et al 1978*).

In the rat mesenteric vascular bed dipyridamole inhibited noradrenaline but not potassium pressor responses. Unlike adenosine and theophylline, dipyridamole did not inhibit responses in preparations perfused with indomethacin plus PGE2 (Figs. *53, 60, 63*). Thus the action of dipyridamole was dramatically different from that of adenosine. It is therefore unlikely that its mechanism of action was related to its known blockade of adenosine uptake.

The actions of dipyridamole were more like those of imidazole and 1-benzyl imidazole (thromboxane synthetase inhibitors). In this preparation after exposure to sufficient indomethacin to abolish prostaglandin biosynthesis, and the restoration of vascular reactivity with exogenous PGE2, thromboxane synthetase inhibitors did not inhibit vascular responses whereas prostaglandin antagonists did. In platelets, *Best et al (1978)* and *Greenwald et al (1978)* have shown that dipyridamole inhibited thromboxane synthetase, while *Best et al (1978)* found that adenosine was

inactive. In rat brain *Abdulla and McFarlane (1972)* have shown that adenosine nucleotides (ATP, ADP) enhanced PGE2 synthesis whereas ATP inhibited PGE3 formation. In the presence of sodium ions, ATP, ADP and cyclic AMP had a biphasic effect on prostaglandin biosynthesis, low concentrations stimulating and high concentrations inhibiting prostaglandin biosynthesis. In kidney, spleen, spleen fat pad and heart, *Needleman et al (1974)* have shown that ATP and ADP, but not adenosine, stimulated prostaglandin biosynthesis. In addition neither 5'-AMP, adenosine, cyclic AMP, guanosine triphosphate or dipyridamole inhibited PGE2 release. The stimulation of prostaglandin biosynthesis (PGE2) by either ATP or ADP was unopposed by quinidine (a putative purine antagonist, *Burnstock 1972*, and prostaglandin antagonist, *Manku and Horrobin 1976*) or dipyridamole (adenosine uptake inhibitor, *Stafford 1966*, and thromboxane synthetase inhibitor, *Best et al 1978*). Other thromboxane synthetase inhibitors did not inhibit the synthesis and release of PGE2 (*Sun et al 1977; Nijkamp et al 1977*).

Recently *Ogino et al (1979)* reported that the purine metabolite uric acid, in mM concentrations, activated prostaglandin hydroperoxidase. This enzyme converts the 15-hydroperoxide of PGG to a hydroxyl group to produce PGH (*Samuelsson 1976*). Various other purine compounds tested did not have a similar action (adenosine, 5'-AMP, adenine, xanthine, hypoxanthine, inosine, 5'-inosine monophosphate, guanosine, 5'-guanosine monophosphate, 3-iso-butyl-1-methylxanthine, allopurinol and urea). In addition the pyrimidine compounds tested were ineffective (cytosine, uracil, thymine, barbituric acid, isobarbituric acid, orotic acid and dihydrouracil). It is therefore unlikely that any of the effects of adenosine, its phosphorylated derivatives or its metabolites in the mesenteric

vasculature were the result of stimulation of prostaglandin biosynthesis.

In the rat ileum *Nakatsu and Bartlett (1979)* have demonstrated that dipyridamole anagonized the actions of adenosine, an effect opposite to that predicted. A specific blocker of adenosine uptake, 6-(2-hydroxy-5-nitrobenzyl)thioinosine, did potentiate the actions of adenosine (*Bartlett 1978*), thus the effect of dipyridamole was apparently by some other mechanism of action.

In the mesenteric vasculature bed hexobendine (an adenosine uptake inhibitor) did not inhibit noradrenaline pressor responses. Lidoflazine, a weak adenosine uptake blocker (*Huang and Daly 1976*) with local anaesthetic properties, inhibited both noradrenaline and potassium responses. In this preparation both procaine and quinidine appeared to be prostaglandin antagonists (*Manku and Horrobin 1976*) and possess local anaesthetic properties.

In summary, adenosine was a prostaglandin antagonist in the mesenteric vasculature. This action was independent of activation of the putative "adenosine" receptor. The purine derivative 1,3-dimethyl-2,6-dioxypurine (theophylline), was a more potent prostaglandin antagonist. The differential action of theophylline indicated that low concentrations had an adenosine like action whereas high concentrations inhibited potassium pressor responses. Dipyridamole had actions inconsistent with either inhibition of phosphodiesterases or adenosine uptake but consistent with the inhibition of thromboxane synthetase. Dipyridamole, unlike adenosine or theophylline, was not a prostaglandin antagonist.

SECTION IV: PROSTACYCLIN

INTRODUCTION

Prostacyclin (PGI2) can be synthesized by a wide variety of tissues including rat stomach (*Pace-Asciak 1977*), blood vessels (*Bunting et al 1976*), placenta (*Myatt and Elder 1977*), fetal blood vessels (*Terragno et al 1978*), mesenteric artery (*Wolfe et al 1979*), pericardium, pleura, peritoneum, aorta, dura mater (*Herman et al 1978*), lung (*Lackeman and Herman 1978*), iris, ciliary body, conjunctiva (*Bhattacherjee et al 1978*), rat and rabbit hearts (*De Deckere et al 1977*) and ileac artery (*Sinzinger et al 1978*).

PGI2 was a potent dilator of blood vessels *in vivo* (*Smith et al 1978*) and *in vitro* (*Dusting et al 1977*). In some blood vessels PGI2 had a biphasic effect, examples are: rat and rabbit coronary vessels (*Karmazyn et al 1978*), guinea pig coronary vessels (*Dusting et al 1977*) and human umbilical artery (*Pomerantz et al 1977*).

In platelets, PGI2 was a potent inhibitor of aggregation *in vivo* and *in vitro* (*Crane et al 1978; Gorman 1979*). Its mechanism of action was thought to involve stimulation of platelet adenylate cyclase (*Gorman et al 1977*) and thus alteration of calcium availability and thromboxane biosynthesis. Recently *Gorman et al (1979)* reported that the action of PGI2 in platelets was on the contraction secretion coupling mechanism, independent of its stimulation of adenylate cyclase.

The effect of PGI2 in the guinea pig heart was to decrease coronary perfusion pressure while it relaxed isolated strips of bovine coronary artery. In these preparations PGI2 decreased the intracellular levels of cyclic AMP and inhibited the release of adenosine. The PGI2 mechanism

of action appeared to be associated with a decrease of adenylate cyclase activity. This therefore leaves a possible inhibition of the release of or action of calcium in the smooth muscle as the mechanism of action of PGI2.

In the mesenteric vascular bed PGI2 blocked noradrenaline and angiotensin contractions and had no effect on potassium induced contractions. The actions of noradrenaline and angiotensin were independent of extracellular calcium, therefore PGI2 could have prevented the release of calcium from intracellular or loosely bound membrane stores.

RESULTS

Effect of indomethacin on prostacyclin action

Because exogenous PGI2 could have been acting as an antagonist of an endogenously synthesized prostaglandin, the effect of a low concentration of indomethacin (10 µg/ml. 2.8×10^{-5} M) on PGI2 (2.5×10^{-10} to 8×10^{-9} M) action was tested; the results are shown in Figure *65*. As can be seen, partial inhibition of prostaglandin biosynthesis did not shift the PGI2 concentration response curve. This suggested that PGI2 was not exerting its inhibitory action by antagonizing an endogenous prostaglandin. It is however possible that if the ratios of the endogenously synthesized prostaglandins had been maintained, then no antagonism would have been detected by examination of the PGI2 dose response curves.

An alternative way to test for a PGI2-PG interaction was to add a fixed amount of prostaglandin (PGE2), which potentiated noradrenaline pressor responses, and then to determine the effects of PGI2. As shown in Figure *65*, PGE2 (2.8×10^{-8} M) did not shift the PGI2 concentration response curve. When this experiment was repeated using PGE1 (2.8×10^{-11} M), which, in contrast to PGE2 potentiated only noradrenaline pressor responses (Figs. *9*, *10*), a dramatic blockade of PGI2 actions was seen (Fig. *66*). Higher concentrations of PGE1, which by themselves partially inhibited noradrenaline responses, shifted the PGI2 concentration response curve to the left (data not shown). The slope of the PGI2 concentration response curve in the presence of a low concentration of PGE1 (2.8×10^{-11} M, Fig. *65*) was significantly less ($p < 0.001$) than that obtained in its absence.

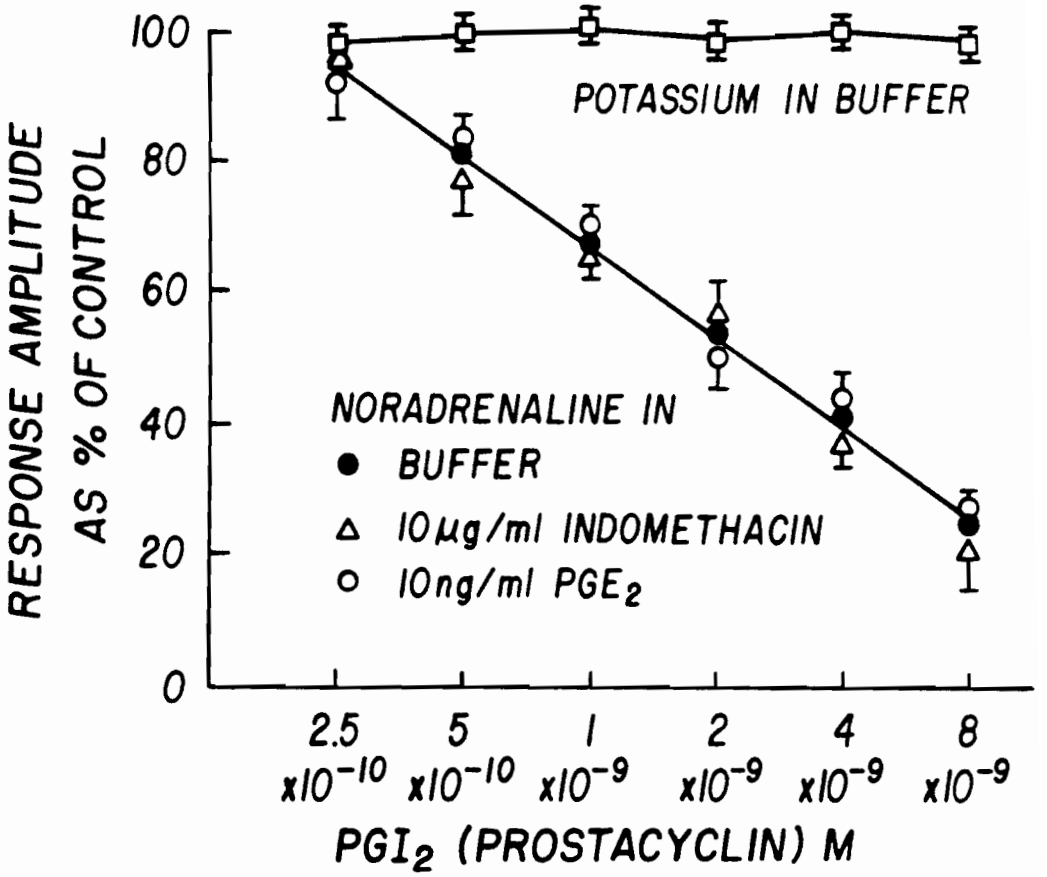

Fig. 65. The influence of PGI2 on potassium and noradrenaline pressor responses in the mesenteric vascular bed. The lack of effect of indomethacin (2.8×10^{-5} M) and PGE2 (2.8×10^{-8} M) on PGI2 inhibition of noradrenaline contractions is clearly shown by the similar concentration effect line. As can be seen PGI2 does not inhibit potassium responses. Each point represents the mean ± SE for six experiments.

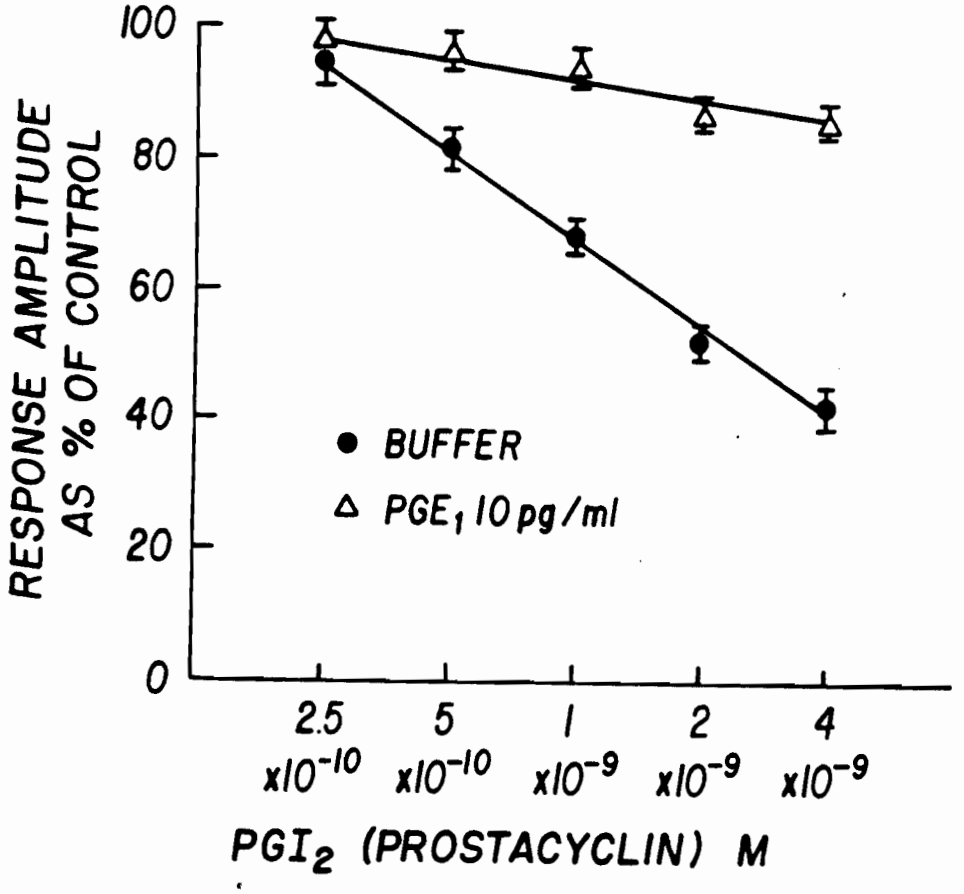

Fig. 66. Antagonism by 2.8×10^{-11} M PGE1 of PGI2 inhibition of noradrenaline pressor responses ($p < 0.001$, ANOVA). PGE1 was added to the buffer reservoir at the fixed concentration shown and control responses determined prior to the addition of PGI2. Pressor responses in the presence of PGI2 + PGE1 are expressed as a % of responses obtained in the presence of PGE1 before the addition of PGI2. Each point represents the mean ± SE for five or six experiments.

These data may be interpreted as the possible result of non-competitive or functional antagonism between PGI2 and PGE1 (at low concentrations) whereas at high concentrations PGI2 and PGE1 appeared to interact in a synergistic manner. Because of the dual nature of PGE1 actions, it was felt necessary to test a prostaglandin antagonist for its action on the PGI2 inhibitory effect.

For these experiments theophylline was selected. In the presence of theophylline (1×10^{-5} M) in normal buffer or in buffer containing indomethacin plus PGE2, the PGI2 concentration response curve obtained was shifted rightwards, parallel to the control curve (Fig. 67).

Effect of theophylline on the reversibility of PGI2 inhibition of vascular reactivity

In preliminary experiments it was noted that upon completion of a cumulative PGI2 dose response experiment that the effects of PGI2 were not readily reversed by perfusing with buffer alone. An example of the trace from such an experiment is shown in Figure 67. After perfusing the preparations with buffer for one hour, vascular reactivity had returned to 50% of normal (Figs. 68, 70). In contrast, if the preparations were perfused with a fixed concentration of theophylline (1×10^{-5} M) and a similar dose response experiment done, upon switching to buffer alone and perfusing for 20 minutes, vascular reactivity returned to approximately 100% of predrug levels (Figs. 69, 70). Note that neither theophylline nor PGI2 altered the base line perfusion pressure.

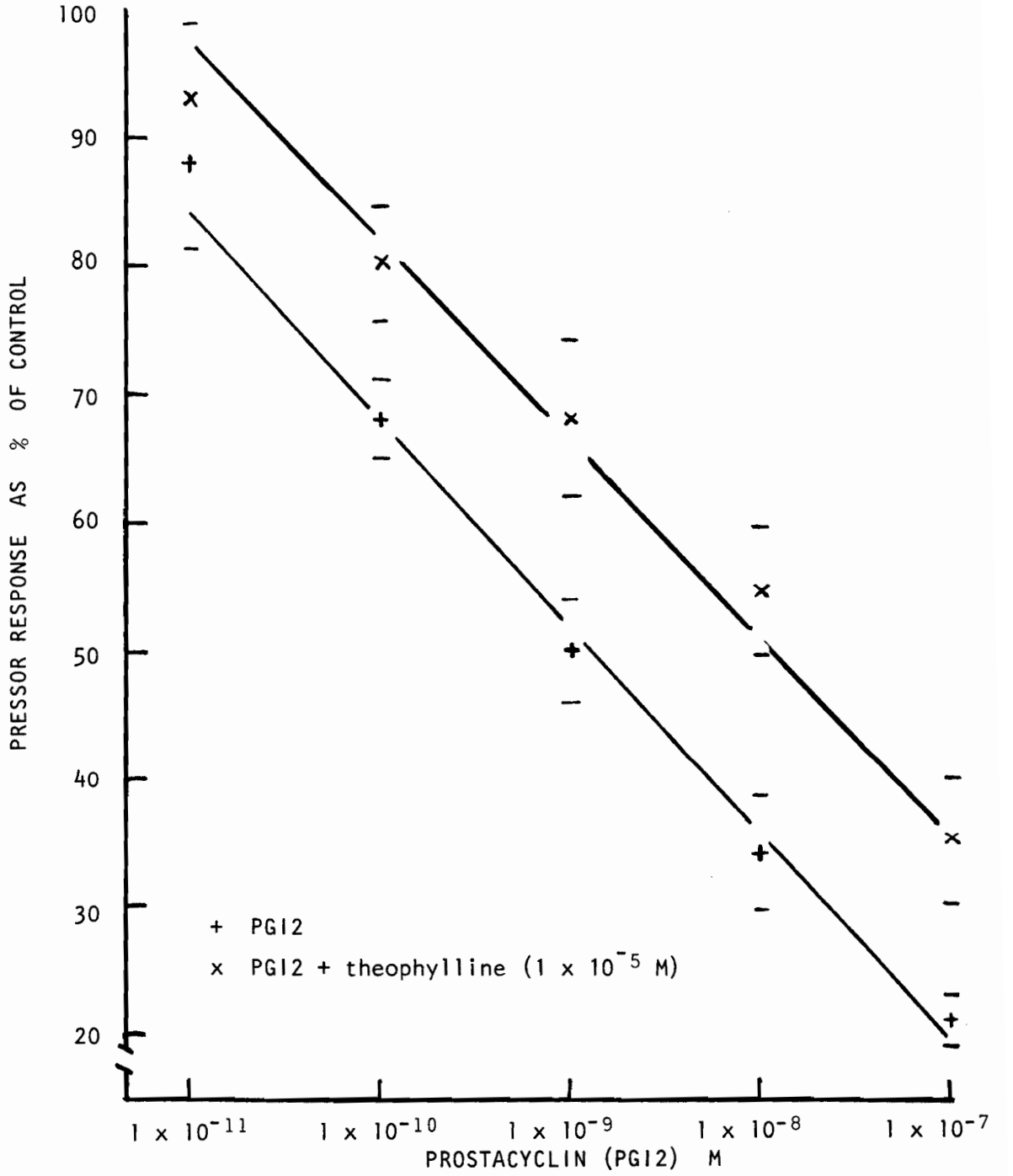

Fig. 67. The antagonism of PGI2 inhibition of noradrenaline pressor responses in the mesenteric vascular bed by theophylline (1×10^{-5}M). Each data point shown represents the mean ± SE, n = 6.

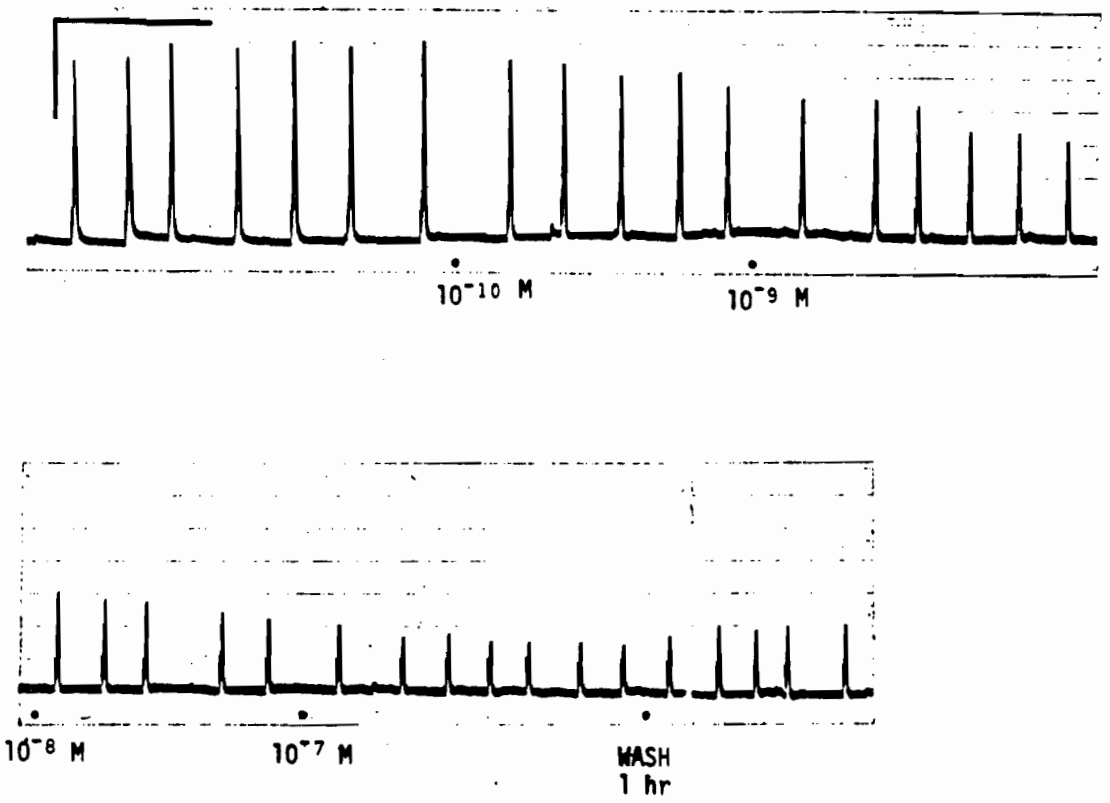

Fig. 68. Influence of prostacyclin (PGI2) on noradrenaline pressor responses in the mesenteric vascular bed. PGI2 was added to the buffer reservoir in a cumulative fashion and noradrenaline responses determined at 4-5 minute intervals. Note the potent inhibition of contraction amplitude without any changes in baseline perfusion pressure. After one hour of perfusion with buffer alone, pressor responses were still depressed. The vertical bar represents 30 mm Hg (1 mm Hg = 133.3 pascals) and the horizontal bar 10 mins.

