Investigation into subtypes of the prostacyclin and prostaglandin E receptors present in smooth muscle.

by

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Statement in terms of PhD Regulation 2.4.15 of the Postgraduate Regulations of the University of Edinburgh.

I declare that this thesis was composed by myself and that all the experimental work described herein was performed by myself with the exception of the TP receptor radioligand binding studies in chapter 5 which were carried out in collaboration with Dr R.L. Jones.

R.L. Jones

R.A Lawrence

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PUBLICATIONS

R.A LAWRENCE & R.L JONES (1988). Prostacyclin analogues contract the guinea-pig ileum by more than one mechanism. *Br. J. Pharmacol.* **94**, 403P.

R.A LAWRENCE & R.L JONES (1988). Thromboxane receptor agonists and antagonists: radioligand displacement and pharmacological activity on human platelets. *Br. J . Pharmacol.* **95**, 680P.

R.A LAWRENCE & R.L JONES (1988). AH 6809 distinguishes between two types of direct contractile action for prostanoids on the guinea-pig ileum longtitudinal muscle. *Br. J. Pharmacol.* **96**, 63P.

R.A ARMSTRONG, R.A LAWRENCE, R.L JONES, N.H WILSON & A. COLLIER (1989). Functional and ligand binding studies suggest heterogeneity of prostacyclin receptors. *B. J . Pharmacol.* **97**, 657-668.

R.L JONES, N.H WILSON & R.A LAWRENCE (1989). EP171: a high affinity thromboxane mimetic whose actions are slowly reversed by receptor blockade. *Br. J. Pharmacol.* **96**, 875-887.

R.A LAWRENCE, R.L. JONES & N.H. WILSON (1989). Relaxant properties of prostaglandin E analogues on rabbit jugular vein. *Br J. Pharmacol. (in press)* .

CONVENTIONS AND NOMENCLATURE

Trivial names of prostaglandins and their analogues have been used throughout this thesis. The systematic names for some of the C20 prostanoids and analogues referred to in this thesis are shown below and those not listed may be deduced from these examples.

Trivial name	*Systematic name*
Prostaglandin H_2	15(S)-Hydroxy-9α,11α-peroxidoprosta-5cis, 13 trans dienoic acid.
Prostaglandin I_2	11α,15(S)-Dihydroxy-9-deoxy-6,9-epoxy-5Z,13-trans-prostadienoic acid.
11,9-epoxymethano PGH_2	15(S) Hydroxy-11α,9α–(epoxymethano) prosta-5 cis, 13 trans dienoic acid.
EP 171	ω-tetranor-16-*p*-flurophenoxy-9α,11α-oxa-10a-homo PGH_2.
STA_2	9,11-Epithio-11,12-methano TXA_2.

In drawing chemical structures stereochemistry is not implied but a thickened or dotted line denotes a substituent located above or below the plane of the paper respectively.

ABSTRACT

This thesis describes attempts to characterize prostacyclin (IP) and prostaglandin E (EP) receptors in smooth muscle preparations using agonist potency ranking and susceptibility to receptor blockade.

Platelet aggregation is inhibited by prostacyclin analogues through activation of IP-receptors present on the plasma membrane. This potentially useful anti-thrombotic action is compromised by powerful vasodilator activity. The relaxant activity of cicaprost, iloprost, carbacyclin and EP157 (a novel prostaglandin endoperoxide analogue which mimics prostacyclin in the human platelet) was measured on isolated arterial rings from man, pig, rabbit and dog: Potency decreased in the order given above and there was little evidence for significant differences between the IP receptors in the different preparations. In man the agonist potency at vascular IP sites correlates well with that of the platelet thus presenting little hope for separation of the two actions.

In addition, the IP receptor present in the guinea-pig ileum has been investigated. This receptor is of particular interest as it has been shown to mediate longitudinal muscle contraction via the release of excitatory neurotransmitters from the myenteric plexus.

Only the action of cicaprost was abolished by morphine and tetrodotoxin: PGI_2, iloprost and carbacyclin were only partially inhibited and the residual contraction in each case was abolished by the EP_1-receptor antagonist, AH6809. These results suggest a high degree of selectivity for cicaprost. However, the analysis is complicated by the discovery of an IP-receptor which inhibits longitudinal muscle contraction (histamine in the presence of AH6809). Antagonist investigations suggest that the excitatory transmitters released by IP-receptor activation are Acetylcholine and Substance P.

Recently, the existence of three subtypes of the PGE receptor have been proposed and pharmacological / chemical efforts have centred on the characterization of these receptors and identification of their physiological actions. A detailed study has been carried out comparing the actions of eleven PGE analogues on three tissues containing EP_3-receptors.

(1) Guinea-pig ileum: In the absence of enteric nerve function this tissue has previously been defined as EP_1- selective. However, the present studies suggest the presence of a second receptor mediating contraction and this has some similarities to the EP_3-receptor found in the guinea-pig vas deferens. In particular sulprostone and MB28767 are highly active in the presence of AH6809.

(2) Chick ileum: Literature studies suggest the presence of an EP_3-receptor mediating contraction. The results obtained confirm this, however, detailed study has revealed differences between agonist activity in this tissue and the guinea-pig vas deferens, the most obvious being the low maximum (~ 50% of that of PGE_2) of the potent EP_3 agonist, sulprostone.

(2) Rabbit jugular vein: This tissue has recently been shown to contain PGD_2 relaxant receptors however, the action of PGE_2 has not yet been studied. Investigations have shown that the preparation contains a

highly sensitive EP_2 receptor mediating relaxation (IC_{50}<1nM). However, the PGE_1 analogue, butaprost, appears to be considerably less active than expected from data obtained in other preparations known to contain EP_2-receptors (guinea-pig trachea and cat trachea).

The rabbit jugular vein has previously been shown to contain a contractile thromboxane (TP) receptor which is highly sensitive to the thromboxane mimetic, U46619 (EC_{50} ~ 3nM). A number of PGE analogues have potent thromboxane-like (TP-receptor agonist) actions thus the above experiments were carried out in the presence of a TP-receptor antagonist. Characterization of this receptor suggests that it has low affinity for TP-receptor antagonists in common with other rabbit tissues.

Chapter 1

General Introduction

GENERAL INTRODUCTION

The first prostaglandin metabolites of arachidonic acid, discovered more than twenty years ago, were the stable species PGE_2 and $PGF_{2\alpha}$ (Bergstrom and Sjovall, 1960 a and b). Subsequently, Bergstrom (1966) announced the structures of several naturally occurring prostaglandins. They have since been shown to arise from unstable intermediates, PGG_2 and PGH_2, the prostaglandin endoperoxides (Hamberg et al, 1974). PGG_2 and PGH_2 also undergo enzymatic transformation to unstable products, thromboxane A_2 (TXA_2) (Piper and Vane, 1969; Hamberg and Samuelsson, 1974) and prostacyclin (PGI_2) (Moncada et al, 1976; Johnson et al, 1976).

Most mammalian cells can release prostanoids in response to various stimuli including hormones, antigen challenge, thrombin and collagen and mechanical trauma. They are synthesised from twenty carbon polyunsaturated fatty acid constituents of membrane phospholipids. Thus, dihomo-γ-linoleic acid, arachidonic acid and eicosopentaenoic acid form the 1-, 2- and 3- series prostanoids respectively (Samuelsson et al, 1978; Moncada et al, 1980; Smith et al, 1985). Arachidonic acid is the most abundant of these three precursors thus the 2- series prostaglandins are formed almost exclusively in most mammalian tissues (Moncada et al, 1980). Before prostanoid synthesis can take place free, unesterified arachidonic acid must be released. Release (see Figure 1.1) is catalysed by the phospholipase enzyme phospholipase A_2 (PLA_2) (Flower and Blackwell, 1976). Although this pathway is thought to be the most important (Mahadevappa and Holub, 1986), there is evidence that in the human platelet, phospholipase C (PLC) may release 1,2-diacylglycerol from which arachidonic acid is liberated by diglyceridelipase (Smith, , 1981).