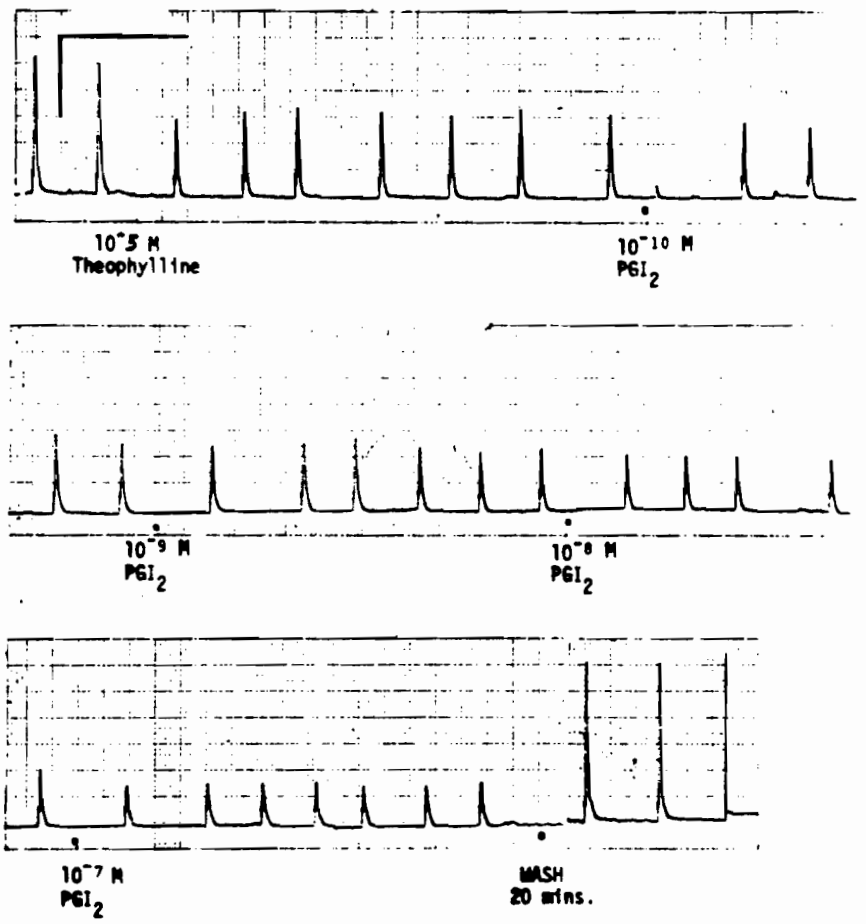

Fig. 69. Influence of theophylline (10^{-5} M) on PGI2 inhibition of noradrenaline pressor responses and the recovery of the vasculature from PGI2 inhibition of pressor responses. The vertical bar represents 30 mm Hg (1 mm Hg = 133.3 pascals) and the horizontal bar represents 10 mins.

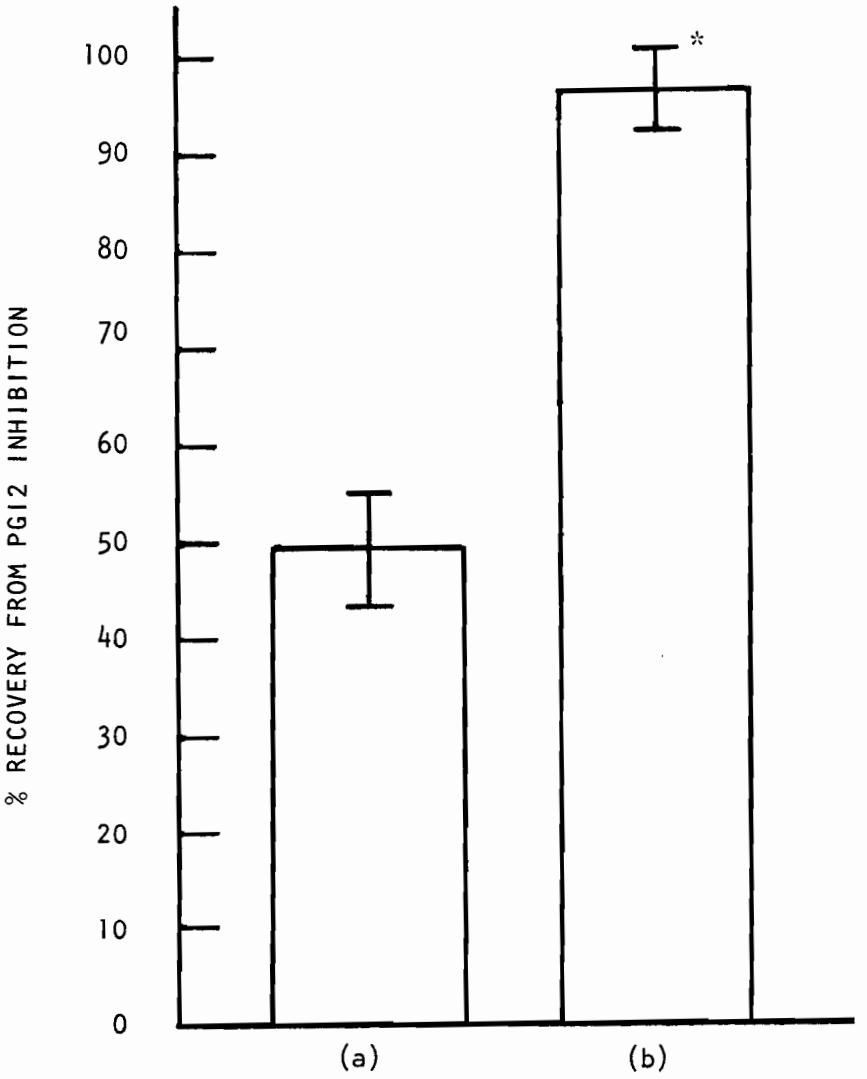

Fig. 70. Recovery of vascular reactivity of mesenteric vascular bed from inhibitory effects of prostacyclin (1×10^{-7} M) in control (a) and theophylline (1×10^{-5} M) pretreated (b) tissues. As shown above within 20 mins. the pretreated preparations had completely recovered whereas up to 1 hour later control preparations had only recovered by approximately 50%. The data shown represent mean ± SE for six experiments. *$p < 0.001$ (ANOVA).

Summary

These data show that PGI2 inhibited vascular reactivity to noradrenaline without altering baseline perfusion pressure and that endogenous prostaglandins synthesized by the vascular smooth muscle did not antagonize the actions of PGI2. Low concentrations of exogenous PGE1 but not PGE2 antagonized the effects of PGI2 in an apparently non-competitive manner. Theophylline antagonized the inhibitory actions of PGI2 and protected the vessels from the residual depressant effect of exposure to high concentrations of PGI2 (1×10^{-7} M) as indicated by rapid restoration of pressor responsiveness upon perfusion with drug free buffer.

DISCUSSION

In this preparation, as noted earlier, the pressor response to potassium ion depolarization depends on the influx of calcium through the plasma membrane to the contractile proteins. In contrast responses to noradrenaline depend partly on the release of intracellular calcium and partly on the entry of extracellular calcium. Indomethacin and other prostaglandin cyclooxygenase inhibitors blocked responses to both types of pressor agents (*Manku and Horrobin 1976; Coupar and McLennan 1978*).

Prostacyclin had effects similar to those of imidazole, 1-benzyl imidazole, nicotinic acid and dipyridamole. The possibility that, like imidazole, PGI2 inhibited TxA2 biosynthesis was ruled out by the observation that in preparations inhibited by 1×10^{-4} M indomethacin and with vascular reactivity restored by the addition of exogenous PGE2, PGI2 was still able to inhibit noradrenaline pressor responses. In this preperation under similar conditions as described above, imidazole, 1-benzyl imidazole and dipyridamole had no effect.

In preparations perfused with one tenth normal calcium containing buffer, the effects of PGI2 were slighty enhanced in that noradrenaline pressor responses were inhibited to a greater degree.

There was no evidence of a competitive type of interaction between exogenous PGE2 or endogenous prostaglandins and PGI2, in contrast to the situation with adenosine, theophylline, papaverine and hydralazine (see previous sections for complete details of the effects of these drugs). The PGI2 concentration response curves were similar in preparations perfused with buffer, 10 ng/ml PGE2 (2.8×10^{-8} M), 10 µg/ml indomethacin (2.8×10^{-5} M) and 64 µg/ml indomethacin (1.8×10^{-4} M) plus either

1 or 5 ng/ml PGE2 (2.8×10^{-9} or 1.4×10^{-8} M).

These data suggested that PGI2 did not interact with PGE2 at a common site. It also ruled out any type of physiological interaction as suggested for TxA2 and PGI2 in human platelets (*Gorman 1979*), where PGI2 had one effect while PGE2 and/or TxA2 might have had an opposite effect.

One possible mechanism would be a noncompetitive interaction of sites which are activated by TxA2. Since occupation and activation of these sites by endogenous TxA2 or exogenous PGE2 may be important in intracellular calcium release (*Gorman 1979; Gorman et al 1979*), their inactivation in a noncompetitive manner could account for the observations made here, those of *Schror et al (1979)* in vascular smooth muscle and the blockade of platelet aggregation observed by *Gorman (1979)* and others. An alternate explanation would be that the interaction between PGI2 and some other substance, for example TxA2, was indirect, perhaps related to the mobilization and sequestration of calcium. Indeed, *Gorman et al (1979)* have elegantly separated the stimulatory effect of PGI2 on the adenylate cyclase and its inhibition of platelet aggregation. Using an intracellular calcium antagonist they showed that the actions of TxA2 synthesized from PGH2 were not inhibited by PGI2 nor was its ability to inhibit the adenylate cyclase enzyme (already stimulated by PGI2) prevented. Increasing the calcium concentration in the presence of PGI2 still resulted in platelet aggregation. This suggested that PGI2 may, in addition, interfere with a calcium dependent process.

In the rat mesenteric vasculature PGI2 appeared to block noradrenaline and angiotensin II stimulation of contraction by preventing the release of intracellular calcium. The contractile proteins remained fully

functional because PGI2 did not inhibit potassium responses. It is therefore possible that PGI2 acted at some site proximal to calcium interaction with the contractile proteins.

It had previously been found that PGE1 antagonized PGE2 actions in the mesenteric vasculature (*Manku et al 1976*). At least part of this antagonism was noncompetitive since the maximal response to PGE2 was reduced. It therefore seemed reasonable to test PGE1 for a possible interaction with PGI2. Low concentrations of PGE1 effectively prevented PGI2 inhibition of noradrenaline responses whereas higher concentrations enhanced PGI2 actions. The slope of the PGI2 concentration response curve in the presence of a low concentration of PGE1 (Fig. 65) suggested a non-competitive antagonism. In contrast, high concentrations of PGE1 enhanced PGI2 actions but did not alter the slope of its concentration response curve.

These data suggested that PGE1 was acting at two sites, each with a different affinity for PGE1, similar to the recent demonstration of two PGE1 binding sites on human platelets by *Siegl et al (1979)*. These workers found that PGE1 was able to bind to both PGI2 sites whereas PGD2 (another inhibitor of platelet aggregation) did not. Their data are compatible with the above interpretation of PGE1 action in the mesenteric vasculature.

Accepting for the moment that TxA2 sites facilitated the release of calcium from intracellular stores, a model for the control of noradrenaline or angiotensin pressor responses can be proposed. PGI2, TxA2 and PGE2 interact to modulate the release of intracellular or loosely bound membrane calcium without compromising the contractile proteins or

the ATP synthesizing enzymes. The precise details of this interaction are presently unknown, however some speculation can be made. The data showed that neither cyclic AMP nor its lipophylic analogue dibutyryl cyclic AMP (up to 10^{-2} M) had any inhibitory effect on noradrenaline contractions. It is therefore unlikely that the effects of PGI2 were mediated by cyclic AMP. In support of the is the recent report by *Schror et al (1979)* showing that PGI2 decreased intracellular cyclic AMP in coronary arteries yet these same concentrations caused relaxation of the vessels. Additional less direct support are the data of *Needleman et al (1979)* showing that PGI3 and TxA3 inhibited both platelet aggregation and adenylate cyclase. In contrast, TxA3 (like TxA2) contracted vascular smooth muscle while PGI3 (like PGI2) caused relaxation.

One would have to conclude that the Δ^{17} double bond (the only difference between PGI2 and PGI3) did not prevent PGI3 from activating the platelet adenylate cyclase. In contrast, the Δ^{17} double bond in TxA3 enabled it, unlike TxA2, to activate adenylate cyclase while preventing it from having TxA2 like actions. In the vasculature such fine discrimination did not appear to exist because TxA3 had effects indistinguishable from those of TxA2 (*Needleman et al 1979*) even though it too might stimulate adenylate cyclase in vascular smooth muscle.

In a comparative study, *Crane et al (1978)* found that analogues of PGI2 were relatively less potent than PGI2 itself in inhibiting platelet aggregation. In contrast, these analogues were equipotent with PGI2 as contractors of gerbil colon and rat uterus. Here again, the conclusion was made that platelet receptors were much more discriminating than the smooth muscle receptors.

In the vascular bed theophylline, (well known as a phosphodiesterase inhibitor, IC50, 1×10^{-4} M, *Amer and Kreighbaum 1975*), antagonized the actions of PGI2. Of particular interest was the finding that this compound, which also acted as a prostaglandin antagonist (*Manku et al 1976;* and data presented earlier), protected the vasculature from the long lasting inhibitiory action of PGI2. It is plausible that exposure *in vivo* to high concentrations of PGI2 for a considerable lenght of time might result in arterial collapse. In this situation a prostaglandin antagonist, if present in early stages, might prevent such a loss of vascular reactivity. Some support for this suggestion is found in the report by *Machiedo et al (1973)* showing that PGE1 was beneficial in hemorrhagic shock. More recently, *Cook et al (1979)* showed that essential fatty acid deficient rats displayed a significant resistance to endotoxin shock (essential fatty acid deficient rats, 18% mortality vs 88% mortality in control rats), while *Korbut et al (1978)* used the prostaglandin cyclooxygenase inhibitor, indomethacin, to prevent fatal hypotension in cats during circulatory shock. These data demonstrated that a product of polyunsaturated fatty acid metabolism via the cyclooxygenase complex was deleterious in shock. A prime candidate for this role is PGI2 since, of the prostaglandins tested, it was the only one which occurred *in vivo* in concentrations high enough to produce a profound fall in blood pressure and perhaps have an irreversible effect on vascular reactivity.

SECTION V: PROSTAGLANDINS AND CALCIUM

INTRODUCTION

It was observed earlier that in preparations perfused with buffer plus indomethacin vascular reactivity to noradrenaline, angiotensin potassium, and calcium deploarizing stimuli was inhibited (Fig. *17*). If prostaglandins were injected as a bolus into the arterial cannula of preparations perfused with indomethacin plus noradrenaline a transitory contraction (similar to noradrenaline contraction in normal preparations) was seen (Figs. *18, 19, 20*). It was also shown that the efficacy of adenosine in inhibiting noradrenaline responses was enhanced by either reducing the extracellular calcium (Fig. *51*) concentration or perfusing with a low concentration of indomethacin. In preliminary experiments the potency of indomethacin as an inhibitor of vascular reactivity was increased by lowering the concentration of calcium in the perfusate; however, the time course for the inhibition was similar to that obtained using normocalcium buffer, as shown earlier (Fig. *34*). For these reasons it was of interest to investigate the effects of calcium in this preparation and attempt to elucidate its mechanism of action using several pharmacological probes.

RESULTS

Effect of the calcium ionophore A 23187 on pressor responses

In preparations perfused with normocalcium buffer A 23187 (1.9×10^{-11} to 1.9×10^{-7} M) inhibited noradrenaline pressor responses in a concentration dependent manner (Fig. 71). In contrast, potassium pressor responses were partially (approximately 20%) inhibited by the ionophore at a concentration of 1.9×10^{-8} M. Higher concentrations of the ionophore had the opposite effect, enhancing potassium pressor responses (Fig. 71). At these higher concentrations of the ionophore, noradrenaline responses were abolished.

Effect of the calcium antagonist, verapamil on pressor responses

Verapamil added to the buffer (normal calcium concentration) perfusing the isolated mesenteric vascular bed inhibited pressor responses to noradrenaline and potassium ion depolarization over a similar concentration range (2.2×10^{-8} M to 1.4×10^{-6} M). At the higher concentrations of verapamil tested (7.02×10^{-7} M and 1.4×10^{-6} M), this compound was significantly more potent against noradrenaline pressor responses ($p < 0.05$, ANOVA, Fig. 72).

Calcium ionophore - calcium antagonist interaction

Adding the calcium ionophore A 23187 (1×10^{-6} M) after verapamil inhibition of noradrenaline pressor responses failed to reverse the inhibition. When the reverse experiment was done verapamil itself did not oppose the inhibitory effect of the ionophore A 23187. Neither compound was able to prevent the actions of the other.

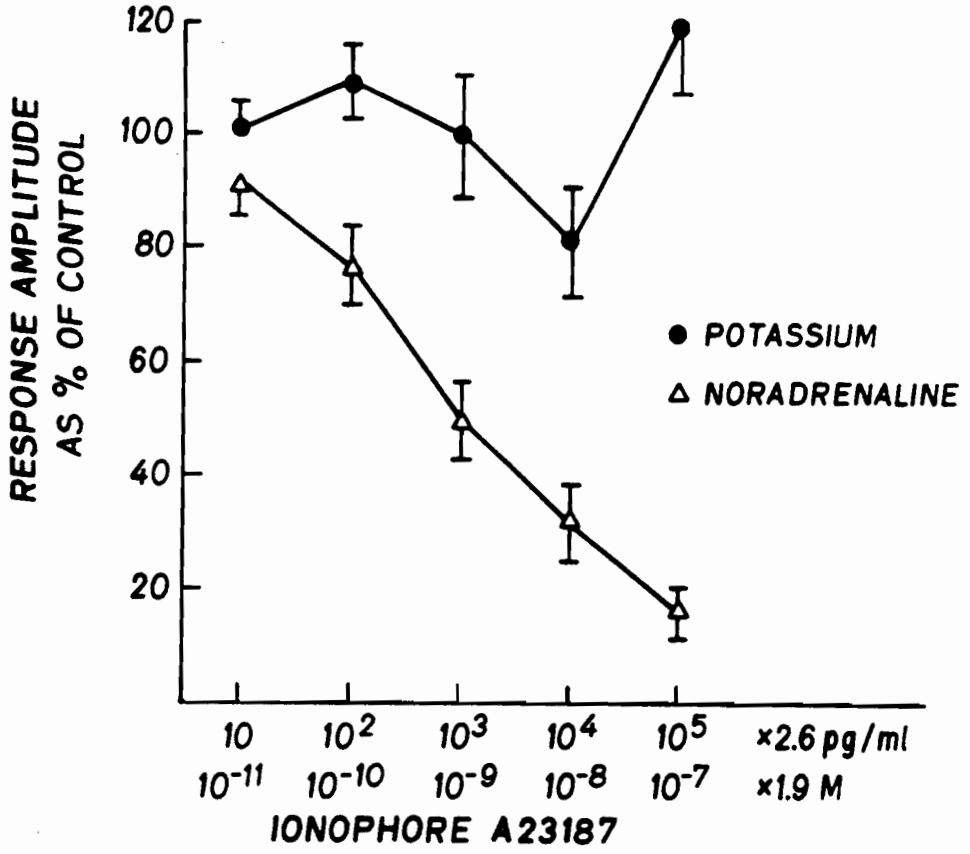

Fig. 71. Effect of the calcium ionophore A23187 on potassium and noradrenaline contractions of the mesenteric vasculature. Preparations were perfused with physiological salt solution to which A23187 was added in increasing cumulative concentrations, final concentrations shown in figure. As can be seen noradrenaline responses were inhibited in a concentration dependent manner whereas potassium responses were partially inhibited over the concentration range 1.9×10^{-11} M to 1.9×10^{-8} M; higher concentrations of the ionophore increased potassium pressor amplitudes. Each point represents the mean ± SE for six experiments.

Verapamil - extracellular fluid calcium interaction

Verapamil as shown above inhibited the pressor actions of both noradrenaline and potassium. In this preparation noradrenaline appeared to mobilize primarily intracellular calcium stores. It was therefore of interest to determine the responses to a fixed concentration of noradrenaline in the presence of 2.2×10^{-6} M verapamil and varied calcium concentrations. This concentration of verapamil was selected because, in the presence of 2.5×10^{-3} M calcium ions, it had no apparent action on noradrenaline responses (Fig. *72* and Fig. *73*). Lowering the extracellular calcium concentration to 5.0×10^{-4} or 1.0×10^{-6} M dramatically enhanced ($p < 0.001$, ANOVA) the inhibitory effect of verapamil (2.2×10^{-6} M). In the presence of 1.0×10^{-6} M calcium, verapamil (2.2×10^{-6} M) inhibited noradrenaline responses by about 90% (Fig. *73*).

Indomethacin potentiation of verapamil action

Indomethacin, a prostaglandin cyclooxygenase inhibitor, at a concentration of 2.8×10^{-5} M, shifted the dose response line for verapamil inhibition of noradrenaline pressor responses to the left, significantly increasing its potency ($p < 0.001$, NOVA, Fig. *74*). Infusion of PGE2 (2.8×10^{-9} to 2.8×10^{-8} M) titrated to a concentration which reversed the indomethacin inhibition of noradrenaline pressor responses, did not prevent verapamil inhibition of noradrenaline contractions. A lower concentration of indomethacin (1.4×10^{-5} M) did not have as significant a potentiating effect on verapamil action ($p < 0.05$, ANOVA, Fig. *72*).