After arachidonic acid is released from tissue stores, conversion into prostaglandins and thromboxanes occurs in two steps (see Fig 1.2). The first step is the formation of the prostaglandin endoperoxides which is catalysed by prostaglandin endoperoxide synthetase (Needleman et al, 1986). The cyclooxygenase activity of the enzyme catalyses the formation of a 15-hydroperoxy compound, PGG_2, while the peroxidase activity

Figure 1.1: Schematic representation of two enzymatic pathways triggering arachidonic acid release from membrane phospholipids

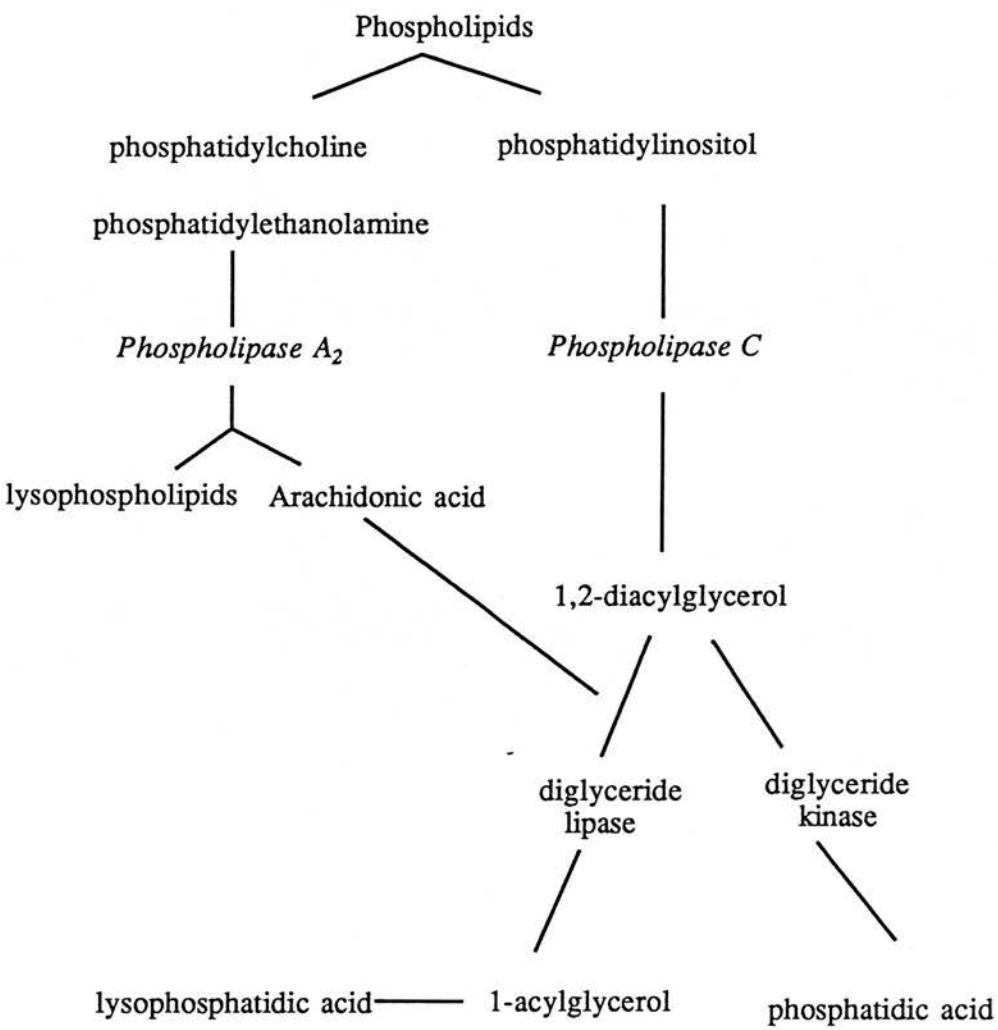
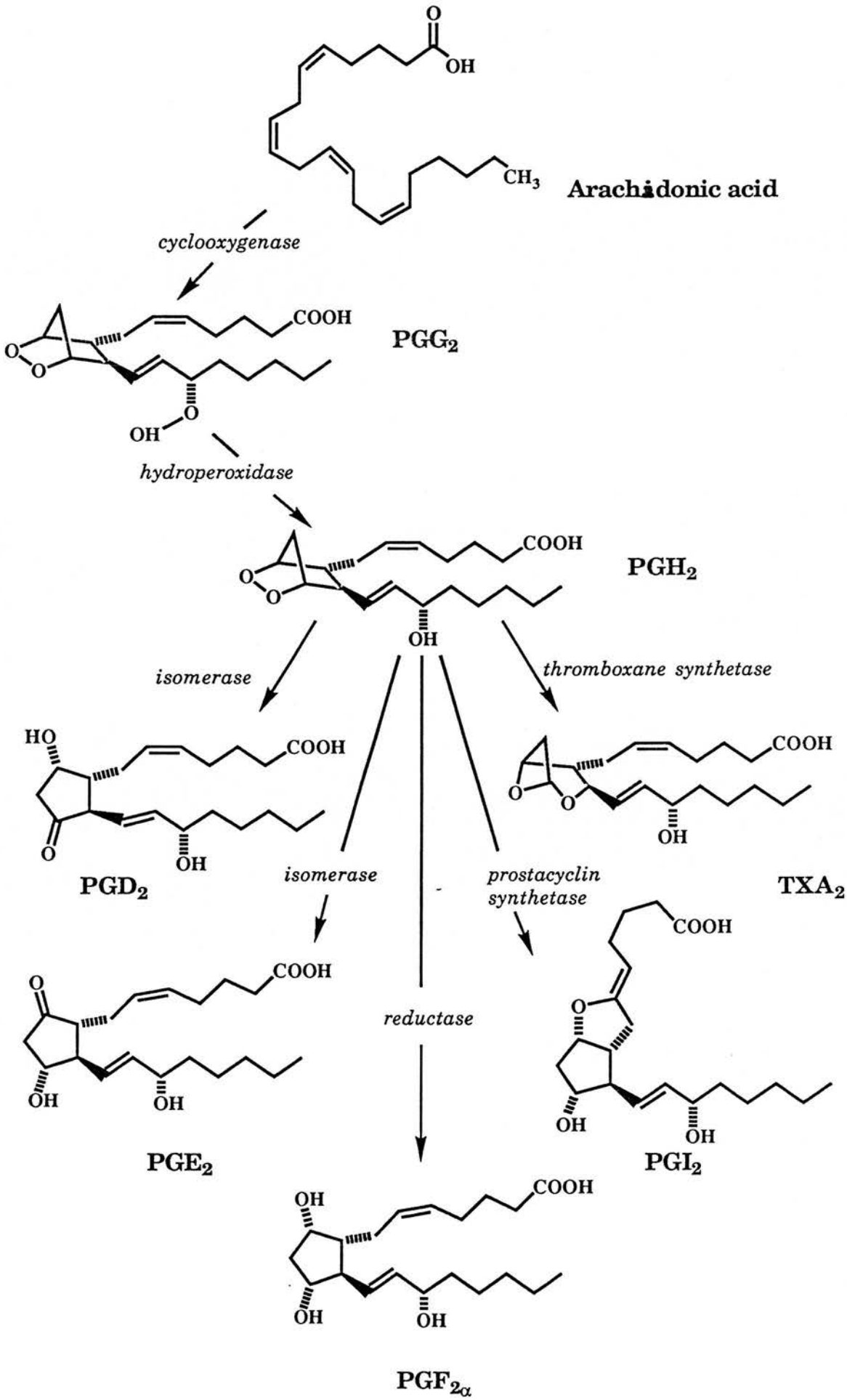


Figure 1.2: Diagram showing the synthesis of the 5 natural prostanoids from Arachidonic acid.


Arachidonic acid
cyclooxygenase
PGG2
hydroperoxidase
PGH2
isomerase
thromboxane synthetase
PGD2
TXA2
isomerase
prostacyclin synthetase
reductase
PGE2
PGI2
PGF2α

reduces the hydroperoxy group to a hydroxy, giving PGH_2. The second step involves enzymes which are tissue specific and result in the formation of products characteristic to a tissue such as thromboxane in platelets (Ho et al, 1976; Ullrich and Haurand, 1976) and prostacyclin in vascular endothelial cells (Gryglewski et al, 1976; DeWitt and Smith, 1983).