To further analyze the actions of calcium in the mesenteric vascular bed it was decided to use dantrolene, a compound which had been

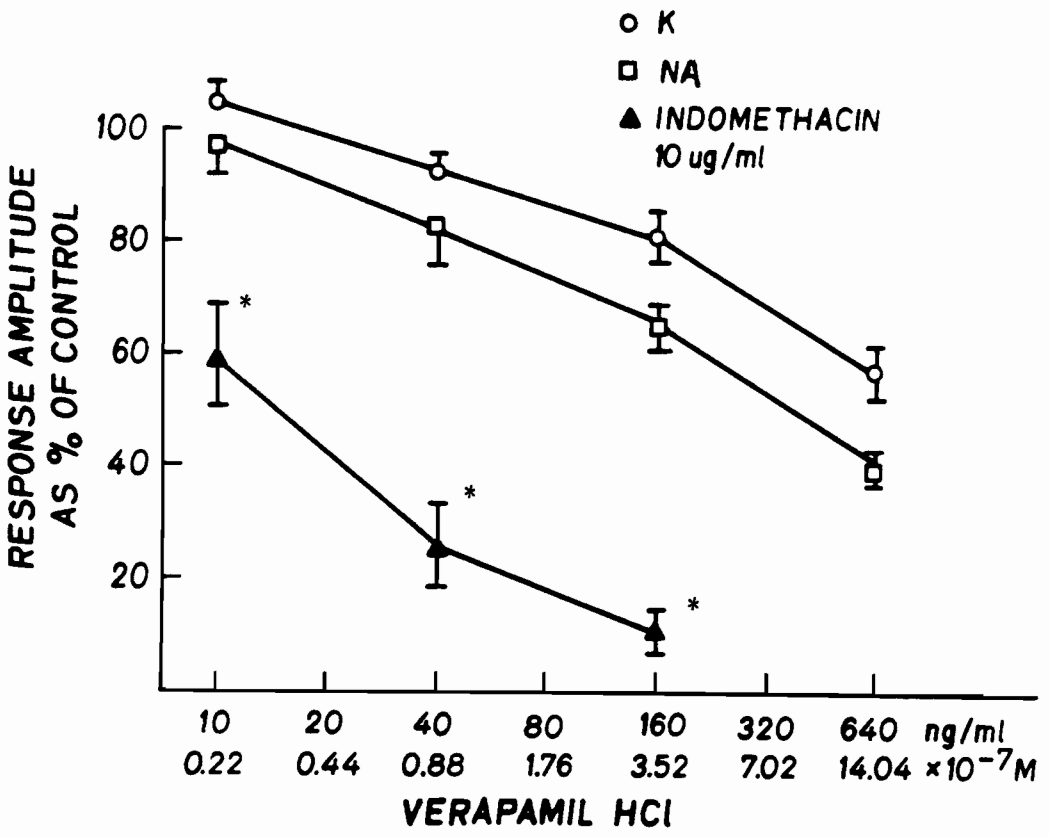

Fig. 72. Effect of the calcium antagonist verapamil on noradrenaline contractions of the mesenteric vasculature in the absence (□) and presence (▲) of indomethacin (2.8×10^{-5} M) and on potassium (o) responses. Each point shown represents the mean ± SE for six experiments. Asterisk significantly different ($p < 0.05$, ANOVA) from noradrenaline responses.

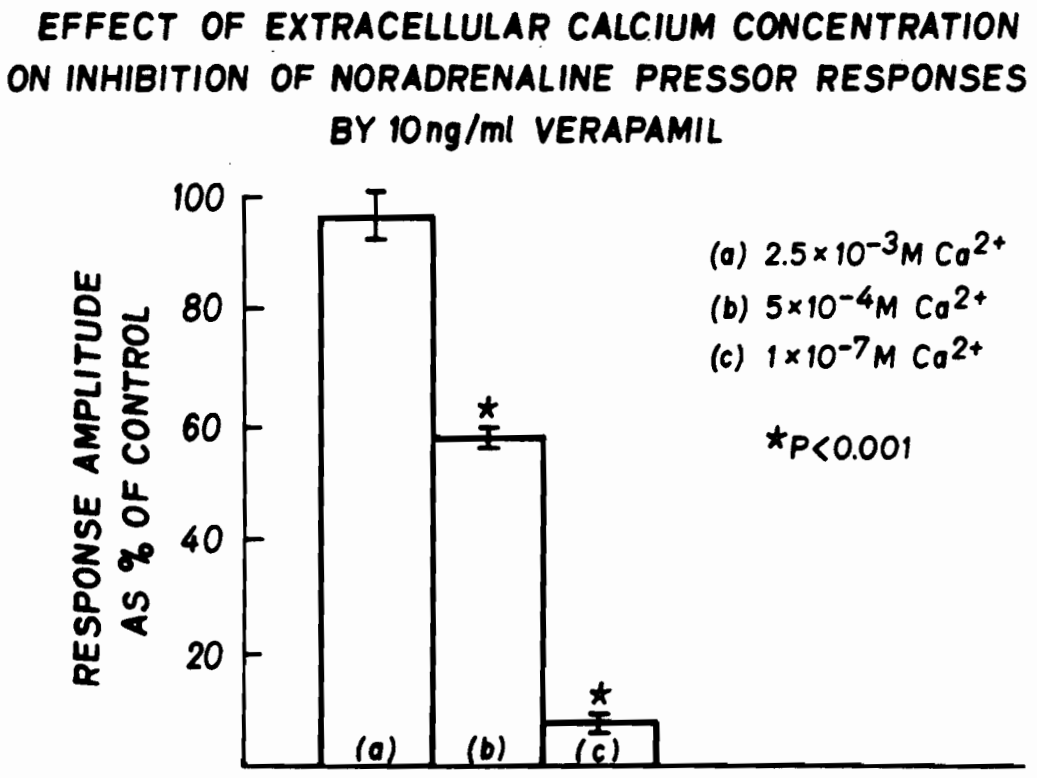

Fig. 73. Effect of extracellular calcium at three concentrations on verapamil (2.2×10^{-6} M) inhibition of noradrenaline pressor responses. Preparations were equilibrated for two hours in buffer of the appropriate calcium concentration and control responses determined prior to the addition of verpamil. All subsequent responses were expressed as a % of mean response amplitudes obtained prior to the addition of verapamil. Each point shown represents the mean ± SE for six experiments.

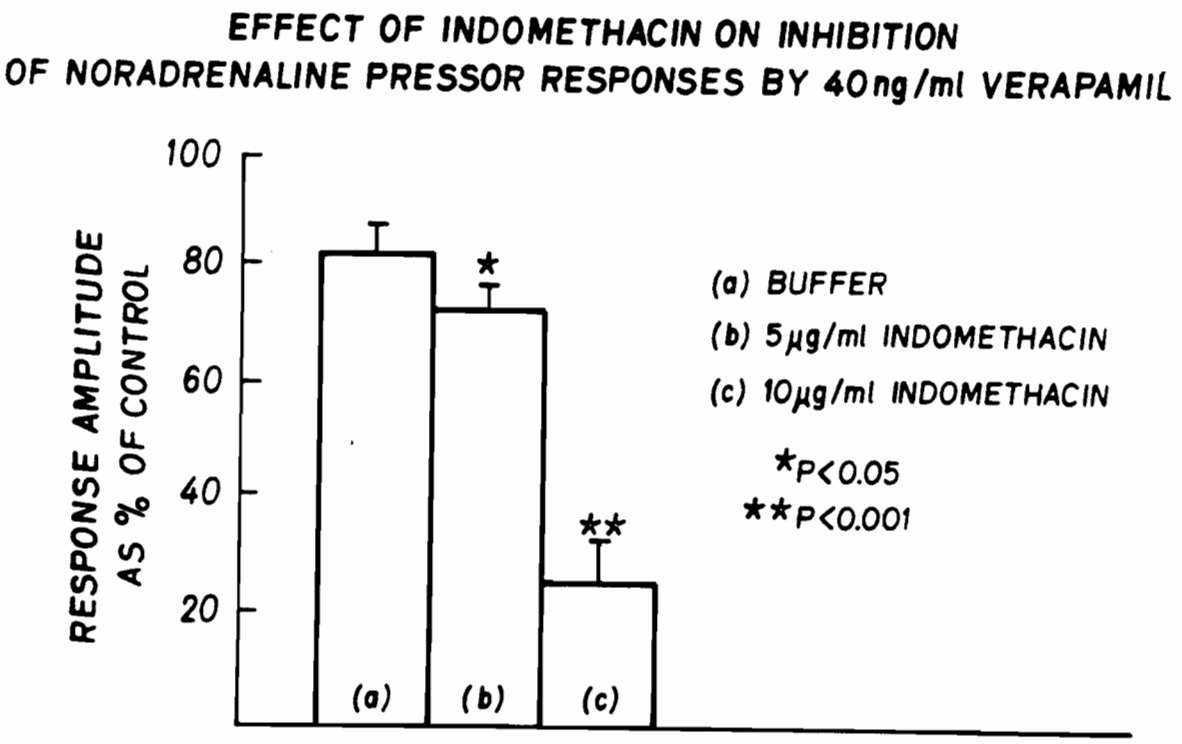

Fig. 74. Effect of pretreatment with indomethacin (1.4×10^{-5} and 2.8×10^{-5} M) on verapamil (8.8×10^{-6} M) inhibition of noradrenaline responses. Preparations were equilibrated for forty minutes in the presence of each concentration of indomethacin and control responses determined prior to the addition of verapamil. Response amplitude to the same concentration of noradrenaline in the presence of verapamil are expressed as a percentage of control responses. Each point represents the mean ± SE for six experiments.

shown to be devoid of any calcium antagonist properties, and which did not inhibit the uptake of calcium (*Desmedt and Hainaut 1977*). This compound is thought to inhibit the release of calcium from intracellular stores by antagonizing the movement, or action of an endogenous ionophore (*Morgan and Bryant 1977*).

Effect of dantrolene on pressor responses

Dantrolene infused through the mesenteric vascular bed inhibited noradrenaline responses in a concentration related manner (Fig. *75*). The concentration of dantrolene causing 50% inhibition of noradrenaline responses was approximately 6.4×10^{-7} M (from Fig. *75*). Similar results were obtained in two experiments in which angiotensin II was used as the pressor substance (data not shown). Unlike the calcium antagonist verapamil, dantrolene had no inhibitory effect on potassium pressor responses (Fig. *75*).

Dantrolene - extracellular calcium interaction

In preparations perfused and equilibrated with buffer containing approximately 1×10^{-6} M calcium (made by omitting CaCl2 from the normal buffer), the effect of dantrolene was significantly enhanced (Fig. *76*). A concentration of dantrolene (3.2×10^{-7} M) which inhibited noradrenaline pressor responses (normocalcium buffer) by 25% now caused an inhibition of approximately 60%. These values were significantly different ($p < 0.001$, ANOVA). In experiments using an intermediate concentration of calcium (2.5×10^{-4} M) a concentration response line intermediate between those shown in Figure *76* was obtained. No significant enhancement

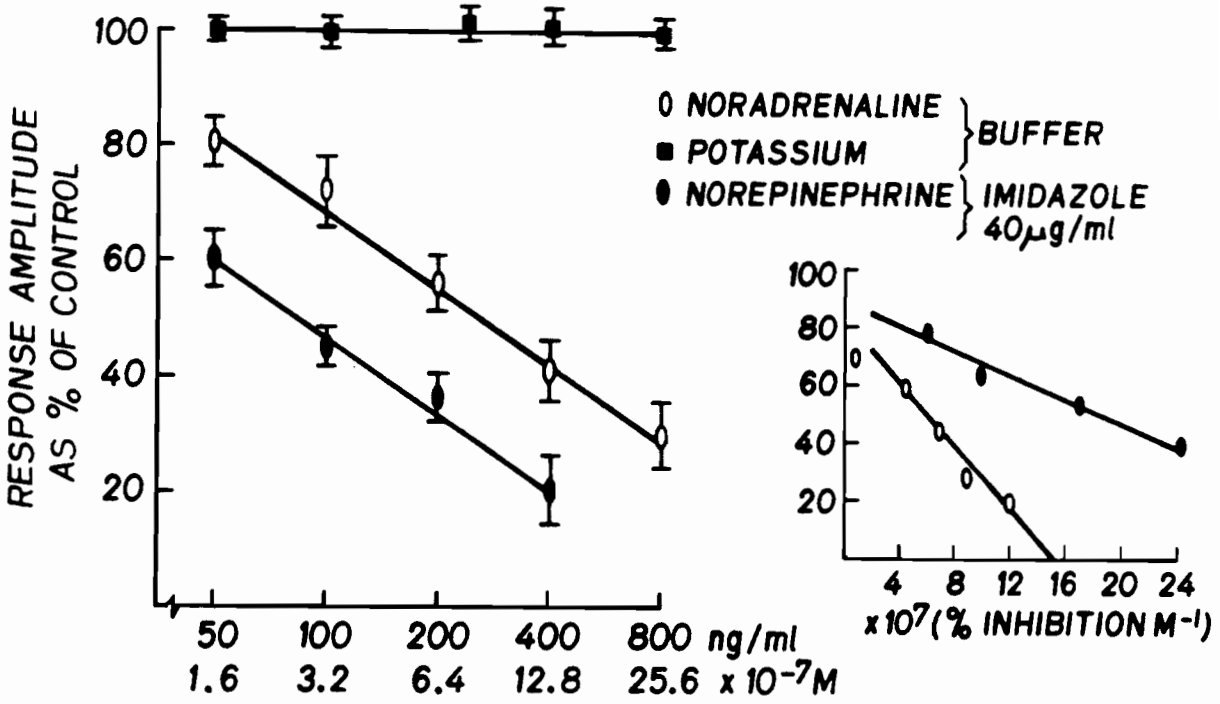

Fig. 75. Effect of dantrolene on vascular responses to noradrenaline in the absence (o) and presence of (●) imidazole (5.6×10^{-4} M), and on potassium contractions (■). Dantrolene was added in increasing cumulative concentrations and its effect determined on pressor response amplitude to intra-arterial injections of fixed amounts of noradrenaline and potassium.

Ordinate: Response amplitude as a percentage of control.

Abscissa: Dantrolene concentration. Inset shows a Hofstee plot for the noradrenaline responses of preparations perfused with increasing concentrations of dantrolene in the absence and presence of 5.6×10^{-4} M imidazole. Ordinate: percentage inhibition of pressor response. Abscissa: percentage inhibition M^{-1}. The respective regression equations are (o) $y = -4.34 \times 10^{-7} \times M + 79$; and (o) $y = -1.6 \times 10^{-7} \times M + 80$.

Each point represents the mean ± SE for six experiments.

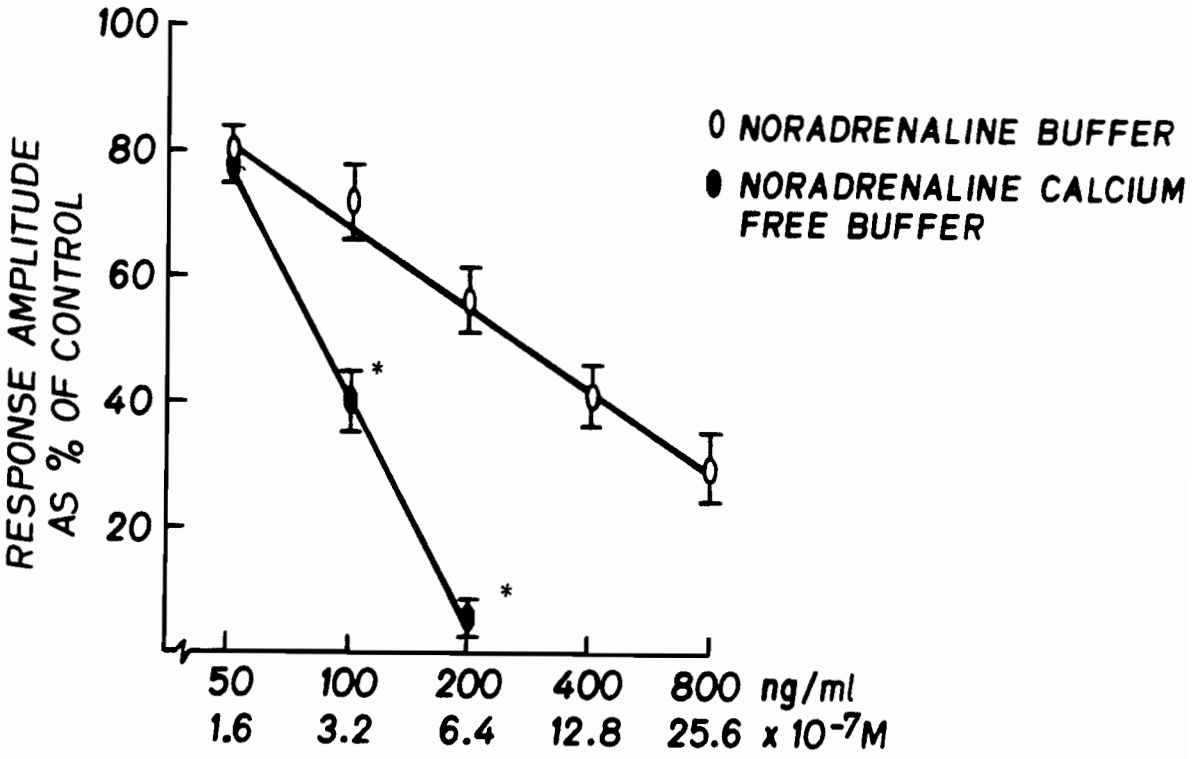

Fig. 76. Effect of dantrolene on noradrenaline pressor responses in preparations perfused with normal calcium (o) containing buffer and those perfused with a calcium free buffer (●, made by omission of calcium from normal buffer). As can be seen, in the latter situation the dantrolene concentration effect line is significantly increased, although the effect of the lower concentration 1.6×10^{-7} M is not increased. Each point represents the mean ± SE for six experiments *$p < 0.001$, ANOVA.

of the effect of the low concentration of dantrolene tested (1.6×10^{-7} M) was seen at any of the two lower calcium concentrations. The significant difference in the slopes of the dantrolene concentration response lines shown in Figure *76* suggest a complex interaction, not necessarily the result of a direct antagonism between dantrolene and calcium, perhaps more of a physiological antagonism via some intermediate.

Dantrolene appeared similar to imidazole, adenosine, and dipyridamole in that it preferentially inhibited noradrenaline pressor responses and, like verapamil, its effects were potentiated by reducing the buffer calcium concentration. It was therefore necessary to determine if it was an antagonist of prostaglandin(s) action or an inhibitor of their biosynthesis.

Dantrolene - prostaglandin antagonism

If dantrolene was an antagonist of prostaglandin action its effect would be expected to be enhanced by decreasing the levels of endogenous prostaglandins. Indomethacin (2.2×10^{-5} M) shifted the dantrolene concentration response line to the left of control (three experiments). The thromboxane synthetase inhibitor imidazole (5.6×10^{-4} M), similarly shifted the dose response line to the left of control (Fig. *75*). A similar parallel intermediate concentration response line was obtained using a lower concentration of imidazole (7.4×10^{-5} M, four experiments). This parallel shift was similar to that obtained for the TxB2 concentration response line in the presence of imidazole (Fig. *49*).

Analysis of these data (dantrolene, Fig. *75* inset) by a graphical method (*Hofstee 1952; Dowd and Riggs 1965*) revealed that in the presence of imidazole, the apparent affinity of dantrolene for its active site

was increased while its maximum inhibitory effect was unchanged. This is consistent with the suggestion of competitive antagonism of TxA2 by dantrolene.

In two experiments, noradrenaline responses were maximally inhibited with indomethacin (1.8×10^{-4} M) and vascular responsiveness restored with PGE2 (2.8×10^{-7} M). After three control reponses were obtained, dantrolene was added to the buffer reservoir in a cumulative fashion and pressor responses determined at each concentration. Under these circumstances dantrolene remained a potent inhibitor of noradrenaline responses unlike imidazole, 1-benzyl imidazole and dipyridamole. In these experiments dantrolene did not inhibit potassium contractions.

These data demonstrated that dantrolene can apparently antagonize the actions of both endogenous and exogenous prostaglandins. The results of the experiment using a thromboxane synthetase inhibitor (imidazole) provided evidence suggesting that it did antagonize TxA2. The demonstration that the effects of dantrolene were opposed by 2.5×10^{-3} M calcium (Fig. *76*) suggested that a more classical prostaglandin antagonist should be examined and its interaction with extracellular calcium documented.

Effect of lowering extracellular calcium on the potency of "NO164"

This compound is a member of a class of prostaglandin antagonists synthesized from phloretin and phosphoric acid whose spectrum of activity was reviewed earlier.