Nonsteroidal anti-inflammatory drugs, such as aspirin and indomethacin, inhibit cyclooxygenase (Vane, 1971), and will therefore prevent the formation of prostaglandin endoperoxides resulting in decreased formation of the final products, the prostaglandins and thromboxanes.

Since the discovery of prostaglandins they have been demonstrated to be involved in a wide range of physiological systems including: contraction/relaxation of smooth muscle, control of neurotransmitter release, aggregation/antiaggregation of blood platelets, control of renal function, structural and functional changes in the ovary, function of the eye and gastrointestinal absorption and secretion. They have also been implicated in the pathophysiology of a number of conditions such as thrombosis, inflammation, asthma, cancer, pyrexia, migraine and renal disease (see Coleman et al, 1990).

Horton (1969) was the first to suggest that discrete receptors for prostaglandins may exist which could be distinguished from those of other agonists by selective antagonists: their action was not blocked by atropine, methysergide, mepyramine, propranolol, phenoxybenzamine and hexamethonium. In addition, he suggested that "from the qualitative and quantitative differences in the biological properties of various natural prostaglandin families several different prostaglandin receptors may exist". PGI_2 and PGD_2 differ from the other 2-series prostanoids in that they potently inhibit platelet aggregation. Studies by Miller and Gorman (1979) and Whittle et al (1978) provided evidence that these prostanoids act via their own distinct receptors: (1) exposure of platelets to high concentrations of PGI_2 blocks the effects of subsequent application of PGI_2 but not PGD_2 and vice versa (2) the antiaggregatory action of PGD_2, but not that of PGI_2, is blocked by the phenyl

Table 1.1: Classification of prostanoid receptors in some isolated smooth muscle preparations. Kennedy et al, 1982.

PREPARATION	GROUP	RANK ORDER OF POTENCY
Guinea-pig ileum	1	$E_2 > I_2 = F_{2\alpha} > D_2 = U\ 46619$
Guinea-pig fundus		$E_2 > I_2 \geq F_{2\alpha} > D_2 = U\ 46619$
Dog fundus		$E_2 > F_{2\alpha} > I_2 > D_2 > U\ 46619$
Chick ileum		$E_2 > I_2 > F_{2\alpha} > D_2 = U\ 46619$
Cat trachea		$E_2 > I_2 > F_{2\alpha} > D_2 = U\ 46619$
Dog iris	11	$F_{2\alpha} > D_2 > U\ 46619 > E_2 > I_2$
Cat iris		$F_{2\alpha} > D_2 > E_2 = U\ 46619 > I_2$
Rat aorta	111	$U\ 46619 >> F_{2\alpha} > E_2 = D_2 = I_2$
Guinea-pig lung		$U\ 46619 >> D_2 > I_2 = F_{2\alpha} > E_2$
Dog saphenous vein		$U\ 46619 >> F_{2\alpha} > E_2 > I_2 > D_2$

phosphonate antagonist, N-0164. Kennedy et al (1982) studied the action of PGE_2, $PGF_{2\alpha}$, U 46619 (a thromboxane mimetic), PGI_2 and PGD_2 on a wide range of preparations. The results showed that the preparations could be divided into three groups on the basis of striking similarities in the rank order of potency of the prostanoid. The authors proposed that the very marked differences between the groups reflected the existence of three distinct classes of prostanoid receptor (see Table 1.1) Thus, they postulated that distinct receptors existed for each class of natural prostanoids: PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TXA_2 corresponded to DP, EP, FP, IP and TP receptors respectively.

This thesis is chiefly concerned with the further characterisation of IP, EP and TP receptors present in various smooth muscle preparations, a range of agonists and antagonists has been used.

A range of basic pharmacological techniques have been employed to classify these receptors.

The law of mass action is used to describe the binding of drugs to receptors:

$$\frac{[AR]}{R_t} = \frac{[A]}{[A] + K_A}$$

where R_t = total concentration of receptors, [AR] = concentration of drug receptor complex, K_A = equilibrium dissociation constant for the receptor.

Stephenson (1956) introduced the idea that the response to agonists was not necessarily directly proportional to the receptor occupancy. He introduced the concept of stimulus (S) which assumed that the response was some function of the stimulus.

$$\frac{E_A}{E_M} = f(S)$$

E_A = Response to a given concentration of A, E_M = Tissue maximal response.

In addition, the parameter relating stimulus to occupancy was termed 'efficacy', (e):

$$\frac{E_A}{E_M} = f\left(\frac{e\,[A]}{[A] + K_A}\right)$$

In this definition efficacy was a drug *and* tissue dependent term. The model was later modified by Furchgott (1966) who defined the term 'intrinsic efficacy', (E):

$$e = E\ [R_t]$$

In these terms intrinsic efficacy was strictly a drug related parameter constant for given drug-receptor pairs across species and tissues. Hence,

$$\frac{E_A}{E_M} = f\left(E\ \frac{[R_t]\ [A]}{[A] + K_A}\right)$$

Thus, the tissue related factors are (1) f, the function relating stimulus and response and (2) R_t, the total receptor concentration. And receptor related parameters are (1) K_A, the equilibrium dissociation constant of the drug for the receptor and (2) E, intrinsic efficacy.

If one assumes that K_A's for full agonists significantly exceed the concentration required for response (K_A < [A]), then stimulus can be given by $S = E\ [R_t]\ [A] / K_A$. Thus, in each individual tissue:

$$S_1 = \frac{E_1\ [A_1]}{K_{A1}} \quad \text{and} \quad S_2 = \frac{E_2\ [A_2]}{K_{A2}}$$

The potency ratio or equi-effective molar ratio (EMR) of these two full agonists in producing equal responses ($S_1 = S_2$) is:

$$EMR = \frac{[A_1]}{[A_2]} = \frac{E_2\ [K_{A1}]}{E_1\ [K_{A2}]}$$

This ratio reflects only the drug-receptor parameters, E and K_A, and is independent of tissue factors. It can thus be used in the classification of receptors.

It is notable that differences in receptor coupling may cause differences in the position of the log concentration-response curves of full agonists (those whose intrinsic efficacy is sufficient to produce a maximal response) but not partial agonists (those with lower intrinsic efficacy). Instead differences in receptor coupling will produce changes in the

maximal response of partial agonists but little change in the position of the log concentration-response curve. Thus, in two different tissues with identical receptors but different stimulus-response coupling a full and partial agonist may have different potency ratios.

The potency of a competitive antagonist depends upon its equilibrium dissociation constant (K_B) for the receptor. This is a chemical term which is independent of receptor function, location and animal species. Schild (1949) developed the pA scale in which pA_2 defines the negative logarithm of the molar concentration of antagonist which produces a two fold shift to the right of a concentration response curve. Schild also derived a useful equation to calculate the K_B of a competitive antagonist:

$$\text{Log}\,(dr\text{-}1) = n\text{Log}\,[B] - \text{Log}\,K_B$$

dr refers to the ratio of equi-active concentrations of agonists in the presence and absence of antagonist, [B]. This equation predicts a linear regression (Schild plot) with a slope of unity when a simple competitive antagonist binds to a homogeneous population of receptors. The resulting K_B under these conditions is a constant which reflects only antagonist receptor interaction and is independent of the agonist used. It is, therefore, useful in receptor classification.

Chapter 2

Experimental Methods

CHEMICALS AND MATERIALS

IP receptor studies

PGI_2	Schering, A.G Berlin.
cicaprost	"
iloprost	"
carbacyclin	Wellcome, U.K
MMM-I-135	Prof. J. Fried, University of Chicago.

EP receptor studies

16,16-dimethyl PGE_2	Upjohn Diagnostics, U.S.A
ICI 80205	ICI Pharmaceuticals Division
ICI 81008	"
17-phenyl PGE_2 -ω-trinor	Cayman Chemicals, U.S.A.
MB 28767	May and Baker, U.K Ltd.
sulprostone	Shering AG, Berlin
AH 6809	Glaxo U.K, Ltd.
oxoprostol	May & Baker, U.K. Ltd.
misoprostol	Searle Ltd.
(±) 11-deoxy PGE_1	Dr P. Crabbe, INSERM, Grenoble.
(nat) 11-deoxy PGE_1	Cayman Chemicals, U.S.A.
11-deoxy-PGE_2-1-alcohol	Synthesised from nor PGA_2 in this Department.
butaprost	Miles Laborotories

TP receptor studies

[^{125}I]-PTA-OH (75 TBq mmol^{-1})	Amersham International, U.K
STA_2	ONO Ltd, Japan
ONO 11120	"
GR 32191	Glaxo, U.K
U 46619	Upjohn Diagnostics U.S.A

Others

substance P	Cambridge Research Chemicals
SP150	Fering, Sweden
hyoscine	Sigma Chemicl Co., U.K.
mepyramine	"
phenoxybenzamine	"
propranolol	"
methysergide	"
indomethacin	"

All EP compounds were prepared by chemical synthesis in this department by N.H Wilson, C. Marr and G.M Muir.