In the mesenteric vascular bed perfused with normal buffer (2.5×10^{-3} M calcium) NO164 selectively inhibited noradrenaline pressor

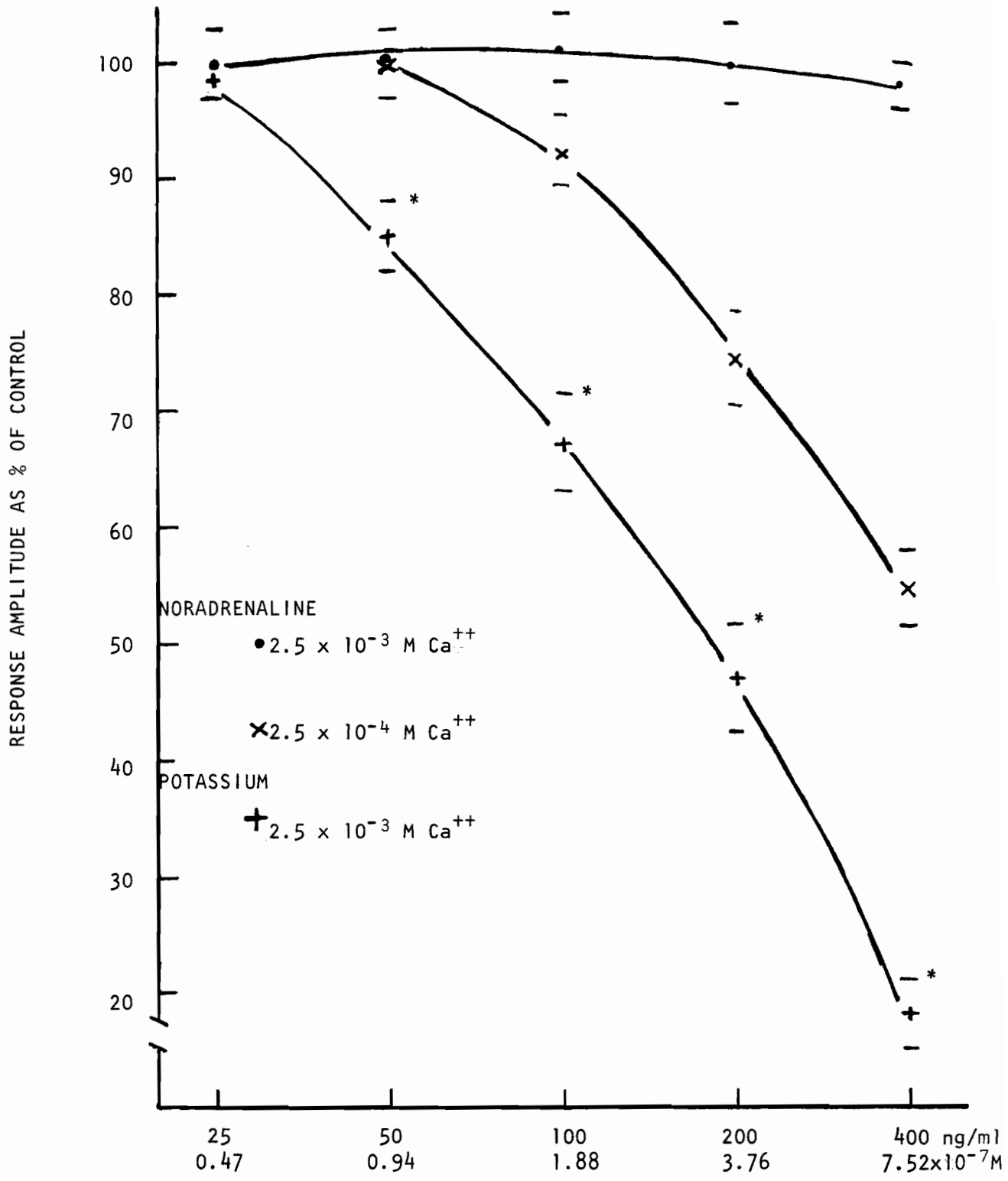

Fig. 77. Effect of N0164 (a prostaglandin antagonist) on vascular responses to noradrenaline in normal calcium (2.5×10^{-3} M) buffer and one tenth normal calcium (2.5×10^{-4} M) buffer. Also shown is the lack of effect of N0164 over this concentration range 4.7×10^{-8} to 7.52×10^{-7} M on potassium responses. Each point shown represents the mean ± SE for six experiments. Asterisk * $p < 0.001$, ANOVA.

responses, the effective concentration range being 4.7×10^{-8} M to 7.52×10^{-7} M (Fig. *77*). This compound also inhibited potassium pressor responses but at much higher concentrations (7.52×10^{-5} M), similar to the findings for theophylline (Fig. *59*). In preparations perfused with low calcium buffer (2.5×10^{-4} M), the N0164 concentration response line was shifted to the left of that obtained using normal calcium buffer (2.5×10^{-3} M). These results are shown graphically in Figure *77*. In one experiment the preparation was perfused with very low calcium containing buffer (1×10^{-6} M calcium) and the effect of a single concentration of N0164 (3.6×10^{-7} M) determined. This concentration of N0164 abolished noradrenaline responses. In normal calcium buffer (2.5×10^{-3} M calcium) this concentration of N0164 inhibited noradrenaline responses by approximately 25% (Fig. *76*).

These data show that noradrenaline pressor resonses can be inhibited by a calcium antagonist (verapamil) and paradoxically by a calcium ionophore (A23187). These responses are also inhibited by PGE1, PGI2, indomethacin, imidazole and dantrolene. Of these latter compounds, indomethacin is a cyclooxygenase inhibitor, imidazole a thromboxane synthetase inhibitor and dantrolene an apparent thromboxane A2 antagonist.

It was decided to compare the effect of these three drugs on calcium concentration response curves using noradrenaline as pressor agent. The results of these experiments are shown in Figure *78*. As can be seen in the control bell shaped curve (buffer alone), calcium had a biphasic effect on pressor responses, the point of inflexion occurring at a calcium concentration of about 1×10^{-4} M. At this concentration pressor responses were maximally potentiated by about 120%. Further increases in calcium concentration led to progressive decreases in

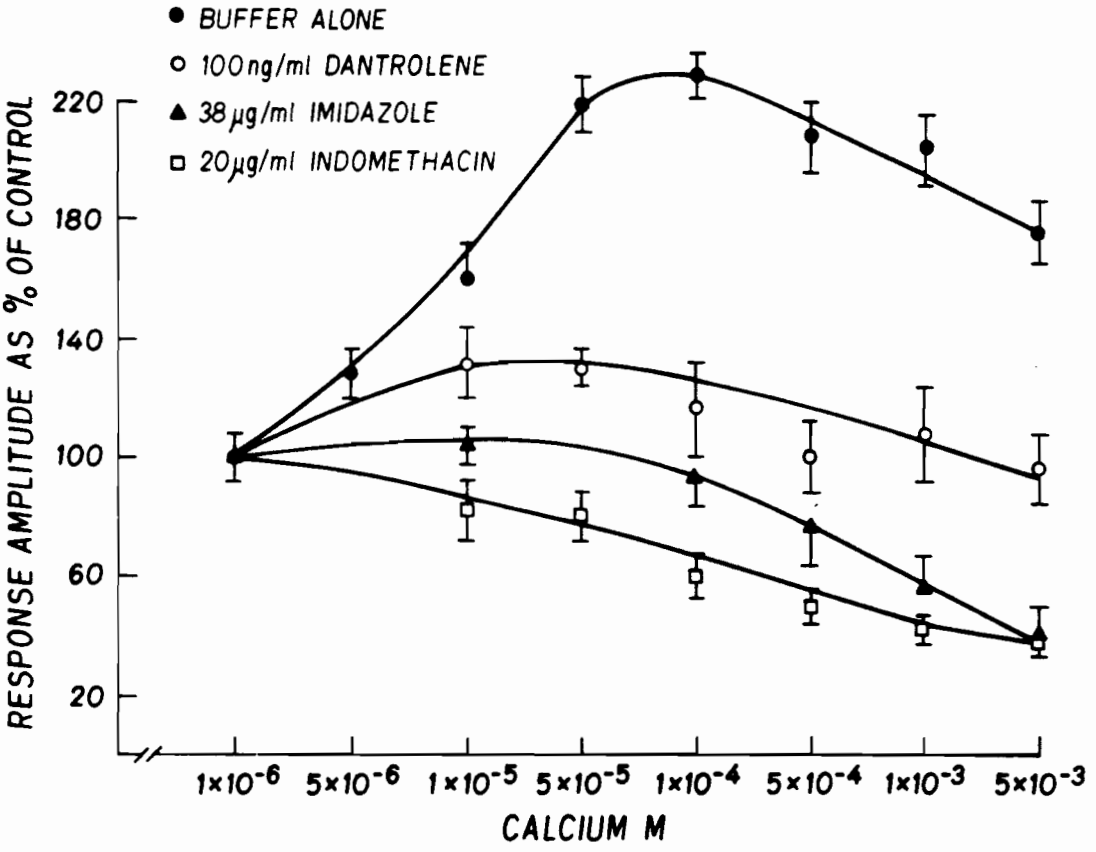

Fig. 78. Influence of increasing concentrations of extracellular calcium (1×10^{-6} M to 5×10^{-3} M) on noradrenaline contractions in the absence (●) and presence of 3.2×10^{-7} M dantrolene (○), 5.6×10^{-4} M imidazole (△) and 5.6×10^{-5} M indomethacin (□). The potentiating action of calcium on noradrenaline contractions was significantly attenuated by all three drugs ($p < 0.05$, ANOVA). Each data point shown represents the mean ± SE, n = 6.

pressor amplitude. At a calcium concentration twice normal (5×10^{-3} M) pressor responses were now only potentiated by about 80% (above responses obtained in 10^{-6} M calcium buffer), a 40% reduction from the maximum stimulation seen at 1×10^{-4} M calcium.

Each agent tested, dantrolene (3.2×10^{-7} M), imidazole (5.6×10^{-4} M) and indomethacin (5.6×10^{-5} M), was able to prevent the calcium induced increase in noradrenaline pressor response amplitude but not the inhibitory effects of high calcium concentrations.

Dantrolene prevented the increase in pressor amplitude in response to increasing concentrations of buffer calcium. The inflexion point for the concentration response curve was shifted from 1×10^{-4} M to 1×10^{-5} M calcium. The pressor responses at the highest concentration of calcium tested were equilivalent to those at 1×10^{-6} M calcium. The thromboxane synthetase inhibitor imidazole abolished the rising phase of the calcium concentration curve (1×10^{-6} M to 1×10^{-4} M) whereas the falling phase (1×10^{-4} M to 5×10^{-3} M) was unaffected. The cyclooxygenase inhibitor, indomethacin, also abolished the rising phase of the calcium concentration response curve and had no effect on the falling phase.

Summary

These data, using pharmacological agents acting at differing stages of prostaglandin biosynthesis or action, suggested that the potentiation of noradrenaline pressor responses by increasing the calcium concentration from 1×10^{-6} M to 1×10^{-4} M was dependent on the action of a prostaglandin. The blockade by both imidazole and indomethacin indicated that the calcium ions either facilitated or stimulated prostaglandin biosynthesis.

The inhibitory actions of dantrolene are consistent with the view that the action of a prostaglandin was responsible for the potentiation seen. The inhibitory or falling phase of the calcium curve at concentrations greater than 1×10^{-4} M appear to be independent of either prostaglandin synthesis or a dantrolene-sensitive prostaglandin action.

DISCUSSION

The calcium ionophore A 23187 is being increasingly utilized because of its selectivity for the divalent calcium cation. This allows the researcher to bypass the normal calcium translocating mechanisms in a number of cellular systems (*Reed and Lardy 1972; 1973; Knapp et al 1977; Lapentina et al 1978; Diamant et al 1978; Rosenberger and Triggle 1979*). The data from many of these studies show a dependence of A-23187 action on extracellular calcium and are consistent with the demonstrated ionophore mechanism of action (*Triggle et al 1975; Knapp et al 1977*). A number of studies have however, shown that A 23187 can initiate calcium-dependent events in the absence of extracellular calcium (see *Rosenberger and Triggle 1978*), indicating that A 23187 may act directly at intracellular calcium stores.

The data presented here showing A 23187 inhibition of noradrenaline but not potassium responses is consistent with the view that it depleted the intracellular activator calcium stores. In experiments using low calcium (10^{-6} M) buffer, similar data, i.e. inhibition of noradrenaline responses (contraction) were obtained.

A 23187 lowers ATP levels in some cells (*Reed 1976*); however, *Knapp et al* (*1977*) have shown that A-23187 up to 5×10^{-6} M did not decrease renomedullary ATP synthesis. The effective A 23187 concentration range in the mesenteric vasculature was 1×10^{-11} to 1×10^{-7} M. The inability of A 23187 to inhibit potassium responses argues against a toxic effect on the contractile proteins or the ATP synthesizing machinery.

It has been shown that some of the actions of A 23187 are dependent upon the stimulation of prostaglandin biosynthesis via activation of the

calcium sensitive phospholipase A2 (*Feinstein et al 1977; Rickett et al 1977; Knapp et al 1977; Waelbroeck and Boeynaems 1977*).

In the mesenteric vasculature, of the natural prostaglandins tested, only PGE1 and PGI2 inhibited noradrenaline contractions. Neither PGE1 nor PGI2 altered potassium responses. The biphasic effect of A 23187 on potassium contractions is therefore inconsistent with a prostaglandin mediated action. It appears that low concentrations of A 23187 (1.9×10^{-11} to 1.9×10^{-8} M) selectively released intracellular calcium stores (*Janis et al 1977; Johansen 1978*) while having no effect on calcium translocation from the extracellular fluid. If this were the case it would provide a rational explanation for the slight inhibition (20%) of potassium responses, since during a contraction initiated by membrane depolarization calcium from the extracellular fluid and intracellular stores is utilized (*Fabiato and Fabiato 1977*). If the second source (intracellular stores) was depleted, potassium contractions will be absolutely dependent on the influx of extracellular calcium and since the duration of depolarization remained constant, the same fixed amount of extracellular calcium enters the muscle cells. Therefore, an apparent inhibition due to the unavailability of intracellular calcium stores is seen. High concentrations of the ionophore which augment potassium responses (see at 1.9×10^{-7} M, A 23187, Fig. 7*l*) will also increase the influx of calcium, as was seen as an increase in basal perfusion pressure. At these concentrations noradrenaline responses were abolished. Thus even in the presence of normal extracellular calcium concentrations noradrenaline responses were abolished, because its responses are primarily dependent on the mobilization of intracellular calcium (*Hudgins and Weiss*

1968; Altura 1978; Kikta and Davis 1979; also see *Tada et al 1979* for review and discussion of molecular mechanism of active calcium transport). In support of this is the demonstration by *Kikta and Davis (1979)* that noradrenaline, in concentrations up to 1×10^{-6} M, did not utilize extracellular calcium during a contraction.

The calcium antagonists verapamil and D600 are thought to selectively block voltage sensitive and insensitive calcium channels (*Felckenstein et al 1971; Bassingthwaigthe et al 1976*). Verapamil blocked vascular responses to both noradrenaline and potassium over a similar range of concentrations. This suggests that verapamil blocks those calcium channels in the plasma membrane activated by potassium, in addition to those intracellular channels activated by noradrenaline.

In the rat aorta (*Peiper et al 1971*) have shown that verapamil inhibited potassium contractile responses but did not influence noradrenaline responses. In the mesenteric vasculature this was not so; noradrenaline responses were more sensitive ($p < 0.05$, ANOVA) than potassium responses, to verapamil inhibition. In a recent study *Mikkelsen et al (1977)* have found that in isolated human peripheral veins, noradrenaline responses were more sensitive than potassium responses to verapamil inhibition. In rat portal vein, *Bilek et al (1974)* found that verapamil effectively blocked both potassium and the noradrenaline evoked contractures.

Thus while there are differences in calcium dependency between preparations from different species, differences also exist between smooth muscles from the same specie. An elegant study by *Sutter et al (1977)* in perfused rat hind quarters showed that in the larger proximal vessels (arterioles and arteries) dependence on extracellular calcium was

decidely less than that of the much smaller peripherial arterioles. They further suggested that rat aortic smooth muscle was not a good model of the true resistance vessels of the vascular tree, as has been previously suggested by *Ljung (1970)* and *Rhodes and Sutter (1971)*.

The inhibition of noradrenaline responses by verapamil could be the result of blockade of calcium binding and reuptake at sites from which noradrenaline releases activator calcium. This would not be very surprizing because verapamil with four $-O-CH_3$ groups is much more lipophylic than hydrophlic. Its high lipid affinity (*Appel 1962*) and the value obtained for the verapamil space by *Bondi (1978)* which was higher than that reported for water space (*Bianchi and Bolton 1967*) suggested distribution into membrane structures. Furthermore *Bondi (1978)* reported that verapamil released intracellular calcium during the slow phase of calcium efflux studies. It has previously been reported that verapamil inhibited calcium binding in preparations of cardiac sarcoplasmic reticulum (*Naylor and Szeto 1972; Watanabe and Besch 1974*). Whatever the explaination, verapamil did abolish pressor responses to both noradrenaline and potassium stimulation.

In addition to the above properties, verapamil appears to also possess local anaesthetic properties (*Balzar 1972; Singh and Vaughan-Williams 1972*). Its action like that of procaine, chloroquine and quinidine, was potentiated by the cyclooxygenase inhibitor. These latter compounds have been shown to possess prostaglandin antagonist properties (*Manku and Horrobin 1976*). These data suggested that part of the mechanism of action of verapamil was due to antagonism of both calcium and prostaglandin at similar or independent sites. Indomethacin (a cyclooxygenase

inhibitor) did not inhibit calcium uptake by the rat mesenteric artery (*Northover 1968*). It is therefore more likely that its potentiation of verapamil action was related to its inhibition of prostaglandin biosynthesis.

The potentiation of verapamil's action by lowering the concentration of calcium present in the extracellular fluid would suggest that verapamil's predominant action was blockade of calcium uptake, not antagonism of prostaglandins. This paradox may be resolved by the observations of *Diegel and Coburn (1979)*, who found that extracellular calcium stimulated smooth muscle prostaglandin biosynthesis. Therefore reducing the calcium in the extracellular fluid perfusing the mesenteric vasculature, a preparation which has a high basal nonstimulated rate of prostaglandin biosynthesis (*Wolfe et al 1979; present data*), will have the same effect as a low concentration of indomethacin, i.e. a reduction of prostaglandin biosynthesis. Verapamil in either situation will then be a more potent antagonist of noradrenaline contractions, as was found to be the case (Figs. *72,73,74*).

In a number of systems (platelets, stomach, trachea, polymorphonuclear leukocytes, lymphoma cells, thyroid and endothelial cells) A 23187 stimulation of prostaglandin biosynthesis was found to be dependent on the presence of extracellular calcium ions (*Knapp et al 1977; Waelbroeck and Boeynaems 1977; Weksler et al 1978*). This then underscored the influence of extracellular calcium ions on prostaglandin biosynthesis.

A separation of calcium antagonism and prostaglandin antagonism was achieved using dantrolene, a muscle relaxant (previously thought to be specific for skeletal muscle, *Pinder et al 1977*) which did not block calcium uptake (*Desmedt and Hainaut 1977*). Dantrolene specifically inhibited only noradrenaline and angiotensin contractions; it did not

inhibit potassium responses. The concentration of dantrolene which inhibited noradrenaline responses by 50% was approximately 0.2 µg/ml (Fig. 75). This concentration is well within the therapeutic plasma level (0.7 - 1.7 µg/ml, *Monster et al 1973*).

Its specificity for noradrenaline and angiotensin responses indicated an intracellular locus of action. This was confirmed in experiments using essentially calcium free buffer (approximately 1×10^{-6} M Ca^{++}). In these latter experiments the slope of the dantrolene concentration response curve was radically increased when compared to that obtained using normal calcium containing buffer. If the actions of dantrolene were simply due to the blockade of calcium release as suggested by *Desmedt and Hainaut (1977)*, then it is difficult to explain these observations.

As discussed above it has been shown that extracellular calcium can stimulate prostaglandin biosynthesis; it was therefore plausible that dantrolene acted as an antagonist of a specific prostaglandin since it is not itself a calcium antagonist. This view was supported by its specificity of action against those pressor agents which primarily mobilized intracellular calcium. This hypothesis was confirmed by demonstrating that the thromboxane synthetase inhibitor imidazole potentiated the actions of dantrolene in an apparently competitive manner as suggested by the graphical analysis (inset Fig. 75). Furthermore, indomethacin also potentiated the actions of dantrolene in preparations in which prostaglandin biosynthesis was maximally inhibited and vascular reactivity restores with a moderate concentration of PGE2. These data show that dantrolene is a prostaglandin antagonist, acting at some site involved in the release of activator calcium stores. This conclusion is in aggreement

with that of *Morgan and Bryant (1977)* who suggested as a result of electro-physiological studies on excitation-mechanical coupling in skeletal muscle, that dantrolene prevented the release of intracellular calcium by antagonizing and/or decreasing the mobility of an endogenous calcium ionophore. In their studies, cooling, a procedure which would decrease the synthesis and the mobility of this ionophore, potentiated the actions of dantrolene. In experiments using the calcium ionophore A23187, they were able to demonstrate antagonism of a competitive nature between the ionophore and dantrolene.

In platelets, there is evidence that TxA2 is essential for intracellular release of calcium (*Gerrard et al 1977; Gorman et al 1979*) and its synthesis has been localized to the platelet dense tubular system, an endoplasmic reticulum membrane system, which represents an intracellular calcium storage compartment (*Gerrard et al 1976; White 1973*).

In the only study in which an effect of dantrolene was reported in smooth muscle (nonvascular), a similar but less potent action of dantrolene was found (*Graves et al 1978*). Unlike the situation in skeletal and mesenteric vascular smooth muscle, dantrolene (3×10^{-7} - 3×10^{-6} M) could not block contractions by more than 50%. In skeletal muscle similar concentrations resulted in a 75% blockade of contractions (*Ellis and Bryant 1972*) while in the mesenteric vasculature these concentrations abolished contractions. These differences are probably the result of structural differences unique to each muscle type and a differntial dependence on extracellular calcium.

Using a classical type of prostaglandin antagonist, N0164, it was confirmed that a reduction of the extracellular calcium concentration

resulted in a greater inhibitory effect of the antagonist. A similar potentiation was observed using the prostaglandin cyclooxygenase inhibitor. Calcium therefore appears to play a complex role in the reactivity of the mesenteric vasculature, participating both in the activation of the contractile proteins and the biosynthesis of prostaglandins. In order to further examine the role of calcium, experiments were conducted in which the concentration of calcium in the buffer was manipulated and the effects of several drugs on the actions of calcium determined.

When the mesenteric vascular bed was perfused with buffer in which the calcium concentration was approximately 1×10^{-6} M (made by omitting the calcium chloride from the buffer formulation), noradrenaline responses remained stable for up to four hours (*Ally et al 1977; 1978*). In bovine and rabbit vascular smooth muscle *Sands et al* (1977) demonstrated that calcium uptake was independent of the extracellular calcium concentration but was dependent on ATP. This explained the maintenance of noradrenaline responsiveness since the activator stores of calcium could be replenished from the low amount of calcium present in the buffer. If a calcium chelator (EDTA or EGTA, 1mM) was added to the buffer, essentially producing a calcium free buffer, vascular contractions to noradrenaline became progressively smaller with time, disappearing within 20 minutes.

In preparations equilibrated in 10^{-6} M calcium buffer, the cumulative addition of calcium caused an initial stimulation of noradrenaline responses followed by an inhibition at higher calcium concentrations (Fig. *78*). One possible explanation would be that calcium from the extracellular compartment was recruited directly by noradrenaline thus accounting for the potentiation, while the inhibition with higher

concentrations of calcium was the result of a stabilization of the cell membrane and a decrease in its permeability to calcium (*Fleckenstein 1970/1971*).

As noted above, potassium contracted the vascular bed in the presence of low concentrations of ionophore unlike noradrenaline. This suggested that the concentration of noradrenaline used in these studies did not depolarize the plasma membrane to any significant degree. It was therefore unlikely that noradrenaline directly recruited the extracellular calcium to activate the contractile proteins. This conclusion is in agreement with the results of *Kikta and Davis (1979)* who showed that noradrenaline at concentrations less than 1×10^{-6} M did not utilize extracellular calcium.

While it was possible that higher concentrations of calcium decreased the permeability of the plasma membrane, it was also possible that it inhibited the release of calcium from intracellular sites such as the inner surface of the plasma membrane. Some support for this explanation was provided by the data of *Makinose (1975)* who found that 10^{-3} M calcium ions in the extracellular fluid inhibited the release of calcium from sarcoplasmic reticular vesicles.

In the experiments in which either dantrolene, imidazole or indomethacin was present in the perfusate during the determination of the calcium concentration response curve, the stimulatory effect of calcium ions was prevented, whereas the inhibitory action was unaffected. Neither of these drugs blocked the uptake of calcium (see above) but each prevented the synthesis or action of prostaglandins. It would appear that the rising portion of the calcium curve (Fig. 78) was the result of stimulation of prostaglandin biosynthesis whereas the falling phase was

independent of either prostaglandin biosynthesis or action.

In summary, low concentrations of a calcium ionophore A23187 inhibited noradrenaline pressor responses by depleting the intracellular calciumstores. Verapamil, a calcium antagonist, appeared to prevent the uptake of calcium through the plasma membrane and the release of calcium from intracellular stores. The interaction of verapamil with indomethacin suggested that verapamil, in addition to its blockade of plasma membrane calcium channels, might possess prostaglandin antagonist properties. Dantrolene, a muscle relaxant, appeared to be a thromboxane A2 antagonist, specifically inhibiting noradrenaline contrations. In experiments in which calcium concentration response relationships were determined in the presence and absence of either dantrolene, imidazole or indomethacin, the data were consistent with a stimulatory effect of calcium on prostaglandin biosynthesis. It was also shown that the auto-inhibitory effects of calcium were independent of prostaglandin action or biosynthesis.

SECTION VI: PROSTAGLANDINS, PAPAVERINE AND HYDRALAZINE

INTRODUCTION

The mechanisms whereby hydralazine reduces mean arterial blood pressure are presently poorly understood (*Gross 1977*). This drug is an effective vasodilator in the femoral, mesenteric, renal, carotid and coronary arteries (*Gross et al 1950; Bein and Brunner 1965*).

Extensive studies by *Stunkard et al* (*1954*) and *Walsh et al* (*1977*) have identified dilation of the peripheral arteriole as the cause of the decreased blood pressure. Since this early work, little progress has been made in developing more efficacious hydralazine analogs, and in working out its mechanism of action (*Gross 1977*).

At various times it has been suggested that hydralazine was acting in a manner similar to papaverine (as a phosphodiesterase inhibitor). Some early data (*Ablad 1963*) showed that both papaverine and hydralazine inhibited noradrenaline contractions during aerobic conditions whereas during anaerobic conditions hydralazine paradoxically potentiated noradrenaline contractions while papaverine was still inhibitory. These data surely argue against any similarity in action.

In guinea pig and dog, *Inatomi et al* (*1975*) demonstrated that hydralazine did not act in a manner similar to papaverine. They concluded that its action was independent of the cyclic AMP phosphodiesterase system.

It has been shown that hydralazine binds tightly to vascular smooth muscle *in vivo* and *in vitro* (*Perry et al 1962; Moore-Jones and Perry 1966; Keberle et al 1973*). This compound antagonized the action of a variety of agents which contracted smooth muscle (*Druey and Tripod 1967; Gross 1977; McLean et al 1978*). This latter group

(*McLean et al 1978*) reported that hydralazine, hydralazine acetone hydrazone and hydralazine butanone hydrazone relaxed established potassium and noradrenaline contractures and inhibited the development of contractures on preincubation. They showed that in the rabbit aortic strips the calcium dependent and calcium independent components of the noradrenaline contractures were both inhibited. They proposed that hydralazine and its hydrazone derivatives inhibited calcium flux through the plasma membrane. However, in human studies using verapamil as an antihypertensive agent, a less than moderate decrease in blood pressure was realized. It was therefore concluded that this calcium antagonist did not have a clinically significant antihypertensive effect (*Pederson 1978*). In a study which investigated the action of hydralazine, *Diamond* (*1979*) found that hydralazine caused relaxation without lowering cytoplasmic calcium. A previous study by *Zsoter and Wolchinsky* (*1978*) showed that hydralazine prevented the development of hypertension in pretreated young genetically hypertensive rats but did not alter the kinetics of calcium uptake or efflux. In the same study neither propranolol nor timolol prevented the development of hypertension. This is in opposition to the report by *Goldberg and Triggle* (*1978*) who found that timolol prevented the development of hypertension but did not, as noted above, prevent the vascular smooth muscle from exhibiting a defect in calcium translocation.

The defect in, or alteration of, the plasma membrane in hypertension was not restricted to the vasculature; platelets became hyperactive (*Hamet et al 1978*) as did vas deferens (*Caulfield et al 1977*)

and non-vascular visceral smooth muscle (*Altman et al 1977*).

In platelets antihypertensive agents inhibited aggregation (*Greenwald et al 1978*) while hydralazine pretreatment of hypertensive animals prevented the development of platelet hyperactivity (*Saunders et al 1977*).

It has been postulated that cyclic AMP inhibits platelet aggregation. Recent studies using platelets from normotensives and hypertensives demonstrated that these observations were not correct (*Hamet et al 1978; Wang et al 1978*). No direct correlation of the level of cyclic AMP, adenyl cyclase or phosphodiesterase activity could be made with either the actual inhibition or activation of the aggregation mechanism, nor was it possible to demonstrate differences in these parameters between platelets from normotensives and hypertensives. In studies in vascular smooth muscle *Bhalla et al* (*1978*) and *Donnelly* (*1978*) did not find any differences in cyclic AMP content of aortas from normal and hypertensive animals.

It has been shown that platelet and vascular hyperactivity preceded the gross manifestation of the hypertensive state (*Sivertsson and Olander 1968; Saunders et al 1977; Lais and Brady 1978*). This has been demonstrated to be the result of an increased membrane permeability to ions. The aorta from spontaneously hypertensive rats exhibited a greater dependency on extracellular calcium. These vessels relaxed in calcium free solution, and contracted to 60% of maximum upon the reintroduction of calcium. Aortic strips from normotensive animals were not similarly affected (*Noon et al 1978; Pedersen et al 1978*). The antihypertensive agents diazoxide and propranolol had no significant

effect on active calcium uptake by mesenteric artery subcellular fractions from normotensive and hypertensive animals, whereas the calcium antagonist SKF525A decreased calcium uptake by subcellular fractions from hypertensive animals more than those from normotensive animals (*Wei et al 1976*).

In a study on the time for maximal relaxation from a contracted state (*Levy 1975*) found no difference in the effect of verapamil (calcium antagonist) on aortas from spontaneously hypertensive and normotensive animals. This suggested that the failure of the aorta from hypertensive animals to relax to the same degree as normotensive aortas (*Weissinger and Bloor 1979*) could have been due to factors other than calcium uptake and resequestration. *Diamond* (*1979*) suggested that hydralazine caused relaxation without lowering cytoplasmic calcium by a possible direct action at the contractile proteins.

One complication of hypertension is the structural changes which take place, secondary to the rise in arterial pressure, resulting in about 80% increase in flow resistance (*Bereck and Bohr 1977*). This hypertrophy in the vessel wall can account for a significant degree of the elevated blood pressure. Another problem is that isolated arteries from genetically hypertensive rats did not show the same hypersensitivity to neurotransmitters (*Hallback et al 1971; Field et al 1972; Shibata et al 1973; Hansen and Bohr 1975*) that isolated perfused vascular beds exhibit (*Lais et al 1974; Tobias et al 1974; Lais and Brody 1978*).

In a recent study *Henrich et al* (*1978*) exhibited the morphological

changes of the microvasculature in established hypertension. They found that in the mesenteric microcirculation a lower flow resistance existed in individual arterioles, associated with a gradual disappearance of the hypertrophized smooth muscle cells towards the capillary bed; the elevation in the resistance to blood flow was due to a reduced number of resistance vessels. It is possible that in other vascular beds a similar condition exists. Therefore, a reduced number of resistance vessels may ultimately be responsible for the increased resistance to flow in established hypertension. This is consistent with the findings of *Hutchins and Darnell (1974)* who also observed a decreased number of arterioles in the cremaster muscle of spontaneously hypertensive rats. In the isolated perfused mesenteric vascular bed of spontaneously hypertensive rats we have found an increased basal perfusion pressure when compared to age matched controls (*unpublished data*). This observation is in agreement with the suggestion of *Henrich et al (1978)* that the elevated flow resistance was due to vessel wall hypertrophy in the series coupled section and a reduction in the number of resistance vessels in the parallel coupled sections of the mesenteric bed.

These studies indicate that spontaneous hypertension in its established phase has different effects on the micro- and macro-circulation. Indeed, *Furuyama (1962)* found that vessel wall hypertrophy was restricted to large arterioles and arteries (diameter > 100μ). In the isolated mesenteric vascular bed, the primary resistance vessels were retained (see Methods). These vessels are less than 100μ in diameter; therefore, muscle cell hypertrophy would not be present in these

resistance vessels in beds from either spontaneously hypertensive or normotensive animals. However, the alterations in the structural make-up of the bed (*Henrich et al 1978*) would persist. *Gross* (*1977*) has said that "there is also no method available that would give a higher degree of probability for detecting a new blood-pressure lowering drug than screening in the normotensive or in the spontaneous hypertensive rat". It is therefore, equally valid an argument that an understanding of the mechanism of action of a direct acting vasodilator can be achieved using preparations from normotensive animals (mesenteric vascular bed). In this vascular bed are found vessels similar to those which determine peripheral vascular resistance, whereas large arteries such as the aorta, used by many investigators (e.g. *Rioux et al 1977; McLean et al 1978*) may not be representative of resistance vessels. This is supported by the recent findings of *Deragon et al* (*1978*), that the sensitivity to the vasodilating effect of nitroprusside in the perfused hindlimb preparations was no different in preparations from normotensive and spontaneously hypertensive rats. These data do not support the hypothesis suggesting the existence of a deficient ability to relax in hypertensive rats. Indeed it showed that the arteriolar resistance vessels in the vascular bed of both groups were equally sensitive to the muscle relaxant. Much of the data suggesting a decreased relaxation was obtained using large arteries.

In this section the effects of papaverine (a classical smooth muscle relaxant) are compared with those of hydralazine on vascular reactivity.

RESULTS

Papaverine Inhibition of Noradrenaline, Angiotensin II and Potassium Pressor Responses

Papaverine (3.3×10^{-8} M to 4.2×10^{-6} M) progressively diminished the pressor responses to both noradrenaline and angiotensin. Papaverine (1.3×10^{-6} M to 8.48×10^{-5} M) was an effective inhibitor of potassium pressor responses. A sample trace for papaverine effectiveness in inhibiting noradrenaline and potassium pressor responses is shown in Fig. *79*. As can be seen papaverine did not decrease the baseline perfusion pressure which indicated that this preparation was virtually atonic. Complete dose response curves for inhibition of noradrenaline and potassium pressor responses are shown in Figure *80*. There was virtually no overlapping of the respective dose response curves for each pressor substance. Papaverine was definitely much more potent against noradrenaline and angiotensin pressor responses with IC50 concentrations $\pm$ 20% of each other (n = 4). The IC50 against noradrenaline responses was 5.8×10^{-7} M and that against potassium responses was almost 20 times greater (1.06×10^{-5} M, Fig. *80*).

The Effectiveness of Papaverine on Noradrenaline Responses in the Presence of Exogenous PGE2

At the beginning of each experiment, PGE2 (5 ng/ml, 1.4×10^{-8} M) was added to the buffer reservoir and control responses determined until they were $\pm$ 10% for three successive responses. The papaverine dose response relationship was then determined by cumulative addition of

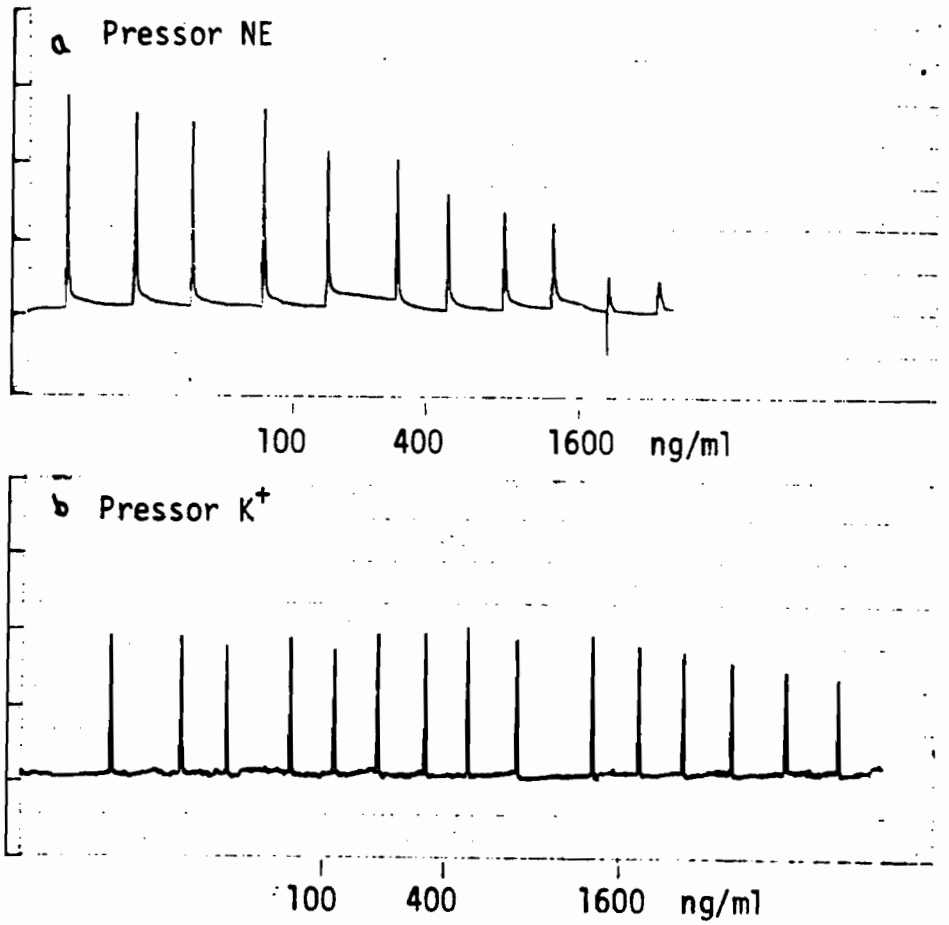

Fig. 79. Influence of papaverine on vascular responses to (a) noradrenaline and (b) potassium. Pressor agent was injected as a 100 µl bolus intra-arterially; papaverine was added to the buffer reservoir in a cumulative fashion. As can be seen papaverine had a rapid effect on pressor responses but did not alter base line perfusion pressure, nor did it influence the duration of the contractions. 100 ng/ml papaverine = 2.6×10^{-7} M. Each division of the vertical scale shown = 20 mm Hg (1 mm Hg = 133.3 Pa).

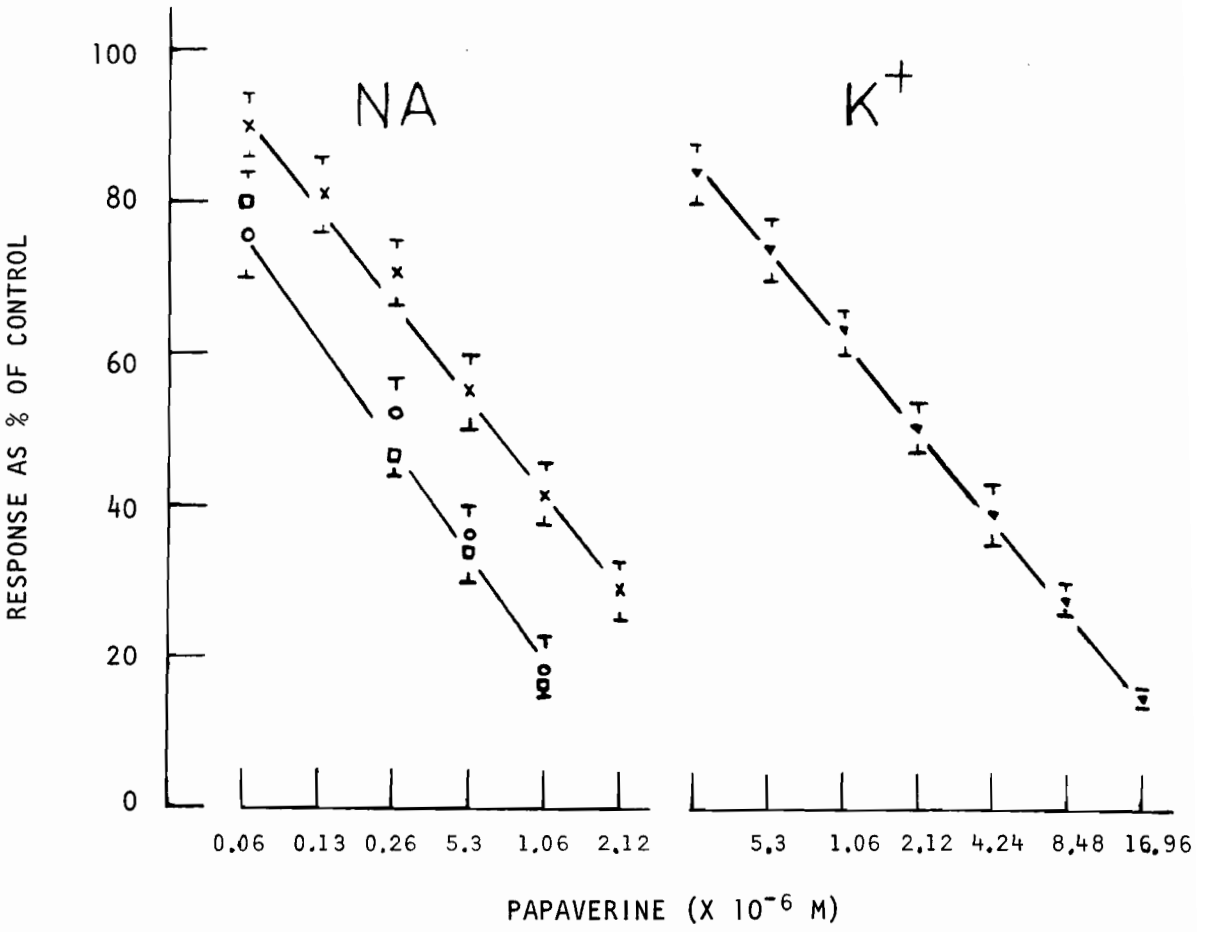

Fig. 80. The effects of papaverine on noradrenaline and potassium responses. The vascular beds were prepared as described in methods. Each pressor agent was injected as a 100 µl bolus into the superior mesenteric artery contractions of the vascular smooth muscle were recorded as an increase in perfusion pressure (See Fig. 79 for sample trace). (x) buffer alone, (□) buffer plus 3.6×10^{-4} M imidazole, (o) buffer plus 6.4×10^{-7} M dantrolene. Each point represents the mean ± SE, $n \geq 6$. The effect of papaverine on noradrenaline responses were significantly increased $p < 0.05$, ANOVA in the presence of imidazole and dantrolene.

papaverine to the buffer reservoir. In the presence of exogenous PGE2 the dose response line was shifted to the right of the control line and the IC50 was increased approximately 4-fold (Fig. *8l*). Since the addition of exogenous PGE2 shifted the dose response line to the right, then inhibition of endogenous prostaglandin biosynthesis would be expected to have the opposite effect.

Papaverine Inhibition of Noradrenaline Pressor Responses in Indomethacin Perfused Preparations

Preparations were prepared and equilibrated as described in Methods; at the end of the equilibration period indomethacin (5.6×10^{-5} M) was added to the buffer reservoir. As before papaverine was added to the buffer in a cumulative manner. As anticipated the dose response curve was shifted leftwards, parallel to those obtained from both control and PGE2 perfused preparations (Fig. *8l*), and the IC50 was now approximately 25% of control (1.3×10^{-7} M). These data suggested that papaverine was a prostaglandin antagonist.

Effect of Papaverine on Noradrenaline Responses in Preparations Perfused with 1.7×10^{-4} M Indomethacin and Either 1.4×10^{-8} M or 2.8×10^{-9} M PGE2

The preparations were perfused with a concentration of indomethacin which abolished vascular responsiveness and presumably prostaglandin biosynthesis. Vascular responsiveness was partially restored by the addition of a fixed concentration of PGE2 to the buffer. Each preparation was used for a single indomethacin plus PGE2 combination and then

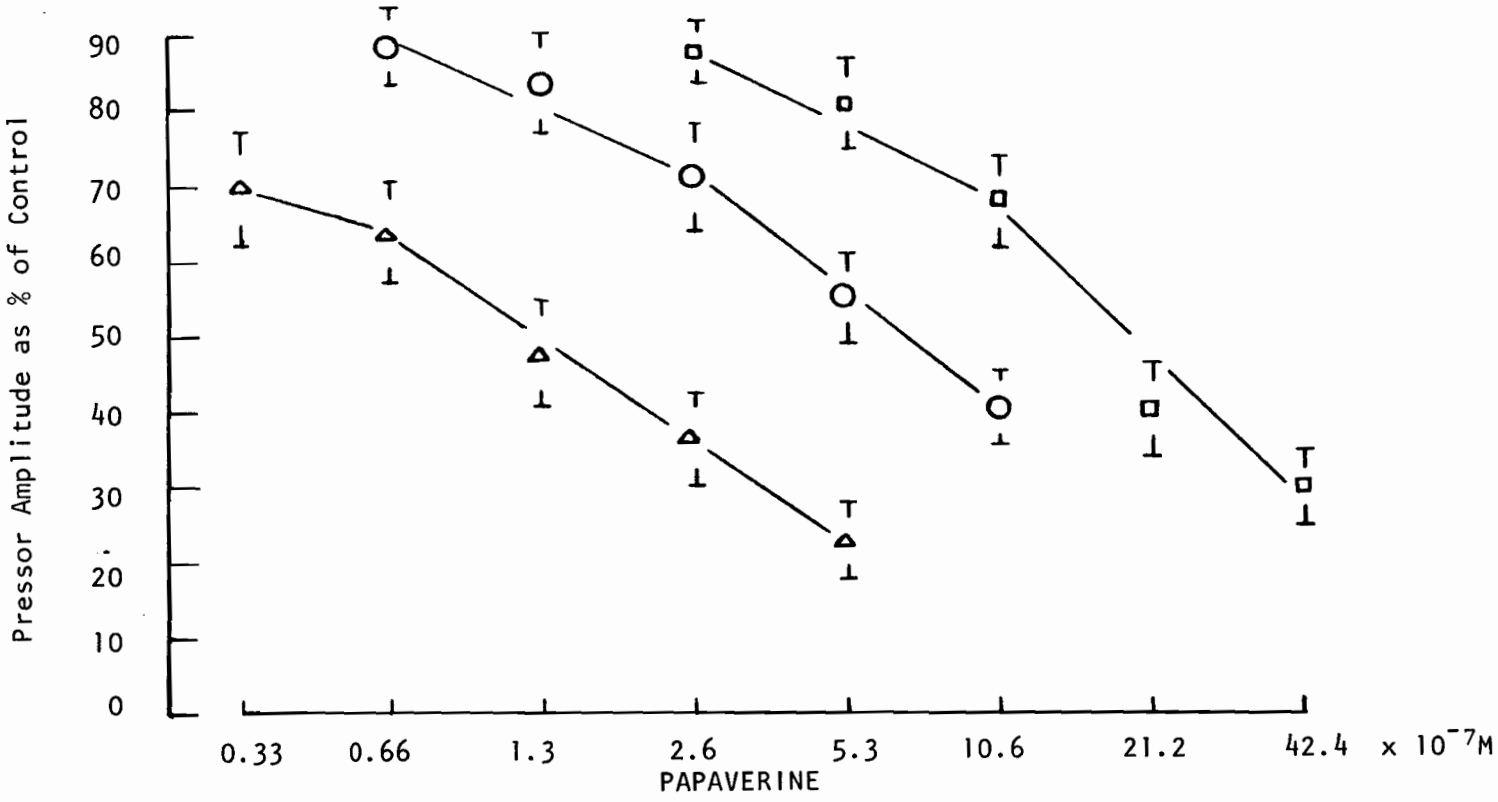

Fig. 81. Changes in response to a fixed dose of noradrenaline in the presence of increasing concentrations of papaverine. The experiments were carried out using plain buffer (o), buffer containing 2.8×10^{-8} M PGE2 (□) or buffer containing 5.6×10^{-5} M indomethacin (Δ). Note the shift to the right caused by the presence of PGE2, and the shift to the left upon partial inhibition of the cyclo-oxygenase with indomethacin. The effects of papaverine in the presence of PGE2 and indomethacin were significantly ($p < 0.05$ ANOVA) different from its effect in plain buffer (o).

discarded upon completion of the experiment. As shown in Figure *82*, the dose response curves are linear, parallel to each other, the curve generated in the presence of 2.8×10^{-9} M PGE2 lying to the left of that generated in the presence of 1.4×10^{-8} M PGE2. These data clearly indicated a relationship between the concentration of PGE2 present and the potency of papaverine. The parallel nature of the dose response curves suggested that the interaction was probably competitive.