Stresnil (Azaperone, 40mg/ml) Janssen

SOLUTIONS

Acid-Citrate dextrose (ACD) - (made up to 120 mls distilled water)

3 g	D-glucose	B.D.H Chemicals
2 g	Disodium Hydrogen citrate	"

Krebs (made up to 5 litres distilled water)

10.5 g	Glucose	"
10.5 g	Sodium Hydrogen Carbonate	"
34.5 g	Sodium Chloride	"
17.5 ml	Potassium Chloride (10%)	"
7 ml	Magnesium Sulphate (10%)	"
8 ml	Potassium Dihydrogen Orthophosphate (10%)	
12.6 ml	Calcium Chloride (1 molar)	"

Tyrodes (made up to 5 litres distilled water)

5 g	Glucose	"
5 g	Sodium Hydrogen Carbonate	"
40 g	Sodium Chloride	"
10 ml	Potassium Chloride (10%)	"
13 ml	Magnesium Sulphate (10%)	"
2.5 ml	Sodium Dihydrogen Phosphate (10%)	"
9 ml	Calcium Chloride (molar)	"

Tris Buffer, pH 7.4 (made up to 1 litre distilled water)

6.06 g Tris taken to pH7.4 with concentrated HCl.

METHODS

ISOLATED SMOOTH MUSCLE PREPARATIONS

Guinea-pig ileum

Male guinea-pigs (300-500 g) were killed by a blow to the head followed by exsanguination. The terminal portion of the ileum was excised after discarding the 8 - 10 cm portion nearest the ileo-caecal junction. The tissue was cleaned and kept in Tyrodes solution of the following composition (mM): NaCl 136, KCl 2.7, $CaCl_2$ 1.4, $MgCl_2$ 0.49, NaH_2PO_4 0.32, $NaHCO_3$ 12 and glucose 5. Segments of ~2 cm were mounted vertically in 10 ml organ baths under 0.5 - 0.75 g tension. Changes in tension were measured by means of a Grass FT03 isometric transducer connected to a Grass polygraph recorder. The bath solution was bubbled with 95% O_2 and 5% CO_2 and maintained at 37°C. Bathing solution was changed by upward displacement and overflow. The tissues were allowed to equilibrate for ~1 hr prior to testing with a dose of 14 nM PGE_2.

Concentration-response curves to all agonists were non-cumulative and drugs were added in 1, 3, 10, 30 or 1, 5, 10, 50 sequence. The preparations were exposed to doses of drug for 30-60 seconds and a minimum of 15 minutes was allowed between additions. Responses were calculated as a % of the acetylcholine maximum obtained in each preparation at the end of the experiment.

Effect of inhibitors

(1) *Against single doses*: Two control responses to the agonist were established, the tissue was then exposed to the inhibitor 20 minutes prior to addition of a third dose of agonist. The effect of the antagonist was then expressed as a % inhibition of the control response (mean value) and the % inhibition obtained from single preparations from 5 different animals averaged and s.e.m (standard error of the mean) calculated.

The differences in response levels before and after exposure to inhibitor were tested for significance using an unpaired Student's t-test.

(2) *Against concentration-response curves:* Concentration-response curves to any one agonist were established simultaneously in 2 preparations from the same animal, one of the tissues being bathed in Tyrodes solution containing the blocker added 20 minutes prior to the start of agonist addition. Dose-ratio was calculated as follows: EC_{25} in the presence of inhibitor / EC_{25} in the absence of inhibitor. The ratios obtained from experiments in 5 different animals were averaged and s.e.m calculated. pD_{25} values (- log EC_{25}) in the presence and absence of the inhibitor obtained from single preparations were calculated and the difference tested for significance using an unpaired Student's t-test.

Inhibition of histamine - induced contractions

Preparations were bathed in Tyrodes solution containing 1 μM morphine and 2 μM AH6809. The tissue maximum was established initially with 1.5 μM histamine. Subsequently, each tissue was exposed to a dose of histamine producing a contraction of ~60% of the maximum at 2 minute intervals. Inhibitors were added immediately after wash-out of histamine. The effect of the inhibitor was calculated as a % inhibition of the control response to histamine.

Chick ileum

5 - 20 day old chicks were killed by decapitation. The dissection and experimental procedure was identical to that used for the guinea-pig ileum. The tissues were allowed to equilibrate for ~1 hr prior to testing with a dose of 14 nM PGE_2.

Concentration-response curves to all agonists were non-cumulative and drugs were added in 1, 3,10, 30 or 1, 5, 10, 50 sequence. The preparations were exposed to doses of drug for 30-60 seconds and a minimum of 15 minutes was allowed between additions. Responses are calculated as a % of the acetylcholine maximum obtained in each preparation at the end of the experiment.

A concentration-response curve for PGE_2 was carried out on one preparation from each animal. The other preparations were exposed to various test prostanoids and EMR values calculated in the following way: EC_{25} of the test prostanoid / EC_{25} of PGE_2 in preparations from the same animal. EMR values from preparations from 5 different animals were averaged and the s.e.m calculated. Similarly pD_{25} values for each agonist were calculated averaged and the s.e.m calculated.

Effect of 'inhibitors '(AH6809, sulprostone and 'cocktail') : A cumulative concentration-response curve for PGE_2 was carried out on one preparation. Following wash-out the 'inhibitor' was added and 10-20 minutes later a second concentration-response curve to PGE_2 was obtained. Control curves were obtained simultaneously in a separate preparation.

Rabbit jugular vein

Male New Zealand White rabbits (2 - 4 kg) were injected with heparin (1000 units) via the marginal ear vein prior to cervical dislocation and exsanguination. The external jugular veins were removed, cleared of fat and adherent connective tissue and cut into rings (~4 mm wide) by transecting the vein. The rings were suspended between two hooks in a 10 ml organ bath containing Krebs solution (mM : NaCl 118, KCl 5.4, $CaCl_2$ 2.5, $MgSO_4$ 1.0, NaH_2PO_4 1.1, $NaHCO_3$ 25, glucose 10 and indomethacin 1μM) under a tension of 0.75 g. Changes in tension were measured by means of a Grass FT03 isometric transducer connected to a Grass Polygraph recorder. The bath solution was bubbled with 95% O_2 and 5% CO_2 and maintained at 37°C. Tissues were allowed a 1 hr period of equilibration during which changes in the resting tension were readjusted. All tissues were initially contracted with 10 μM histamine to establish the tissue maximum.

EP receptor studies:

For investigation of the relaxant actions of prostanoids, stable tone levels between 50 - 60 % of the maximum were obtained in all preparations with ~1μM histamine. Higher levels of contraction were subject to a

considerable degree of fade. In order to block any thromboxane receptor agonist action of the prostanoids tested, dose-response curves were obtained in the presence of the TP receptor antagonist, GR 32191 (10μM) (Lumley et al, 1987). Each preparation was exposed to two cumulative series of PGE_2 doses before testing a prostanoid analogue. The tissues were allowed ~30 minutes of frequent washing between cumulative additions. Responses were calculated as the % inhibition of the histamine-induced tone. PD_{50} values for each agonist were calculated and those obtained from 5 different animals were averaged and the s.e.m. calculated. Equi-effective molar ratios (EMR) were calculated in the following way: IC_{50} for the analogue / IC_{50} for PGE_2 (second curve) in the same preparation. EMR from single preparations obtained from 5 different animals were averaged and the s.e.m. calculated.