Papaverine Inhibition of Potassium Pressor Responses in Preparations Perfused with 1.7×10^{-4} M Indomethacin and 2.8×10^{-8} M PGE2

To six preparations perfused with indomethacin (1.7×10^{-4} M), PGE2 was added to give a final concentration of 2.8×10^{-8} M, which partially restored pressor responses. Papaverine added cumulatively to the perfusate inhibited potassium pressor responses in a concentration dependent manner. The concentration response curve was shifted to the right of control in a non-parallel manner. The approximate papaverine IC50 concentrations were: control preparations, 1.06×10^{-5} M and indomethacin plus PGE2 preparations 2.1×10^{-5} respectively (determined from their dose response curves, Figs. *80* and *84*). The maximum effective concentration of papaverine tested under both conditions (8.44×10^{-5} M) gave indistinguishable results. In normal preparations, responses were $15 \pm 2\%$ and in indomethacin plus PGE2 perfused preparations they were $16 \pm 4\%$ of pre-papaverine pressor amplitudes.

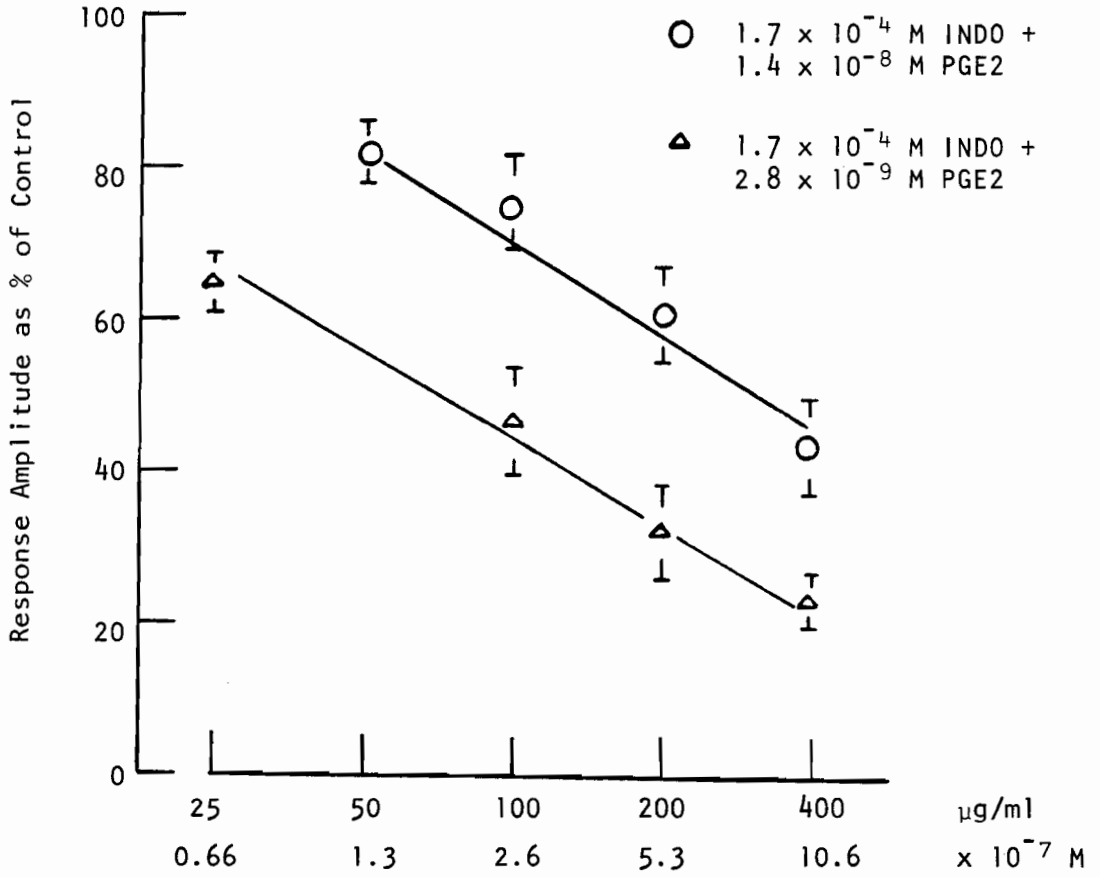

Fig. 82. Inhibition of noradrenaline pressor responses by increasing concentrations of papaverine, in preparations in which responses had been abolished with 1.7×10^{-4} M indomethacin and reactivity restored with either 1.4×10^{-8} M PGE2 (○) or 2.8×10^{-9} M PGE2 (△). Each point represents the mean ± SE for six experiments and are significantly different ($p < 0.01$, ANOVA) between concentration response curves.

Effect of Papaverine in Preparations Perfused with 1.8 x 10^{-3} M Imidazole and 2.8 x 10^{-8} M PGE2

Because of the demonstration that papaverine inhibited noradrenaline and potassium pressor responses over dissimilar concentration ranges (Fig. *80*) and the apparent antagonism between papaverine and endogenous or exogenously added prostaglandins (Figs. *81* and *82*) it was of interest to examine this interaction in preparations in which only the noradrenaline responses were inhibited with a TxA2 synthetase inhibitor (imidazole) and vascular reactivity restored with exogenous PGE2. As before papaverine effectively inhibited noradrenaline pressor responses (Fig. *83*). This inhibitory effect was readily reversed upon switching to papaverine free buffer. The effective papaverine inhibitory concentration range was 0.66 x 10^{-7} M to 2.12 x 10^{-7} M.

Effect of Papaverine in Preparations in which Noradrenaline Pressor Responses were Inhibited by either Imidazole (5.6 x 10^{-4} M) or Dantrolene (6.4 x 10^{-7} M)

These concentrations of imidazole and dantrolene inhibited noradrenaline pressor responses to a similar degree. Three control pressor responses to noradrenaline were obtained in the presence of either compound, papaverine was then added to the perfusion reservoir in a cumulative fashion and pressor responses to the same fixed concentration of noradrenaline were determined. The dose-response curves generated in the presence of either imidazole or dantrolene were virtually identical (Fig. *80*) and were to the left of and parallel to the control curve. Both imidazole and dantrolene increased the potency of papaverine by an

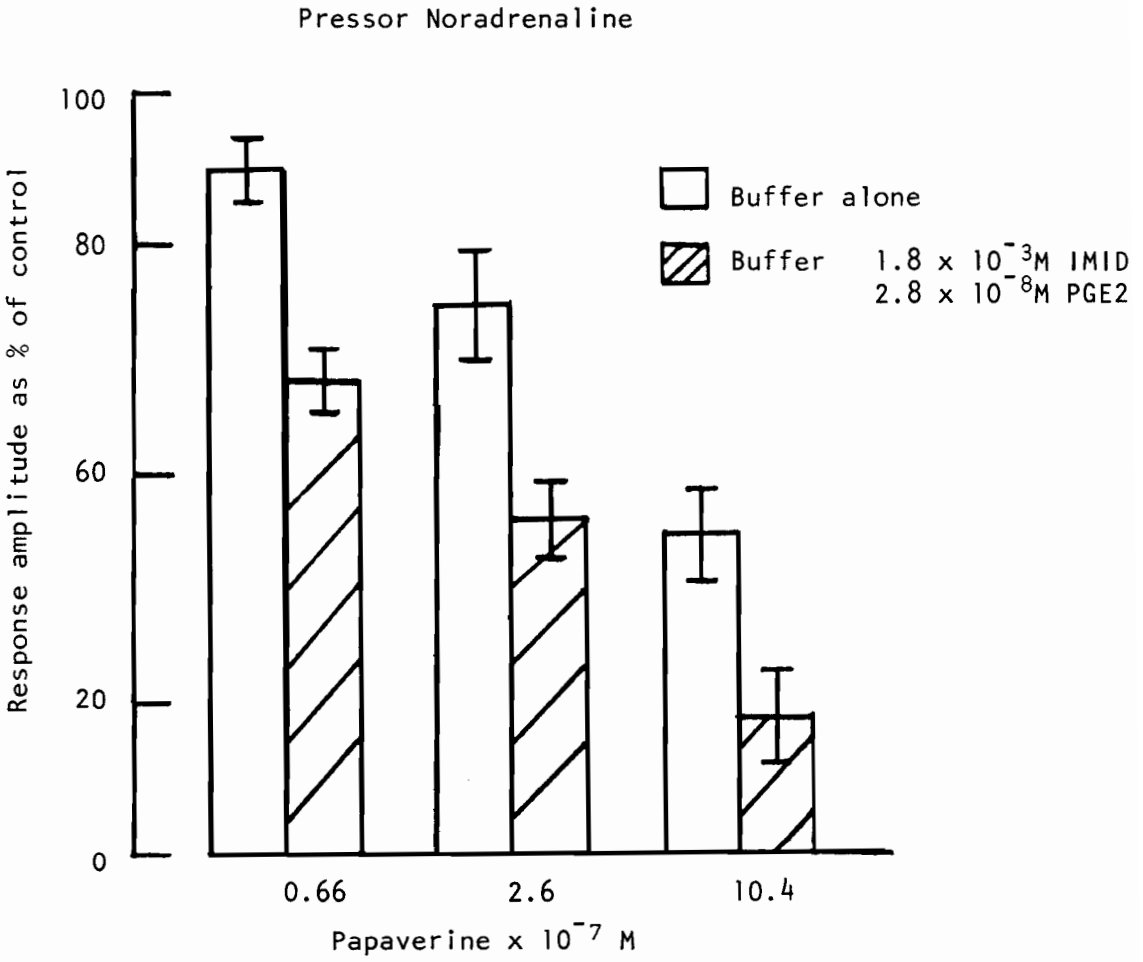

Fig. 83. Papaverine inhibition of noradrenaline contractions of the mesenteric vasculature in preparations perfused with buffer alone (□) or buffer plus 1.8×10^{-3}M imidazole and 2.8×10^{-8} M PGE2 (▨). Each response shown represents the mean± SE for six experiments. * $p < 0.01$ ANOVA.

equivalent amount.

Comparison of Papaverine Inhibition of PGE2 in the Rat Fundic Strip and Mesenteric Vascular Preparation

The fundus of male Wistar rats was cut away from the rest of the stomach, opened out longitudinally and placed in a beaker containing Kreb's solution (composition given in general methods). A 2.5 cm long by 0.5 cm wide strip was cut out and a thread was attached to each end. The preparation was mounted in a 50 ml muscle bath and superfused with Kreb's solution at 37° C and aerated with 95% O_2, 5% CO_2 mixture. One end of the strip was anchored to an L-shaped aluminium rod immersed in the buffer and the other end to an isometric force transducer. Contractile responses were recorded on a Devices recorder. The muscle was stretched to give a resting tension of 0.8 gram. The preparation was contracted by adding PGE2 (5 ng/ml, 1.4×10^{-8} M) directly to the muscle bath; papaverine was added at the plateau of the PGE2 contracture. The new plateau established at a lower tension in the presence of papaverine was noted and was expressed as a % of the initial PGE2 contracture. These data were plotted as % of control response vs papaverine concentration. The range of papaverine concentrations inhibiting PGE2 contractions was similar to that found effective against potassium pressor responses in the perfused mesentery (Fig. *84*). The papaverine dose response curve obtained using mesenteric vascular beds perfused with indomethacin (1.7×10^{-8} M) is shown for comparison.

Papaverine at concentrations which inhibited noradrenaline contractions in the vasculature had no PGE2 antagonist action in the rat

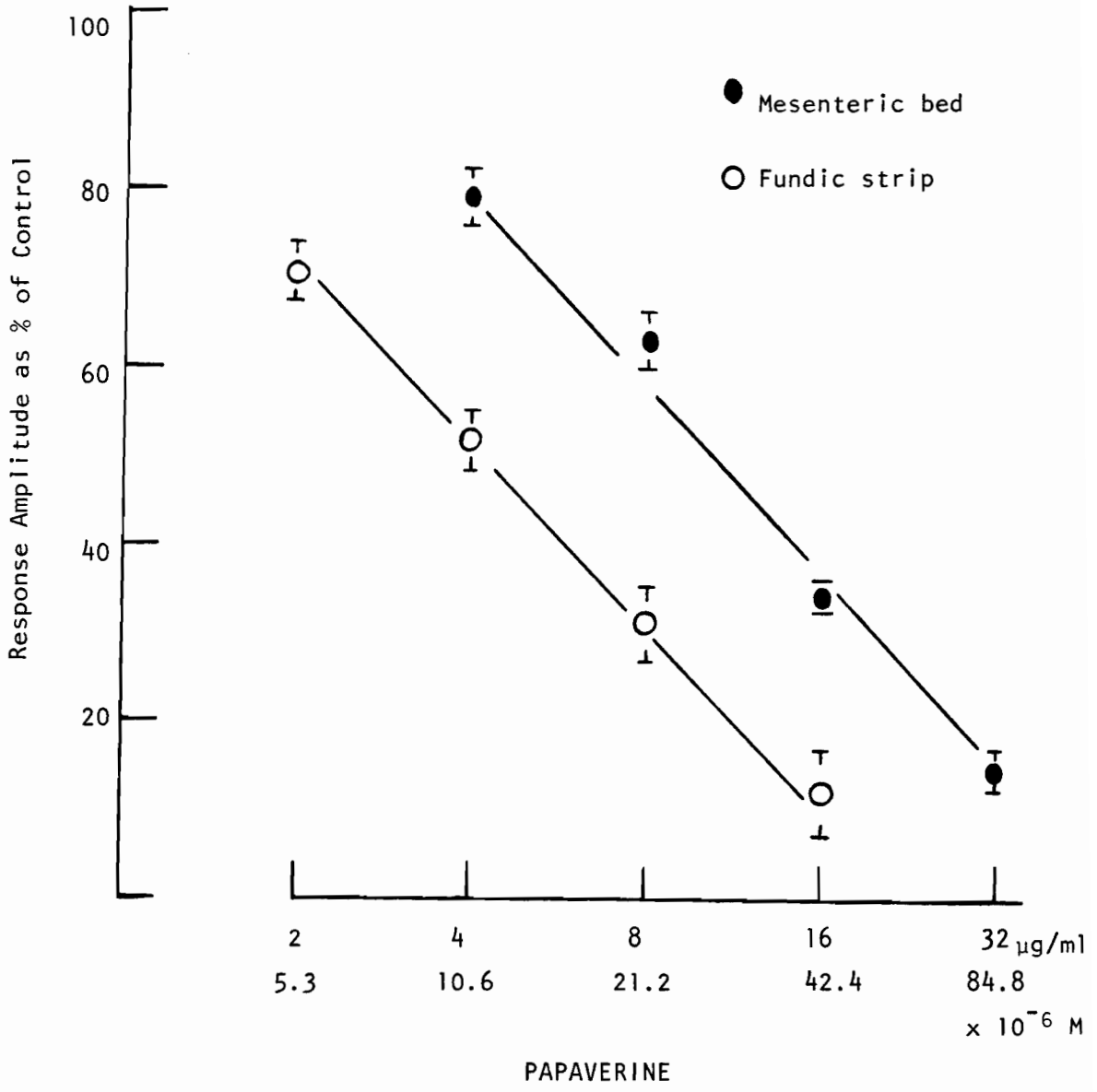

Fig. 84. Papaverine inhibition of potassium contractions in the rat mesentric vascular bed in which vascular responses had been abolished with 1.7×10^{-4} M indomethacin and reactivity restored with 2.8×10^{-8} M PGE2 (●) and PGE2 (1.4×10^{-8} M) contraction of the rat fundic strip (○). Each point represents the mean ± SE for five or six experiments.

fundic strip. The concentration effect curves shown (Fig. *84*) clearly indicate that papaverine antagonized the direct contractile effects of PGE2 on the fundic strip at concentrations which inhibited PGE2 facilitated potassium responses in the mesenteric vascular bed.

Summary

Papaverine acted as an effective prostaglandin antagonist blocking potassium and noradrenaline contractions in the mesenteric vascular bed. Papaverine exhibited a concentration dependent selectivity in its actions; low concentrations blocked noradreanline contractions whereas high concentrations were required to block potassium contractions. These high contractions were also required to block the direct contractile actions of PGE2 in the rat fundic strip. The actions of papaverine were opposed by the addition of PGE2 to the buffer and potentiated by either a thromboxane synthetase or a cyclooxygenase inhibitor. In the absence of endogenous prostaglandin biosynthesis, papaverine inhibited vascular contractions which had been restored by the addition of exogenous PGE2. Papaverine antagonized both the modulatory effects of prostaglandins in the mesenteric vascular and the direct contractions caused by PGE2 in the fundic strip preparation.

Effect of Hydralazine on Vascular Responsiveness

Preliminary experiments established that hydralazine (1.6×10^{-6} M to 4.09×10^{-4} M) inhibited both noradrenaline and angiotensin pressor responses. Potassium pressor responses were not affected by any concentration of hydralazine tested (up to 1×10^{-3} M).

Effect of Hydralazine on Noradrenaline Responses in Preparations Perfused with either Indomethacin or Imidazole

In preparations perfused with either indomethacin (5.6×10^{-5} M), or imidazole (5.6×10^{-4} M) the inhibitory effects of hydralazine on noradrenaline pressor responses were significantly increased (Figs. *85* and *86*). Indomethacin potentiated the inhibitory effects of hydralazine over the concentration range (1.6×10^{-6} M to 1.02×10^{-4} M), so that at a concentration of 1.02×10^{-4} M hydralazine was twice as effective in indomethacin treated preparations as compared to normal buffer perfused preparations (ANOVA $p < 0.001$). In contrast, in imidazole perfused preparations the potency of lower concentrations of hydralazine was enhanced so that the lowest concentration tested (1.6×10^{-6} M) was now 4 times as effective in inhibiting noradrenaline pressor responses (Fig. *86*). At the highest concentration of hydralazine tested (2.05×10^{-4} M) there was no difference in pressor responses between the imidazole treated and the normal preparations (Fig. *86*).