TP receptor studies

Agonists: Two cumulative concentration-response curves for U46619 were obtained on each tissue before testing the TP-receptor agonists. Tissues were allowed 30 minutes of frequent washing in between concentration-response curves. Responses were calculated as a % of the U 46619 maximum which was established in each preparation at the end of the experiment. The pD_{50} values for each of the agonists were calculated and those obtained from 5 different animals were averaged and the s.e.m calculated. EMR values for each agonist were calculated in following way: EC_{50} for the test compoud / EC_{50} for U 46619 (second curve) in the same preparation.

Antagonists: The TP-receptor antagonists were added 20 minutes prior to establishing a third concentration-response curve to U 46619. Dose ratios were calculated as follows: EC_{50} for U 46619 in the presence of antagonist / EC_{50} for U 46619 in absence of the antagonist. Dose-ratios obtained from preparations from 5 different animals were averaged and s.e.m calculated.

Arterial preparations

Rabbit superior mesenteric artery and was removed after exsanguination of animals and placed immediately in Krebs solution (as above). Pig gastro-epiploic artery was obtained after exanguination of pigs (25-35 Kg) under Stesnil ($20mgkg^{-1}$ intramuscularly) and pentobarbitione ($20mgkg^{-1}$ intravenous) anaethesia being used for other purposes. Dog mesenteric artery was obtained from dogs under pentobarbitone anaesthesia being used for other purposes. Human mesenteric artery was obtained from patients undergoing gastro-intestinal surgery for a number of conditions. In all cases connective tissue and fat were removed from apparently healthy sections of artery and 4 rings (2-4 mm wide) obtained by transecting the artery. The rings were set up in 10 ml organ baths for isometric tension recording by Grass FT03 force displacement transducers coupled to a Grass polygraph. Arterial rings were streched to an optimal tension of 1.5 g and readjusted to this level throughout the equilibration period (1 hr). All rings were initially contracted with 100 μM phenylephrine (PE) to establish the tissue maximum. Stable tone levels of 60-80% of the maximum were obtained in all preparations with 1-3 μM PE. Responses of the dog mesenteric artery to PE were more variable and KCl, 10 or 15 mM, was used instead giving stable tone levels of 40-60 % of maximum. On each preparation two cumulative concentration-response curves to iloprost were obtained followed by one of the test compound. Since experiments showed no change in the responsiveness to iloprost between second and third exposures, equi-effective molar ratios (EMR) were calculated from IC_{50} values for iloprost (second curve) and the test compound. EMR derived from single preparations obtained from 5 different animals were averaged and s.e.m. calculated.

Graphs

All graphs in this thesis show the arithmetic mean of responses. The bars represent ± the standard error of the mean.

RADIOLIGAND BINDING ASSAY

Preparation of platelet suspension

Blood was collected by venepuncture from a forearm vein of human volunteers and mixed with ~20% volume of acid citrate-dextrose solution. The blood was then centrifuged at 250 g for 20 minutes. The platelet rich plasma (PRP) was then transferred to a clean tube and 5-10 ng/ml of PGI_2 added. A platelet pellet was prepared by further centrifugation of the PRP at 450 g for 20 minutes. The supernatant plasma was removed with a pasteur pipette before the pellet was carefully resuspended in ~50 mls of assay buffer (pH = 7.4): NaCl (100 mM), glucose (5 mM), Tris HCl (50 mM) with indomethacin (1 μM). The platelet suspension was kept at 37°C until use.

Radioligand binding assay

The method used follows closely that of Narumiya et al (1986). Aliquots of the platelet suspension (100 μl) were added to Eppendorf tubes which already contained 0.02 pmol [^{125}I]-PTA-OH (50 μl) and increasing concentrations of the displacing agents (50 μl). Each displacer was tested at six different concentrations in a 1, 3, 10, 30 etc. sequence and each concentration was tested in triplicate. Each tube was incubated at 37°C for 30 minutes. In the experiments in which the displacing agent was a TP receptor agonist, 4 nM cicaprost was included in the incubation mixture in order to prevent aggregation. Three tubes containing vehicle instead of displacing agent were also included, thus allowing measurement of the total binding of the radioligand. An additional three tubes contained 1 μM ONO11120 - which was used to determine the non-specific binding of the radioligand.

Following incubation, the tubes were centrifuged at 16,000 g for 1 minute in a MSE 'Centaur 2' centrifuge. and the supernatant removed by tapping the tube on absorbent paper. The pellets were then gently washed with 1 ml of cold (0 °C) assay buffer, which was discarded and any remaining solution removed from the tubes using a cotton bud. The

radioactivity present in each tube was measured by a gamma counter (LKB, Universal Gamma Counter).

Results

The displacement to each concentration of displacer is expressed as a % of the maximum specific binding, calculated by subtraction of the non-specific binding from the total binding obtained in each assay. The data was plotted as % specific binding versus log of the concentration of displacer and the IC_{50} (concentration of displacer to cause 50% inhibition of specific binding) calculated from the graph. The relationship between IC_{50} and displacer K_d is given below

$$IC_{50} = \frac{Kd}{1 + [L]/K_L}$$

Where L is the radioligand concentration and K_L is the dissociation constant if the radioligand.
When [L] is << $[K_L]$, then the IC_{50} of each displacer can be taken to represent its K_d value. In this study this study the K_d of the ligand of ~20 nM (Mais et al, 1985a; Narumiya et al, 1986; Halushka et al, 1987) is considerably higher than the ligand concentration of 0.1 nM.

Responses and K_d values are expressed as arithmetic means (± s.e.m) .

Chapter 3
IP-Receptor Studies

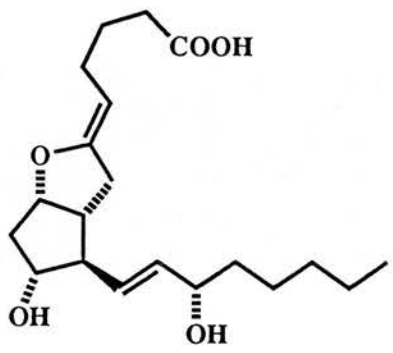
Platelet and vascular actions

Moncada et al (1976) found that microsomal preparations of pig and rabbit aorta and pig mesenteric artery transformed prostaglandin endoperoxides into an unstable substance (then named PGX), which differed from all currently known prostaglandins and thromboxanes (or their metabolites). PGX was found to have potent platelet antiaggregatory properties and relaxed strips of rabbit coeliac and mesenteric artery (Bunting et al, 1976a and b). Subsequently, Johnson et al (1976) elucidated the structure of PGX and it was renamed prostacyclin (PGI_2). Since then prostacyclin has been shown to be a potent inhibitor of platelet aggregation (0.5-20 nM) *in vivo* in a wide range of species (Sturzebecher et al, 1986). Prostacyclin elevates cyclic AMP which in turn prevents platelet aggregation independently of its effects on cytosolic free Ca^{2+} (Gorman et al, 1977). In human platelet rich plasma (PRP), PGD_2 and PGE_1 are 20 and 40 times less active than PGI_2 as inhibitors of aggregation (Whittle et al, 1978). Experiments have shown that PGE_1 inhibits aggregation via the PGI_2 (IP) receptor while PGD_2 has a distinct site of action (Whittle et al, 1978; Miller and Gorman, 1979). In addition, PGI_2 has been shown to dissaggregate white thrombi in a system in which blood from animals or humans is superfused over collagen strips (Bandt et al, 1984). When infused into humans PGI_2 reduces platelet aggregability, dissipates circulating platelet aggregates and prolongs the bleeding time (Gryglewski et al, 1978; Szczeklik and Gryglewski, 1979). In addition, PGI_2 has been demonstrated to exert this anti-thrombotic action in many animal models of venous and arterial thrombosis (Witt et al, 1986).

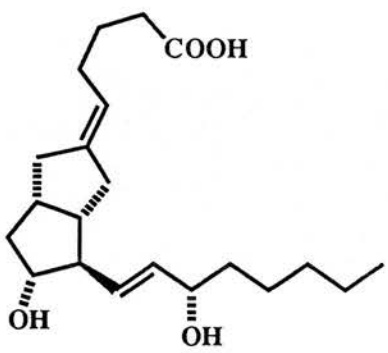
However, prostacyclin also has a potent dilatory action on vessels from a number of species, and *in vivo* it causes a drop in systemic blood pressure and reduces peripheral resistance by dilatation of arterioles. In humans this action causes reddening of the face and neck and unpleasant vascular headaches (see Whittle and Moncada, 1984).