Inhibition of Vascular Responsiveness by Hydralazine in Preparations Perfused with 1.76×10^{-3} M Imidazole plus 2.8×10^{-8} M PGE2

The above data indicated that hydralazine was probably a thromboxane A2 antagonist because of the enhancement of its inhibitory actions by both indomethacin and imidazole. To test the hypothesis that hydralazine was indeed acting as a prostaglandin antagonist, vascular responsiveness was inhibited with 1.76×10^{-3} M imidazole (thromboxane synthetase inhibitor) and noradrenaline pressor responses restored by the simultaneous perfusion with 2.8×10^{-8} M PGE2. Three control responses were obtained

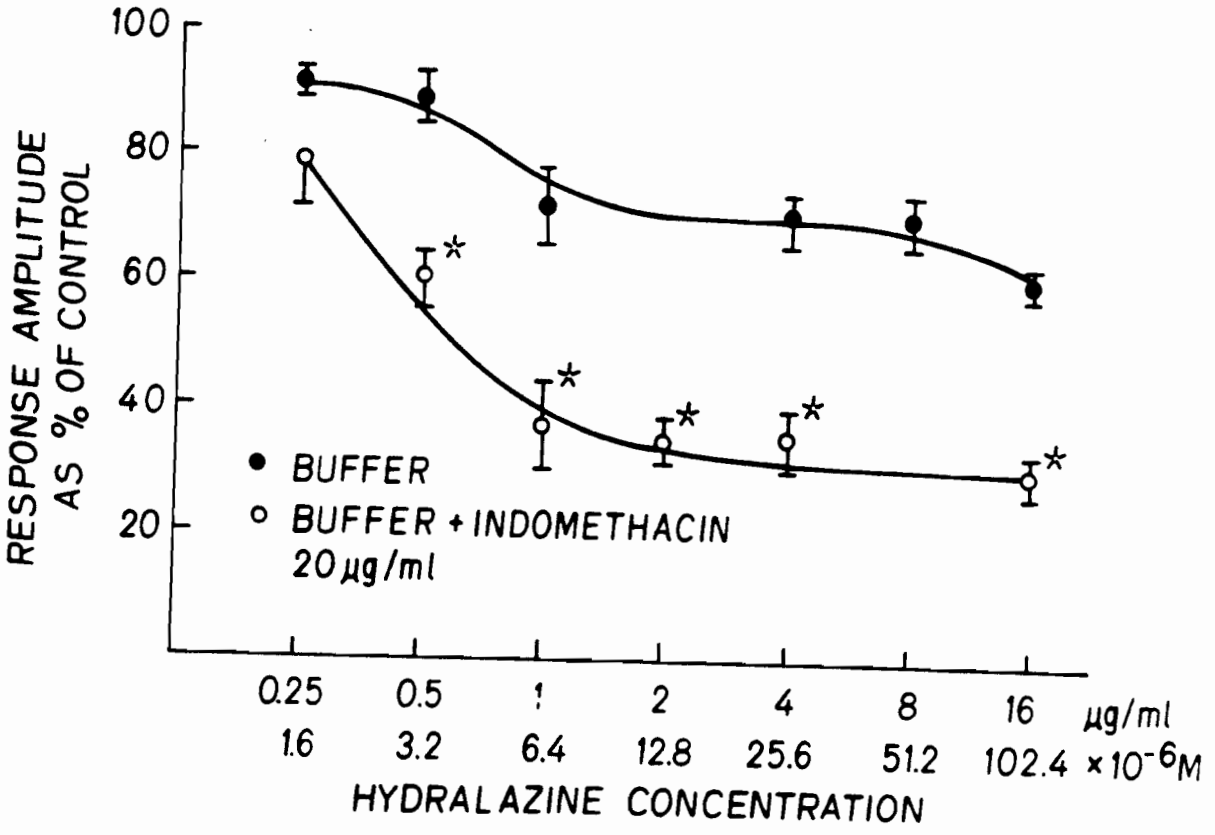

Fig. 85. Influence of indomethacin (5.6×10^{-5} M) on hydralazine's inhibition of noradrenaline pressor responses. As can be seen noradrenaline responses were inhibited to a greater degree in the indomethacin treated preparations (o) than in control (●). Each point represents the mean ± SEM for six experiments.
* $p < 0.001$ (ANOVA)

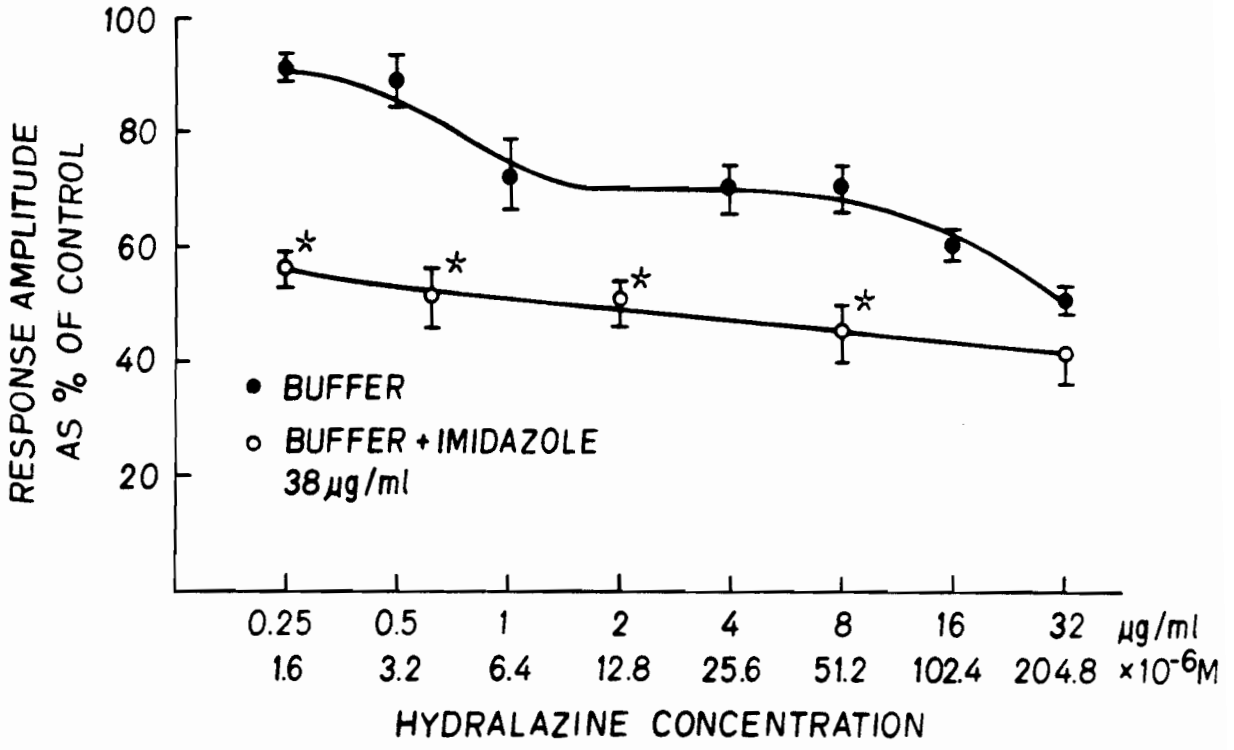

Fig. 86. Influence of partial inhibition of thromboxane synthetase by 5.6×10^{-4} M imidazole on the inhibitory effect of hydralazine on noradrenaline pressor responses. As can be seen (o) hydralazine was now more potent ($p < 0.01$, ANOVA) at low and moderate concentrations (1.6×10^{-6} to 5.12×10^{-5} M) in comparison to its actions in control preparations (•). Each point represents the mean ± SE for six experiments.

and then the effects of the cumulative addition of hydralazine to the buffer on pressor responses were determined. Under these conditions, hydralazine inhibited noradrenaline pressor responses in a concentration dependent manner and the plateau phase seen in normal buffer perfused preparations had vanished (Fig. *87*). Thus, in the absence of thromboxane synthetase activity hydralazine produced maximal inhibition (Fig. *87*).

Potentiation of Hydralazine Action by Low Extracellular Calcium

As shown earlier the effects of calcium ions in this preparation were linked to both stimulation of prostaglandin biosynthesis and action. In preparations perfused with low calcium buffer (1×10^{-6} M, but shown as 0 mM calcium in the Figures) the potency of hydralazine as an inhibitor of noradrenaline pressor responses was significantly enhanced relative to preparations perfused with normal concentration of calcium ions (2.5×10^{-3} M, Fig. *87*) and indomethacin alone (Fig. *88*). In the latter case the effect of hydralazine plateaued at a concentration of 1.28×10^{-5} M. In contrast the inhibitory action of hydralazine showed no similar plateau in preparations perfused with either low calcium or imidazole (1.76×10^{-3} M) plus PGE2 (2.8×10^{-8} M, Fig. *87*).

Reversal of Hydralazine Inhibition by Calcium or PGE2

In two experiments preparations were perfused with low calcium buffer (1×10^{-6} M) and vascular responses inhibited with hydralazine (2.56×10^{-5} M). Upon the readdition of calcium (2.5 mM) to the buffer, the inhibition was partially reversed. A similar reversal was seen upon the addition of PGE2 (2.8×10^{-8} M).

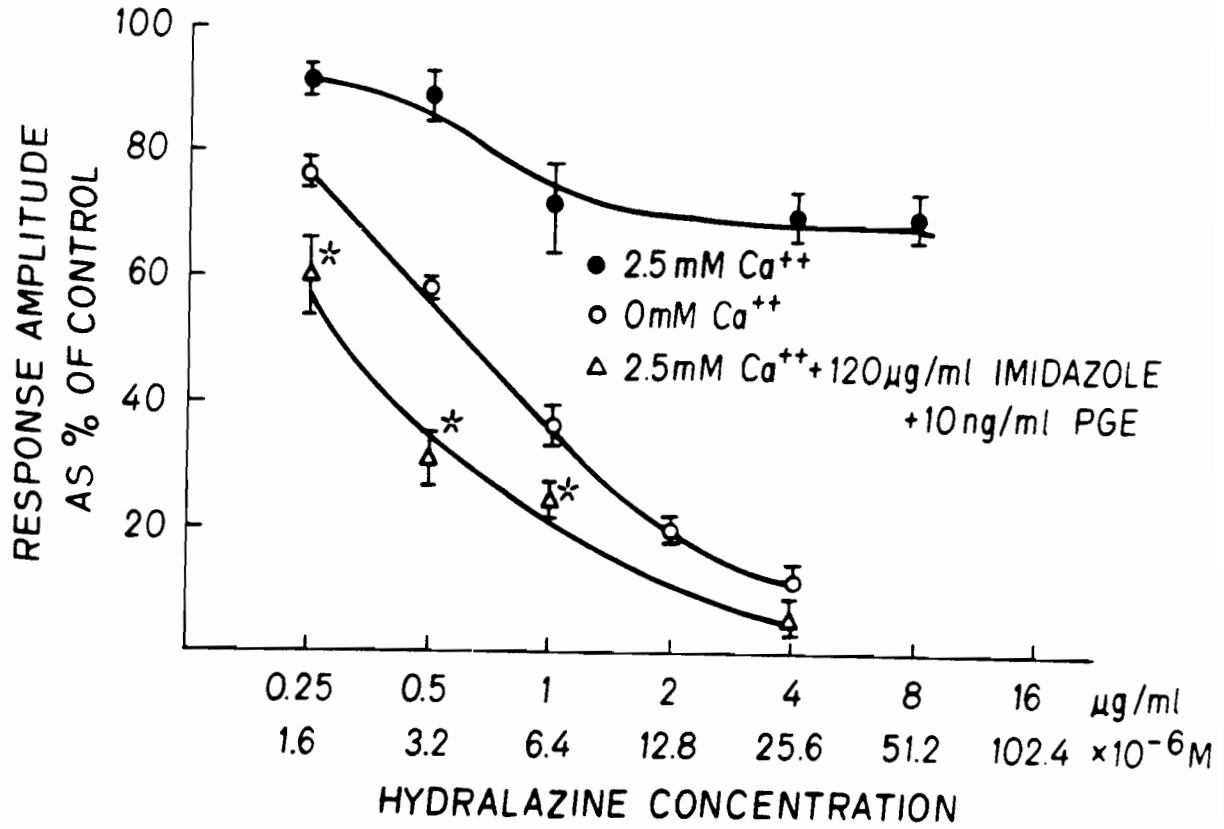

Fig. 87. Effect of hydralazine on noradrenaline contractions of the perfused mesenteric vasculature in control (●), 0 mM Ca buffer perfused (○) and 1.76×10^{-3} M imidazole plus 2.8×10^{-8} M PGE2 perfused preparations. As can be seen vascular effects of hydralazine were significantly enhanced ($p < 0.001$, ANOVA) in calcium free and imidazole plus PGE2 perfused preparations. The effects of hydralazine was also greater ($p < 0.05$, ANOVA) in imidazole plus PGE2 preparations (Δ) at concentrations indicated by asterisk in figure when compared to effects in preparations equilibrated in 0 mM calcium buffer (○). Each point represents the mean ± SE for six experiments.

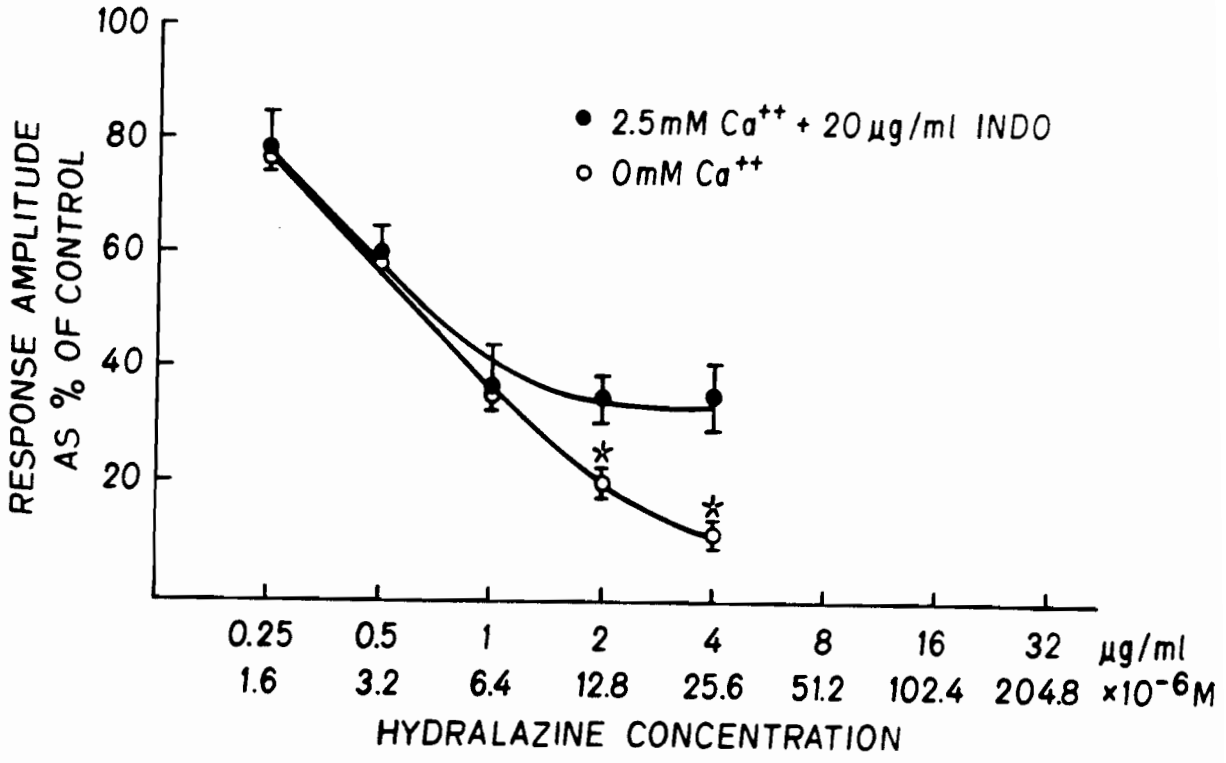

Fig. 88. Comparison of the effect of indomethacin (5.6×10^{-5} M) in preparations perfused with normocalcium buffer and those perfused with 0 mM calcium buffer only on hydralazine inhibition of noradrenaline contractions in the mesenteric vasculature. As can be seen over the hydralazine concentration range 1.6×10^{-6} M to 6. $\times 10^{-6}$ M there is no difference between the two curves whereas at 1.28×10^{-5} and 2.56×10^{-6} M the effect of hydralazine is greater ($p < 0.05$ and $p < 0.001$ respectively, ANOVA) in the low calcium (○) perfused preparations. Each point represents the mean ± SE for six experiments.

Indomethacin Blockade of Calcium Antagonism of Hydralazine

It was possible that the reversal of hydralazine inhibition was independent of prostaglandin biosynthesis. This was tested by perfusing preparations with low calcium buffer and indomethacin (1.1×10^{-5} M). After three control responses were obtained to noradrenaline stimulation, hydralazine (2.56×10^{-5} M) was added to the perfusate. After the noradrenaline pressor responses were maximally inhibited, calcium (2.5×10^{-3} M) was added to the buffer. There was no reversal of the inhibitory effects of hydralazine on noradrenaline responses. In a similar experiment the ability of PGE2 to reverse the effect of hydralazine was not prevented by the indomethacin pretreatment.

Effect of Hydralazine on PGE2 Induced Contractions of Rat Fundic Strip

Hydralazine did not inhibit PGE2 induced contractions of the rat fundic strip. As noted earlier hydralazine did not inhibit potassium contractions of the mesenteric vasculature.

The data above is consistent with the view that hydralazine was a prostaglandin antagonist whose action was tempered by a simultaneous stimulation of an opposing prostaglandin biosynthesis. This latter point could be confirmed by the determination of the levels of prostaglandins before and after exposure of the vasculature to hydralazine.

Hydralazine Stimulation/Facilitation of Prostaglandin Biosynthesis during Noradrenaline Pressor Responses

The mesenteric vascular bed was prepared as described in methods and the preparations equilibrated for 2 hours. Subsequently, three

1 minute fractions of the perfusate were collected at 5 minute intervals. The vasculature was then stimulated by a 100 µl bolus injection (intra-arterial) of noradrenaline and the perfusate collected for the 1 minute period coinciding with the contraction of the blood vessels. After the collection of 4 noradrenaline stimulated fractions, hydralazine was added to the buffer reservoir, and 1 minute samples were collected as described above. The concentrations of hydralazine tested were 6.4×10^{-6} M, 5.12×10^{-5} M and 1.02×10^{-4} M.

The results of the analyses for PGE2 and 6-keto-PGF1α are shown in Figure *89*. It can be seen that noradrenaline caused an increase in PGE2 release of 400% above basal release while 6 keto PGF1α was increased by approximately 66% above basal levels. Hydralazine (6.4×10^{-6} M) added to the perfusate increased PGE2 and 6 keto PGF1α levels by 60% and 100% respectively above normal noradrenaline stimulated release. This effect was detectable within 5 minutes after the addition of hydralazine to the buffer reservoir. Higher concentrations of hydralazine did not produce any further increases in the synthesis of either PGE2 or 6-keto-PGF1α. On the contrary, the level of PGE2 in the perfusate remained constant whereas that of 6-keto-PGF1α fell back to prehydralazine levels (1079 $\pm$ 59 pg/ml, M $\pm$ SD). This argues against an increase in PGI2 levels as being the mechanism of action of hydralazine in this preparation since the inhibitory actions of hydralazine persisted in the presence of control 6-keto-PGF1α levels and in indomethacin plus PGE2 perfused preparations.

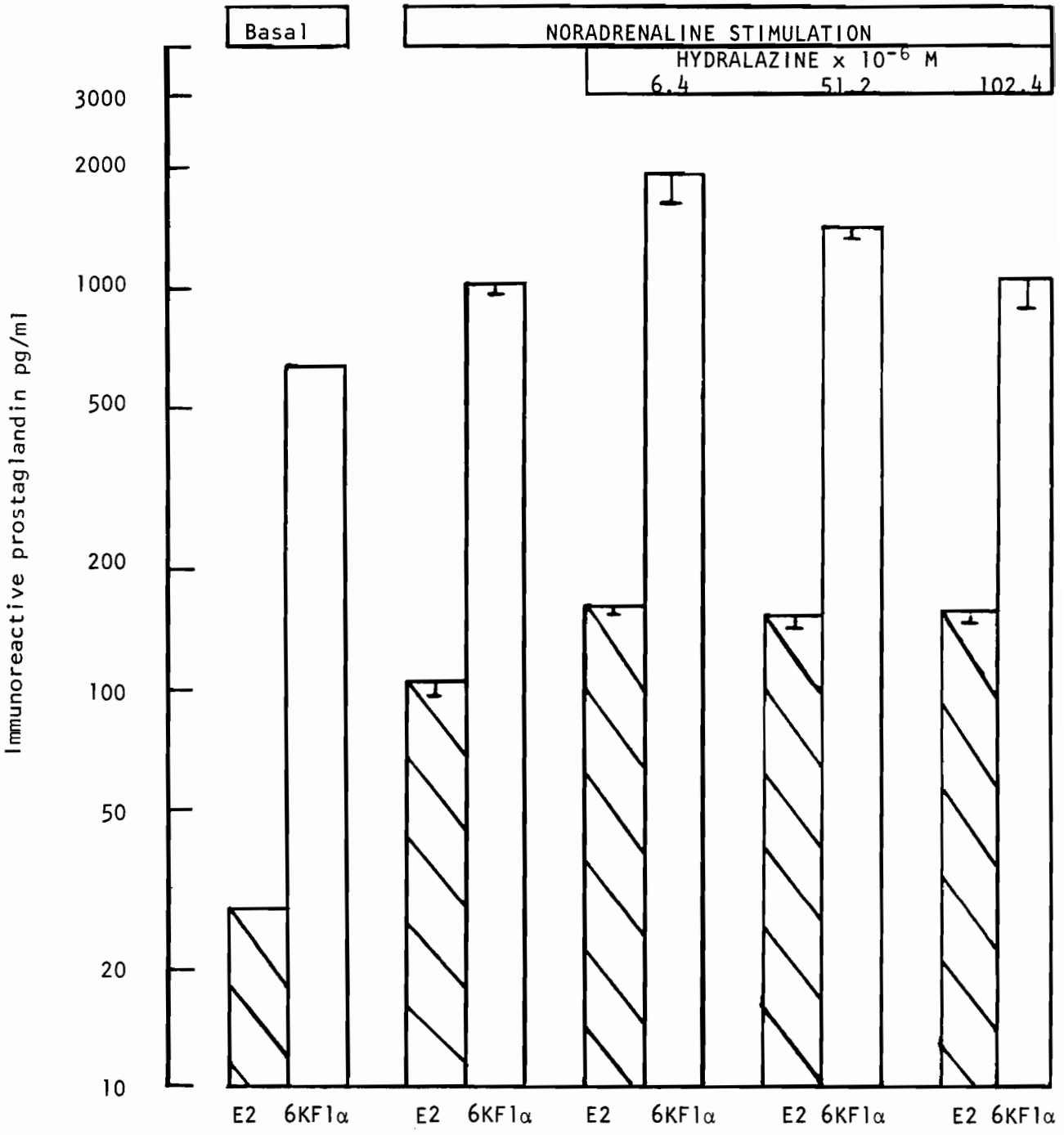

Fig. 89. Influence of hydralazine on noradrenaline stimulated prostaglandin biosynthesis (PGE2 and 6-keto-PGF1α) in the mesenteric vascular bed. Hydralazine significantly increased ($p < 0.01$, ANOVA) PGE2 levels in the effluent. Each vertical bar represents the mean ± SE for 16 samples from 2 separate experiments.

Hydralazine stimulation of basal prostaglandin biosynthesis

While an effect of hydralazine on noradrenaline stimulated biosynthesis was demonstrated (Fig. 89), it was not known whether basal synthesis would also be increased. Consequently, the experiment described above was repeated using a single concentration of hydralazine (5.1×10^{-5} M) but omitting the noradrenaline stimulation. Within 5 minutes of the addition of hydralazine to the perfusate, PGE2 levels had increased from 200 pg/ml to 260 pg/ml, and 6 keto PGF1α had increased from 1400 pg/ml to 2300 pg/ml. After 20 minutes the 6 keto PGF1α levels had plateaued at 1500 pg/ml and PGE2 levels at 230 pg/ml. Thus the release of PGE2 was elevated by 15% and that of 6 keto PGF1α by 7% above prehydralazine levels. These data show that hydralazine had a direct effect on prostaglandin biosynthesis.

DISCUSSION

Hydralazine, unlike many other vasodilators including diazoxide, prazosin, glycerol trinitrate, sodium nitroprusside and papaverine, had little effect on veins *in vivo* (*Collier et al 1978; Ferrari 1974*). Calcium antagonists such as verapamil also showed no distinction in the vasculature and are of little benefit in hypertension (*Pedersen 1978: Gross 1977*).

It has been suggested that hydralazine acted as a calcium antagonist or an inhibitor of phosphodiesterase (*Barron et al 1977; Andersson 1973*). However *Ablad (1963)* demonstrated that hydralazine only inhibited phosphodiesterase activity of vascular smooth muscle at high concentrations (100 µg/ml) whereas it induced relaxation at lower concentrations (0.1 to 10 µg/ml). In addition, under anaerobic conditions hydralazine paradoxically potentiated noradrenaline contractions, whereas papaverine inhibited noradrenaline responses. *Diamond (1979)* reported that hydralazine caused relaxation without lowering cytoplasmic calcium.

In the development of spontaneous hypertension in the rat, decreased prostaglandin catabolism in the kidney and lung preceded the onset of hypertension (*Pace-Asciak 1976; Leary et al 1977*). As a result circulating prostaglandin levels were elevated. These prostaglandins may have contributed to the hypertension. Other researchers have found that isolated vascular tissues from hypertensive rats had elevated levels of prostaglandins (*Rioux et al 1977; Limas and Limas 1977*). *Pace-Asciak et al (1978)* reported that aortic rings and homogenates from spontaneously hypertensive rats had an enhanced biosynthetic capability for prosta-

cyclin, a potent hypotensive agent (*Scholkens 1978*). Similar data have been reported by *Okuma et al (1979)*. It has been suggested that the increased prostacyclin production was an adaptive response attempting to lower the blood pressure. Not all blood vessels synthesized prostacyclin as their dominant prostaglandin (*Skidgel and Prince 1978; Gerritsen et al 1979*). The latter group found that the cerebral microvessels synthesized PGE2 as their dominant prostaglandin. This then suggests that microvessels in other vascular beds may produce amounts of prostaglandins different from that synthesized by large vessels, such as the aorta.

It has been shown that thromboxane synthetase inhibitors and prostaglandin antagonists inhibit platelet aggregation (*Fitzpatrick and Gorman 1978; Needleman et al 1977; Fredholm 1976*). Papaverine and diazoxide, both vasodilators, inhibited platelet aggregation (*Shtacher et al 1976; Glusa et al 1977; Greenwald et al 1978*). Papaverine has been shown to be a prostaglandin antagonist (*Levy 1973; 1974*) and has been used in the past to treat hypertension (*Zozulia 1976*), experimental cerebral arterial spasm (*Kuwayama et al 1972*), coronary constriction (*Parker et al 1977*), experimental myocardial ischemia (*Kisin 1978*) and angina (*Sangiorgi et al 1971*). Since vasoactive prostaglandin metabolites have been implicated in the platelet reaction, it is possible that the antihypertensive property of diazoxide and hydralazine may be due at least in part to their ability to interfere with prostaglandin action or biosynthesis. Because the abnormality in the hypertensive vessels persists in the isolated, synthetic media perfused vascular beds, any biochemical changes must reside within the smooth muscle cell or its surrounding tissues.

In the mesenteric vascular bed papaverine was a potent inhibitor of noradrenaline and potassium contractions. Papaverine appeared to be more specific (in a concentration defined manner) for noradrenaline responses. It inhibited these responses at lower concentrations and this appeared to be selective for an intracellular calcium pool, preventing its mobilization. At a concentraction which maximally inhibited noradrenaline responses, inhibition of potassium responses was just discernable. Different concentrations were presumably required to inhibit both pressor agents either because different substances were being antagonized or the same substance was being antagonized at different sites.

In aggreement with published data the dose response curve for papaverine was potentiated by partial inhibition of prostaglandin synthesis (with indomethacin) and antagonized by the addition of exogenous PGE2 to the perfusate. As before the inhibition of noradrenaline responses was seen at concentrations of papaverine which had minimal effect on potassium responses. It is unlikely that papaverine was behaving as a calcium antagonist, because verapamil inhibited both noradrenaline and potassium responses in this preparation over a similar concentration range. Papaverine did not inhibit prostaglandin biosynthesis (*Ahnfelt-Rønne and Magnussen 1978; Chignard and Vargaftig 1978*). The concentration range of papaverine found effective against noradrenaline responses was similar to plasma levels found in man (133 ng/ml - 592 ng/ml, *Garrett et al 1978*). It is unlikely that levels approximating those which inhibited potassium responses in this preparation are ever achieved during therapeutic management.

In preparations perfused with maximal concentrations of indomethacin and vascular responsiveness restored with exogenous PGE2, papaverine was still able to inhibit noradrenaline responses. At these concentrations of indomethacin (1.7×10^{-4} M) the phosphodiesterase(s) in the

vessels were probable maximally inhibited (*Flower 1974*). The mesenteric vascular bed was atonic thus only those factors which affect responses to pressor stimuli or elevate basal perfusion pressure were detected. Dipyridamole which also possesses phosphodiesterase inhibitory actions was ineffective in indomethacin plus PGE2 perfused preperations. This argues against the hypothesis that increases in intracellular cyclic AMP levels mediated the actions of papaverine.

The parallel nature of the papaverine dose response curves suggested that the interaction with the prostaglandins was of a competitive nature although it does not prove it.

In similar experiments with indomethacin plus PGE2, papaverine effectively inhibited potassium responses. Interestingly, the concentration of papaverine required was not changed by pretreatment of the preparations. This is in agreement with the view that papaverine inhibited noradrenaline and potassium responses by acting at different sites. In this experimental situation the only prostaglandin present in appreciable amounts was that added to the perfusate. The PGE2 added to the buffer was required for vascular reactivity, therefore two sites of action would have to be postulated to account for the observations made here. Further support for this was provided in those experiments in which noradrenaline responses were preferentially abolished with the thromboxane synthetase inhibitor, imidazole, and vascular reactivity restored with exogenous PGE2 (Fig. *83*) and those experiments in which responses were partially inhibited by either imidazole or dantrolene alone (Fig. *79*). As before noradrenaline responses were inhibited over the lower papaverine concentration range. The low concentrations of imidazole and dantrolene shifted the papaverine concentration responsive curves equally to the left (Fig. *79*).

These data can be interpreted to suggest that over the low concentration range papaverine was acting as a thromboxane A2 antagonist, thus preventing the mobilization of intracellular or loosely bound calcium, while at higher concentrations it prevented the influx of extracellular calcium through the plasma membrane. In support of this view is the recent report by *Broekaert and Godfraind (1979)* which showed that papaverine ($<10^{-5}$M) inhibited calcium evoked contractions by an action on intracellular sequestration. In depolarized aortic and mesenteric arteries which are absolutely dependent upon extracellular calcium, papaverine ($<10^{-5}$M) was ineffective. *Demesy-Waeldele and Stoclet (1975)* found that papaverine ($<3 \times 10^{-5}$ M) depressed rat aorta contractility directly, independent of its effects on calcium translocation. They also found that, unlike the inhibition caused by dibutyryl cyclic AMP papaverine's effect could not be reversed by the addition of excess calcium to the buffer.

In experiments presented here using the rat fundic strip, the contractures elicited by PGE2 were absolutely dependent on extracellular calcium. Low concentrations of papaverine did not inhibit PGE2 induced contractions wheras high concentractions abolished the contractures. The concentrations of papaverine required in the fundic strip were similar to those required to inhibit potassium responses in the mesenteric vasculature. High concentrations of papaverine have been shown to have an action similar to cadmium ions and verapamil in canine cerebral and peripheral arteries (*Hayashi and Toda 1977*). In this preparation verapamil inhibited both noradrenaline and potassium responses over a similar range of concentrations (see data in previous section).

Hydralazine, unlike papaverine did not inhibit potassium responses. It had a limited inhibitory action on noradrenaline contractions (Fig. *85*). Its action was not similar to that of indomethacin, papaverine or

verapamil, and seemed to be superficially similar to that of adenosine or dantrolene.

In preparations perfused with a submaximal amount of either indomethacin or imidazole, different effects were seen. In the indomethacin treated group, the levels of all prostaglandins were reduced, theoretically maintaining the ratios between the various prostaglandins. Indomethacin increased the maximum inhibition of noradrenaline pressor responses at all concentrations of hydralazine, with higher concentrations of hydralazine , having a greater effect. In contrast imidazole, dramatically enhanced the effects of low concentrations of hydralazine (1.6×10^{-6}M) while further increases in hydralazine concentrations had a minimal effect (Fig. *85*, *86*). These data were distinctly different from those obtained with any other agent used in this study. Thes concentrations of hydralazine are similar to those found by *Ablad (1963)* to lower blood pressure in man.

These significant different interactions with the cyclooxygenase and the thromboxane synthetase enzymes suggested that hydralazine was not a simple calcium or prostaglandin antagonist. The increased efficacy seen in the presence of these synthetase inhibitors do indicate that endogenous prostaglandin biosynthesis attenuates or modifies the actions of hydralazine.

Indomethacin, as shown earlier, inhibited basal prostaglandin biosynthesis and prevented the stimulation of biosynthesis by noradrenaline, whereas imidazole inhibited thromboxane synthesis and diverted the endoperoxides through other branches of the pathway (*Nijkamp et al 1977*). If the only action of hydralazine was the antagonism of TXA2 then it is difficult to explain the plateau seen in Fig.*85*. *Tai and Yuan (1978)* showed

that imidazole competes with the endoperoxide,PGH2, for the active site on TxA2 synthetase. If hydralazine both stimulated the production of endoperoxides and antagonized TxA2 actions, then this would explain the greater inhibi ory effect seen in experiments in which the cyclooxygenase was partially inhibited.

This hypothesis was tested by maximally inhibiting TxA2 synthetase with imidazole and restoring vascular responsiveness with PGE2, high concentrations of which can apparently activate TxA2 receptors. In this situation hydralazine inhibited noradrenaline pressor responses in a concentration related manner, with no evidence of a plateau similar to that seen in preperations perfused with hydralazine alone. This wasconsistent with the proposed hypothesis.

It has been shown by *McLean et al (1978)* and *Haegele et al (1978)* that hydralazine , its metabolites (hydralazine pyruvate hydrazone and hydralazine ketoglutarate hydrazone) and two derivatives (hydralazine acetone hydrazone and hydralazine butanone hydrazone) were active *invitro* in producing relaxation of noradrenaline and potassium induced contractures of rabbit aorta. They concluded that hydralazine and its hydrazone metabolites would contribute *in vivo* to the hypotensive effects of the parent molecule. These conclusions were not supported by the *in vivo* testing of the metabolite, hydralazine pyruvate hydrazone, which had no hypotensive effect in rats, although these animals were responsive to hydralazine itself. Because the original testing was done on large arteries an incorrect prediction had been made. *Worcel (1978)* had shown that hydralazine inhibited phenylephrine and serotonin induced induced contractions of distal segments of the caudal artery, wheras it was ineffective in the proximal portion (from normotensive animals). In vessels from

hypertensive animals hydralazine inhibited phenylephrine and serotonin contractions in both segments of the caudal artery. This emphasizes that he large arteries from normotensive animals are not suitable for pharmacological studies with vasodilators.

As discussed earlier calcium appears th play a crucial, perhaps pivotal role in determining vascular reactivity. Calcium in the extracellular fluid can stimulate prostaglandin biosynthesis (*Diegel and Coburn 1979, Coburn et al 1977*). Therefore by lowering the concentration of calcium ions in the buffer, a reduction in the basal synthesis of some prostaglandins may be realized. This would be analogous to using an inhibitor of prostaglandin synthesis. In preparations perfused with 1×10^{-6} M calcium containing buffer, in a concentration dependent manner, the dose response curve obtained paralleled that obtained in the presence of maximally effective concentrations of imidazole plus PGE2.

These data support the view that hydralazine inhibited the release of intracellular calcium stores and at the same time stimulated prostaglandin biosynthesis. The responses seen in the presence of submaximal concentrations of indomethacin are qualitatively similar to those obtained using either imidazole plus PGE2 perfused or low calcium perfused preperations suggested that extracellular calcium was necessary for hydralazine stimulation of prostaglandin biosynthesis. Hydralazine unlike papaverine did not inhibit PGE2 induced contractures of the rat fundic strip, demonstrating that its action was perhaps at a different site. Recently *Bennett et al (1978)* reported that SC19220 antagonized PGE2 but not endoperoxide (PGH2) analogue induced contractures of the rat fundic strip. They concluded that several receptor types were present in this prep.ration.

Recently *Haeusler and Gerald (1978)* reported that hydralazine increased prostaglandin E like material in canine venous blood. The data obtained *in vitro*, using the isolated perfused mesenteric vascular bed, showed a similar increase in PGE2 biosynthesis. The levels of PGI2 fell back to prehydralazine concentrations whereas the release of PGE2 remained elevated.

The precise mechanism whereby hydralazine increased PGE2 biosynthesis is not presently known. However it appears to be unique in that it antagonized the actions of endogenously added prostaglandins while either stimulating the general biosynthesis of endoperoxides or diverting the existing endoperoxides to PGE2. *In vitro and in vivo* , hydralazine has been shown to be a thromboxane synthetase inhibitor (*Greenwald et al 1978, 1979*). It has previously been shown by *Nijkamp et al (1977)* that thromboxane synthetase inhibitors diverted the endoperoxides , increasing the synthesis of the other prostaglandins. The increase in PGE2 levels seen in the presence of hydralazine was most likely due to a similar diversion.

Hydralazine administered to young hypertensive rats prevented the manifestation of the hypertensive state but not the development of the altered calcium kinetics seen in hypertensive vessels (*Zsoter and Wolchinsky 1978*). This argued against the hypothesis that hydralazine acted by altering calcium fluxes (*McLean et al 1978*).

It has been suggested that PGI2 released from lungs could function as a circulating vasodilator and contribute to the regulation of blood vessel tone and blood pressure (*Dusting et al 1978*). In studies using infusions of PGI2 *Pace-Asciak et al (1978)* and *Scholkens (1978)* have shown that PGI2 had more potent hypotensive properties than PGE2 in the normal and spontaneously hypertensive rat. In a study using normotensive cats *Smith et al (1978)* found that PGI2 was the only consistent vasodilator.

The data presented here and that of *Haeusler and Gerald (1978)* does not support the hypothesis that hydralazine acts by stimulating PGI2 biosynthesis, which then causes the decrease in blood pressure. In the rat mesenteric vascular bed hydralazine stimulated PGE2 but not PGI2 biosynthesis.

The results presented here suggest that hydralazine acts as a hypotensive agent by both antagonizing the action of a prostaglandin(s) while at the same time inhibiting the biosynthesis of perhaps TxA2. Hydralazine did not behave like the classical smooth muscle relaxant, papaverine and appeared to be quite unique in its actions. Indeed an understanding of its mechanism of action could lead to the systematic development of more efficacious agents since this drug has been unchallenged for over thirty years (*Koch-Weser 1978*) as an antihypertensive.

The value of the mesenteric vascular bed as an experimental system in the development and testing of antihypertensive agent was shown in a recent report by *Doxey (1978)* describing the antihypertensive properties of tolmesoxide *in vivo*. The data obtained *in vivo* correlated with the predictions made from *in vitro* studies in the mesenteric vascular bed, obtained from normotensive animals.

SUMMARY

Prostaglandins (PGs) were tested over the concentration range of 10^{-13} to 10^{-6} M in the perfused rat mesenteric vascular bed (MVB). Nondepolarizing stimuli such as noradrenaline (NA) recruit primarily intracellular or loosely bound calcium ions (Ca^{++}) whereas depolarizing stimuli like potassium ions (K^{+}) recruit primarily extracellular Ca^{++} (ECF Ca^{++}). PGE2, PGF2α and PGA2 enhanced NA and K^{+} phasic contractions. Low concentrations of PGE1 and PGA1 potentiated, although high concentrations inhibited NA responses. None of PGE1, TxB2, 6-keto-PGF1α, PGD2 or PGB2 influenced K^{+} contractions. 16,16-dimethyl PGE2 had actions that were a mixture of those of PGE2, PGE1 and PGF2α. Indomethacin (INDO) blocked pressor resonses to all stimuli (NA, K^{+}, angiotensin (ANGIO), vasopressin, and Ca^{++}) whereas the thromboxane (Tx) synthetase inhibitors (imidazole (IMID), 1-benzyl-imidazole (1-b-IMID), nicotinic acid, and 9,11-azo-prosta-5,13-dienoic acid (AZO) inhibited responses to only NA and ANGIO. The effects of both the cyclooxygenase and Tx synthetase inhibitors were reversed by PGE2 at ng/ml concentrations. Analyses of MVB perfusate and incubation fluid of mesenteric arterial and arteriole rings by direct RIA and combined TLC-RIA procedures, with authentic standards, identified immunoreactive PGE2, PGF2α, 6-keto-PGF1α and TxB2. INDO inhibited the synthesis of all PGs whereas IMID and AZO inhibited only TxB2 biosynthesis. Adenosine inhibited NA but not K^{+} contractions, acting at a site distinct from the classical "adenosine" receptor as defined by structure activity relationships. In both isolated lymphocytes and MVB, adenosine and its analogues were shown to be PG antagonists. In the MVB neither cyclic AMP nor dibutyryl cyclic AMP at concentrations up to 10^{-2} M had any inhibitory

effect. Dipyridamole (DIPYR) preferentially inhibited NA and ANGIO responses, howeverits actions were distinctly different from adenosine, other adenosine uptake blockers or phosphodiesterase inhibitors but similar to those of the Tx synthetase inhibitors. The Ca^{++} ionophore A23187 abolished NA responses by apparently depleting intracellular activator Ca^{++} stores since K^{+} responses were actually enhanced by high concentrations of A23187. Verapamil, a Ca^{++} antagonist, inhibited pressor responses to all stimuli. Its effect was enhanced by low concentrations of INDO, IMID and by a lowering of ECF Ca^{++}. Dantrolene, INDO and IMID prevented ECF Ca^{++} (10^{-6} to 10^{-5} M) stimulation of NA responses but had no effect on the inhibitory effects of higher Ca^{++} concentrations (10^{-5} to 10^{-2} M). Hydralazine (HYD) preferentially inhibited NA and ANGIO responses. Unlike IMID, and DIPYR, HYD inhibited NA responses in INDO + PGE2 perfused preparations an action consistent with antagonism of PGE2 action. In normal preparations the inhibitory effect of HYD plateaued suggesting stimulation of PG biosynthesis. RIA analysis showed that HYD stimulated PGE2 but not 6-keto-PGF1α biosynthesis. In the rat fundic strip HYD did not antagonize the direct PGE2 contraction though it inhibited PGE2 actions in the MVB. HYD therefore apparently acted via a complex interaction with PGs involving their biosynthesis and their action(s) at the receptor level. Vascular reactivity in the MVB was dependent on PG(s) action(s) at sites proximal to the contractile proteins. Various agents acting at the level of PG biosynthesis or action interfered with pressor responses. The possibility therefore existed that PGs and Tx synthesized within the vascular wall play a role in the physiological and pathophysiological responses of the microcirculation.

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B.Sc.	University of Western Ontario, London, Ontario	1974
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AWARDS

Eldon Boyd Fellowship in Pharmacology, Queen's University.	1975
RODA Summer Scholarship, Health and Welfare Canada	1975
Pre-Doctoral Fellowship from the Clinical Research Institute of Montreal.	1976-79

RESEARCH TRAINING

Smooth muscle pharmacology; General instrumentation; Isotopes; Drug metabolism; Bioassay techniques; Chromatography: TLG paper, gas; Physiological recording; Behavioral pharmacology; Enzyme kinetics; Platelet studies; Radioimmunoassay techniques; cAMP, cGMP, PGE2, PGF2α, TXB2, PGI2; Receptor binding studies (crude homogenates); Receptor pharmacology; Structure activity relationships; Protein assays.

TEACHING EXPERIENCE

Laboratory demonstrator, Biology, University of Western Ontario	1974
Laboratory demonstrator/instructor, pharmacology, Queen's Univ.	1975
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Laboratory demonstrator, physiology, McGill University	1977
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SOCIETY MEMBERSHIP

- The Pharmacological Society of Canada
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PUBLICATIONS

1. Ally, A.I. and K. Nakatsu. Adenosine inhibition of isolated rabbit ileum and antagonism by theophylline. J Pharmacol Exp Ther 199: 208-215, 1976.

2. Ally, A.I., Horrobin, D.F., Karmali, R.A., Morgan, R.O., Karmazyn, M., Manku, M.S. Adenosine as a natural prostaglandin antagonist in vascular smooth muscle. Prostaglandins 14: 109-115, 1977.

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6. Ally, A.I., Manku, M.S., Horrobin, D.F., Karmali, R.A., Morgan, R.O., Karmazyn, M. Thromboxane A2 as a natural ligand for benzodiazepine receptors. Neurosci Lett 7: 31-34, 1978.

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CLAIMS OF ORIGINALITY

1. Utilizing stimuli which recruit either primarily ECF or ICF calcium the effects of PGs on vascular reactivity in mesenteric microvessels were documented. It was shown that PGs acted at site(s) which were extremely discriminating between PG molecules. This was exemplified by the effects of 16,16-diMePGE2 which appeared to be a composite of those of PGE2, PGE1 and PGF2α.

2. The characterization of the dependency of vascular reactivity on endogenous PG biosynthesis and the demonstration that noradrenaline responses appeared to be dependent on a TxA2 biosynthetic capability.

3. The identification of TxB2 by combined RIA-TLC procedures in the mesenteric vasculature which was sensitive to known cyclooxygenase and thromboxane synthetase inhibitors. While this thesis was in preparation *Maurier et al (1979)* reported the demonstration of (TxA2) TxB2 biosynthesis by bovine cerebral arterioles.

4. The identification and demonstration that adenosine and its derivatives acted as prostaglandin antagonists in both the mesenteric vasculature and in isolated lymphocytes. The adenosine site of action was shown to be different from the classical "adenosine" receptor.

5. The prediction from data obtained in this system that dipyridamole was a thromboxane synthetase inhibitor (*Ally et al 1977*). Recently *Best et al (1979)* and *Greenwald et al (1978)* confirmed this using platelets as a thromboxane generating system.

6. The first demonstration of an effect of dantrolene on vascular smooth muscle contractility. From data obtained here, it appeared that dantrolene was a thromboxane antagonist (*Ally et al 1978*).

7. The elucidation of the mechanism of action of the antihypertensive agent, hydralazine, in the mesenteric vasculature. Although this compound stimulated PGE2 biosynthesis, its primary action appeared to be antagonism of protaglandin actions. Hydralazine did not stimulate the biosynthesis of the vasodilatory prostaglandin, PGI2.