In view of these two major actions of PGI_2 recent chemical effort has centred on the synthesis of chemically and metabolically stable PGI_2

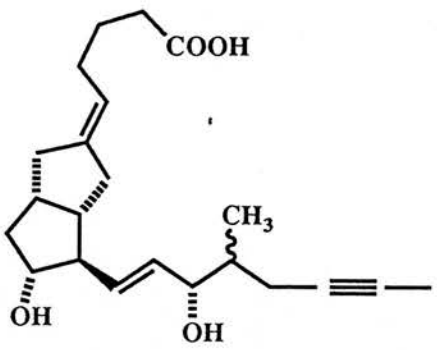
Figure 3.1: Structure of PGI_2 analogues and EP 157



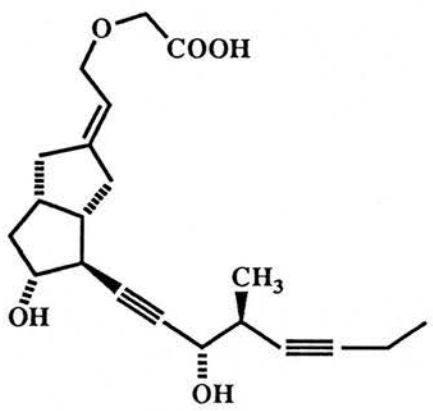
PGI_2



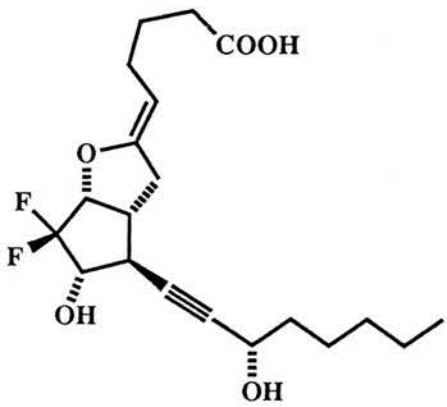
Carbacyclin



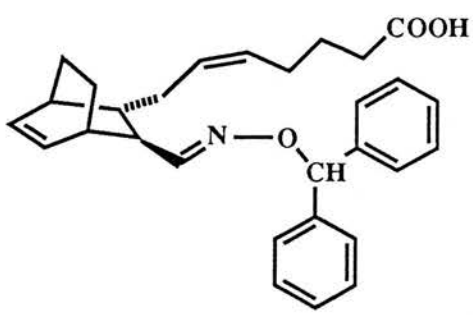
Iloprost



Cicaprost



MMM-I-135



EP 157

analogues which retain the anti-thrombotic actions but have reduced vasodilatory activity.

The chemical instability of PGI_2 (Fig. 3.1) is due to the strained enolether structure which can be protonated by the nearby carboxylic function. Stabilization has been obtained by replacing the oxygen of the enolether with a carbon, sulphur or nitrogen atom (Skuballa et al, 1987). In carbacyclin (Fig. 3.1), the substitution is a carbon atom, and this compound has been shown to be 3-10 times less active than PGI_2 as an inhibitor of platelet aggregation and in reducing systemic arterial blood pressure in a number of species (Whittle et al, 1980). Further modifications of the carbacyclin structure have been shown to produce increased agonist potency at IP receptor sites on both platelet and vessel. In iloprost (ZK 36374) (Schror et al, 1981) there is an additional single methyl group at C16 and an acetylenic group at C18, and this compound has been shown to be equipotent with PGI_2 both as an inhibitor of platelet aggregation and as a vasodilator (Casals-Stenzel et al, 1983).

Iloprost has also been shown to be orally active in man with a half-life of 20-30 minutes (Fitscha et al, 1987), however, in the clinical situation it would be useful to have an even greater duration of action. The relatively short half-life of iloprost is due to rapid metabolism, primarily by ß-oxidation of the upper side chain (Krause et al, 1984). The most recent PGI_2 analogue to be synthesized, cicaprost (Skuballa et al, 1986), (Fig. 3.1) contains an ether oxygen in the 3 position to prevent ß-oxidation. In addition, increased potency has been obtained by: (a) converting the 13,14 double bond into a triple bond (b) introduction of a pure 16(S) methyl group and (c) addition of a further methyl group at C20 (it is notable that unlike cicaprost, iloprost is a mixture (approx. 50:50) of 2 diastereoisomers epimeric at C16: Skuballa and Vorbruggen, 1983). Cicaprost (ZK 96480) has been shown to be 5-12 fold more potent than PGI_2 with respect to *in vivo* hypotensive and antiaggregatory actions, with a duration of action of 5-48 hours (Mueller et al, 1984; Sturzebecher et al, 1986).

There is no indication from the above studies that the IP receptor on the platelet differs from that on blood vessels. However, Whittle and

Moncada (1984) used a 'selectivity ratio' to give an indication of the separation between antiaggregatory and vasodilatory actions of a series of PGI_2 analogues. They found that it was possible to lose platelet activity while retaining vascular actions, and on this basis proposed that there may be differences in the structural requirements for binding to these sites. However, as pointed out by the authors the data have to be interpreted with caution: they have compared *in vivo* (cardiovascular) results with *in vitro* (platelet) and the data have been obtained from different species (rat blood pressure *vs* human platelet).

Later studies have demonstrated that certain PGI_2 analogues display antagonist actions on the vasculature while retaining antiplatelet (agonist) actions (Fassina et al, 1985; Corsini et al, 1987; Olivia et al, 1989). However, the interpretation of these results is called into question by recent findings that a number of PGI_2 analogues (iloprost and carbacyclin) and indeed PGI_2 itself, have significant EP_1 agonist activity (Dong and Jones, 1982; Dong et al, 1986; Sheldrick et al, 1988; Lawrence and Jones, 1988). EP receptors are known to mediate contractile responses in a number of tissues and these may oppose any IP-mediated relaxation via physiological antagonism.

There appears to be a need for comprehensive study of the actions of a number of PGI mimetics in both the platelet and vasculature from a range of species. We have investigated the relaxant action of iloprost, cicaprost and carbacyclin in visceral arterial vessels *in vitro* from man, pig, rabbit and dog and compared this to their antiaggregatory action in platelets from the same species. Since iloprost appears, from the literature (see Gryglewski and Stock, 1987), to be the most widely studied PGI_2 analogue it was used as the standard agonist. Any EP-mediated contractile actions of the compounds could be assessed on the basis of (1) the effect of the EP_1-receptor antagonist, AH 6809 (Coleman et al, 1985) (2) the fact that cicaprost has been shown to be highly selective for the IP receptor with little EP_1 receptor activity (Dong et al,1986).

In addition, we have further investigated the actions of EP 157. Earlier work (Armstrong et al, 1986) has shown that the endoperoxide analogues, EP 157 and EP 035, behave as specific thromboxane receptor

antagonists in isolated smooth muscle preparations which are prostacyclin-insensitive (rabbit aorta, dog saphenous vein and guinea-pig trachea). However, in human platelets EP 157 inhibits aggregation induced by ADP, PAF and thromboxane, maximally activates membrane-bound adenylate cyclase and displaces [^{3}H] iloprost binding. These actions suggest that the compound is an agonist at platelet IP receptors - this is of particular interest in view of the fact that EP 157 bears little structural resemblance to PGI_2 (Fig. 3.1). Thus, EP 157 has been included in the study of vascular IP receptors.

Actions in gastrointestinal smooth muscle of the guinea-pig ileum

Like PGE_2, prostacyclin (PGI_2) is synthesised in the human and animal intestinal tract (Bennet et al,1971; Whittle, 1981). As discussed above PGI_2 appears to be a relaxant agent in a range of vascular smooth muscle preparations from different species. However, Gaion and Trento (1983) have shown that PGI_2 (1 nM-1 μM) produces concentration-dependent contractions of the longitudinal muscle of the guinea-pig ileum. They found that at a concentration near the EC_{50} (20 nM), these contractions were abolished by TTX (10 nM), and inhibited by atropine (1-30 nM) and hemicholinium-3 (20 μM). In addition, PGI_2 potentiated electrically- induced contractions while having no effect on the response to exogenous Ach. On the basis of these results they proposed that PGI_2-induced contractions are mediated by Ach released from cholinergic neurones as a consequence of increased excitability of the cell bodies (see *'Action of TTX*). Further evidence for this mechanism of action was presented by Gaion and Trento (1984b) in finding that the action of PGI_2 was inhibited by noradrenaline (30-300 nM), morphine (10-50 nM) and the purinergic receptor agonist N^6-phenylisopropyl adenosine (PIA) (10-50 nM). Noradrenaline, enkephalins and adenine nucleotides have all been identified in intestinal nerves of the guinea-pig and other species (Furness and Costa, 1982), and are known to presynaptically modulate cholinergic function (Paton and Vizi, 1969; Grintzler et al, 1975; Szerb, 1982; Moody and Burnstock, 1982.).

However, these results appear to be at variance with earlier work showing that PGI_2 has a contractile action in the guinea-pig ileum in

the presence of high concentrations of atropine (Moncada et al, 1976; Kennedy et al, 1982), although under these conditions PGI_2 appears to be considerably less active (EC_{50}= 2.7 μM). Gaion and Gambaratto (1987) later confirmed these results showing that in the presence of TTX (1 μM) or atropine (30 nM) 1 μM PGI_2 retained 20-30% of its contractile response. In addition, this response was unaffected by procaine or by quercetin, although both drugs inhibited contractions in the absence of TTX. There is evidence that procaine interferes with Ach release (Paton et al, 1971) leaving the direct response to some agonists unaffected (Bury and Mashford, 1976). This would also appear to be true for quercetin as it was found to have no effect on direct contractions induced by Ach. The authors suggest that at high concentrations, in addition to a neuronal component, PGI_2 has a direct contractile component of action mediated by IP receptors present on the smooth muscle cells. However, in view of the EP_1 agonist action of PGI_2 demonstrated by Dong et al (1986), an obvious explanation for the TTX/atropine-resistant component of action would be a direct agonist action of PGI_2 on the EP_1 receptors known to be present in the guinea-pig ileum (see Chapter 4).

In view of the unique nature of this IP receptor linked to neurotransmitter release we have investigated the action of a number of PGI_2 analogues in this system. The nature of the transmitters released by IP-receptor activation has been studied using the muscarinic receptor antagonist, atropine and the substance P receptor antagonist, SP150. In addition, EP_1 receptor activity of these analogues was studied using the EP_1 receptor antagonist, AH 6809.

Some details of the physiology of the guinea-pig ileum and the mechanism of action of the compounds used to inhibit enteric nerve function are given below:

Physiology

The gastrointestinal tract of the guinea-pig ileum consists of two muscle layers and embedded between these two layers lies the enteric nervous system. The majority of enteric neurones belong to the myenteric plexus (Auerbach's), which lies just underneath the outermost muscle layer of the gut wall, the longitudinal muscle, being sandwiched

between this layer and the major gut muscle layer, the circular muscle. The main role of these nerves is to bring about the ordered patterns of contraction and relaxation seen during peristalsis.

The neurones of the myenteric plexus contain many neurotransmitters, and neuromodulators including Ach, enkephalin, 5HT, VIP, substance P, ATP, cholecystokinin, somatostatin, neuropeptide Y, galanin and GABA (North, 1982; Jensen et al 1987). Evidence would suggest that Ach is the major excitatory transmitter from the myenteric plexus of the guinea-pig ileum to the cells of the longitudinal muscle layer (Paton, 1957) and also within the plexus (Nishi and North, 1973). Electrical stimulation of the enteric nervous system leads to the release of several substances (North, 1982), of which Ach is the most readily detectable and the most widely studied (Szerb, 1982). In addition, peristalsis and electrically-induced twitches of the guinea-pig ileum are inhibited to a large extent by atropine (Kosterlitz and Lees, 1964 ; Hirst, 1979.). However, there is also evidence that besides Ach, substance P (SP) is a major excitatory transmitter in the gastrointestinal tract. SP occurs within very large numbers of nerve processes and in about 5% of cell bodies in the myenteric plexus (Furness and Costa, 1980). A role for SP in peristalsis is most likely because there seems to be an arrangement of SP neurones capable of maintaining propulsive motility in the presence of atropine (Bartho and Holzer, 1985).

Action of morphine

It has been known for a number of years that morphine inhibits the reflex peristaltic contractions and longitudinal muscle contractions of the isolated guinea-pig ileum (Kosterlitz and Robinson 1957, Gyang et al 1964). Schaumann (1957) found that morphine decreases the spontaneous release of acetylcholine (Ach) from the guinea-pig ileum without inhibiting its synthesis or release from ground ileal tissue. These observations led him to suggest that morphine reduces the excitability of the neurones that release Ach - a conclusion which still stands today. In addition, Paton (1957) showed that while morphine did not affect the contractions of the tissue to Ach, it depressed twitches of the tissue induced by coaxial stimulation. These early observations have been confirmed by later electrophysiological studies. Extracellular

recording has shown that morphine and enkephalin (1 nM-1 μM) will inhibit firing of myenteric neurones, and almost all neurones tested were sensitive to inhibition (North and Williams, 1977). Intracellular recording from myenteric neurones has revealed that most cells are hyperpolarised by morphine (North and Tonini, 1977). Furthermore, the hyperpolarisation takes place primarily on the cell process - this has been shown to prevent action potential propagation along the process of the myenteric neurone (Morita and North, 1981).

While it has generally been thought that endogenous opioids inhibit peristalsis and twitch of the electrically stimulated ileum by inhibiting the release of enteric Ach, there is evidence that additional mechanisms may be involved (Kromer and Schmidt, 1982). It has been shown that effective peristalsis can be evoked in the presence of atropine and this effect is abolished by the [Met] - enkephalin analogue FK33-842 (Bartho et al 1982a). There is evidence that atropine-resistant contractions of the guinea-pig ileum in response to electrical stimulation are mediated by substance P: these have been shown to be inhibited by morphine (Bartho et al, 1982b). In addition, Donnerer et al (1984) could only demonstrate an appreciable release of substance P immunoreactivity during peristalsis in the presence of naloxone. These results may reflect modulation of the release of enteric substance P by opioids (Bartho and Holzer, 1985).

Action of TTX

TTX is known to block the passage of sodium ions through axonal membrane channels, and as sodium currents are important in the generation of action potentials TTX will block impulse conduction. However, TTX is without effect on either the release of transmitter from nerve terminals or the sensitivity of the post-synaptic membrane to the transmitter (Narahashi, 1974). Hence, preparations treated with TTX are useful for studies on the mechanism of transmitter release .

VASCULAR RELAXATION

Concentration-response curves

PGI analogues: Log concentration-response curves obtained on the human mesenteric artery, pig gastro-epiploic artery, rabbit mesenteric artery and the dog mesenteric artery are shown in Fig. 3.2. Sensitivity to iloprost decreased in the order man, dog, rabbit, pig and cicaprost was slightly more active than iloprost on all four preparations. EP 157 showed threshold relaxation at 20 nM in the human (Fig. 3.7A) and 200 nM in the dog, rabbit and pig (Fig. 3.7C). At the highest concentration tested (6.7 μM) it gave relaxations of 70-95% of the iloprost maximum. In the pig gastroepiploic artery, concentration-response curves to carbacyclin were shallow reaching a maximum of ~40% at 100 nM. At concentrations above this carbacyclin produced discrete contractions of the preparation (see Fig. 3.7C).

PGE analogues: In vessels from man, pig and rabbit PGE_2 was highly active as a relaxant, however in the pig and rabbit the curves reversed at concentrations above 50 nM (see Fig. 3.3). 16,16-Dimethyl PGE_2 contracted all three preparations; the pig vessel was considerably more sensitive (EC_{50}, 5 nM) than those of the human and rabbit (see Fig. 3.3).

Action of AH 6809

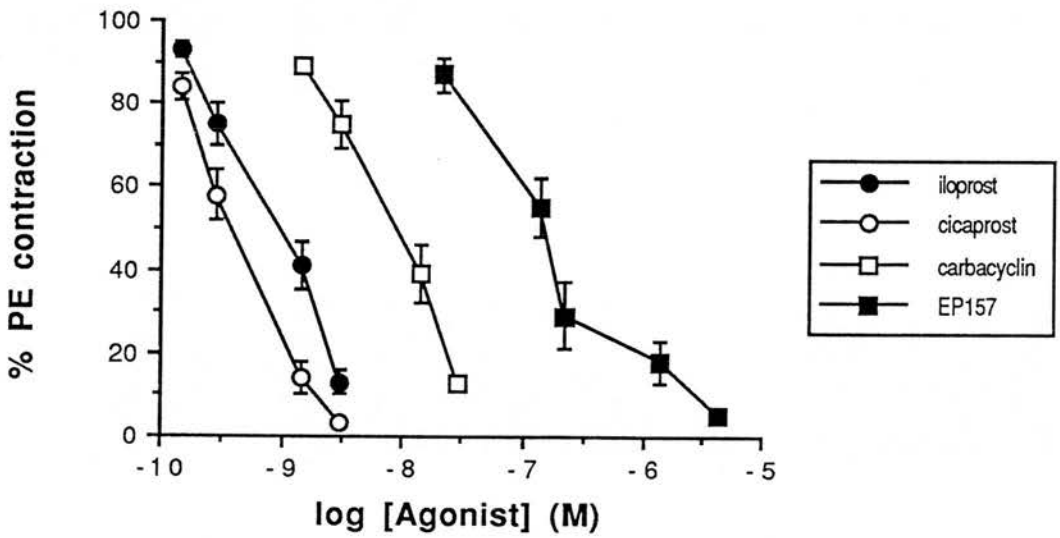
AH 6809 (10 μM) slightly accentuated the relaxant action of PGE_2 in the rabbit mesenteric artery and inhibited the reversal of the curve (Fig. 3.4). However, in the pig vessels (Fig. 3.5) the blocker had no effect on the concentration-response curve of carbacyclin or 16,16-dimethyl PGE_2.

Action of EP 092

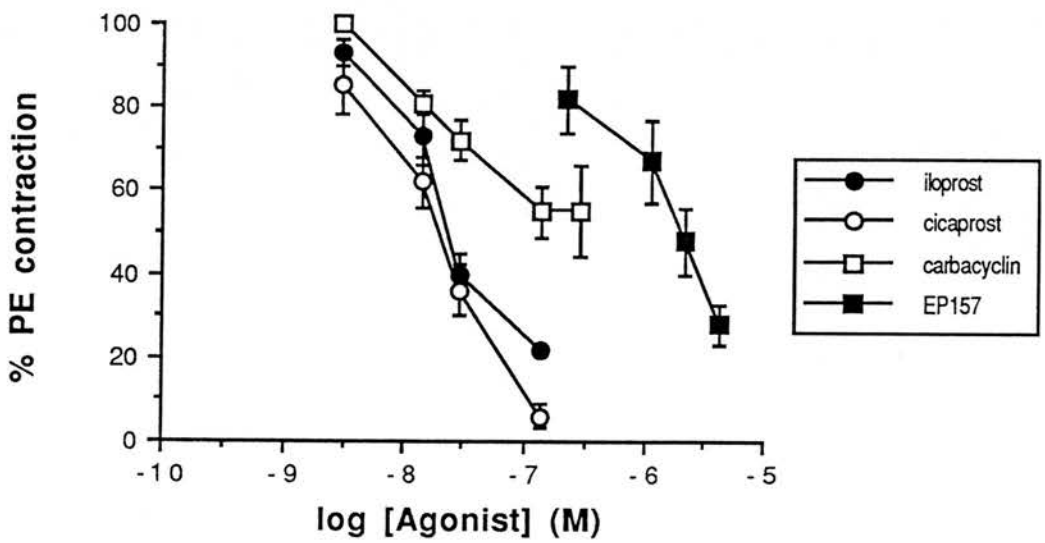
In the pig EP 092 (1 μM) caused a parallel rightward shift in the concentration-response curve of U46619 consistent with a pA_2 of 7.6. However, this concentration of blocker had no effect on the

Figure 3.2: Log-concentration response curves for relaxation of
(a) human mesenteric artery (b) pig gastro-epiploic artery
(c) rabbit mesenteric artery (means of 6-9 experiments) and
(d) dog mesenteric artery (means of 4-6 experiments).

a)

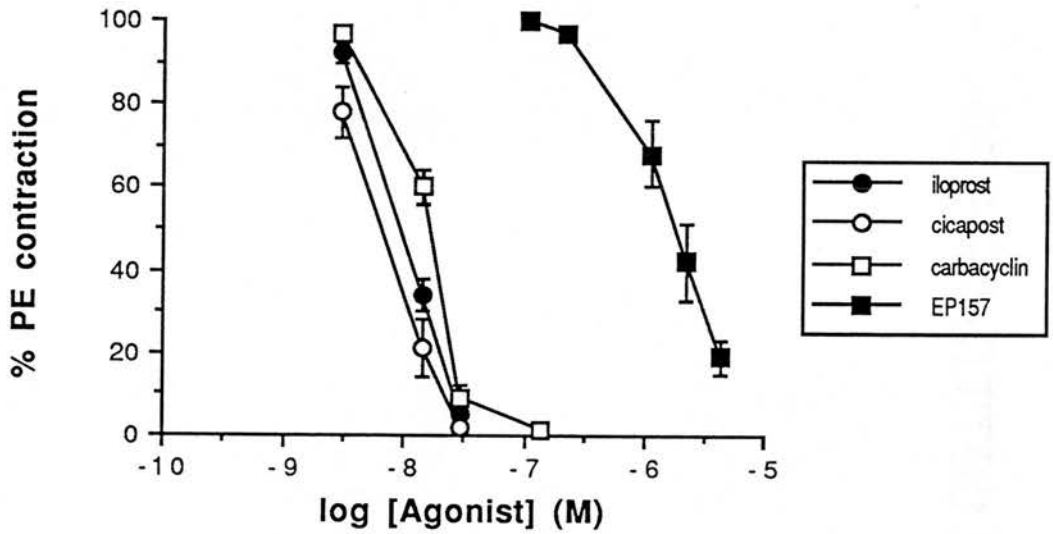


b)



(bars represent the s.e.m)

c)


% PE contraction
100
80
60
40
20
0
-10
-9
-8
-7
-6
-5
log [Agonist] (M)
iloprost
cicapost
carbacyclin
EP157

d)

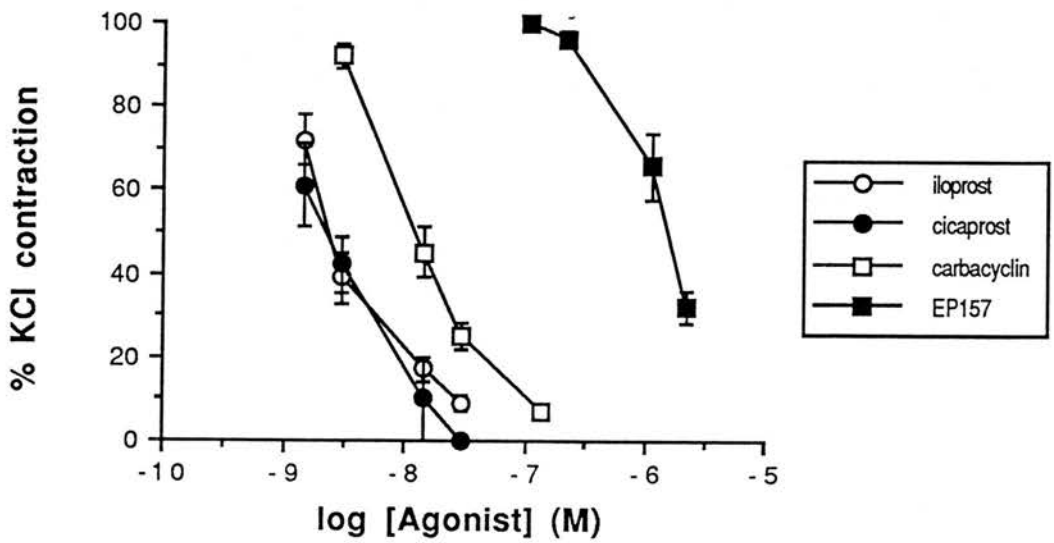

% KCl contraction
100
80
60
40
20
0
-10
-9
-8
-7
-6
-5
log [Agonist] (M)
iloprost
cicaprost
carbacyclin
EP157

Figure 3.3: Log-concentration response curves of (a) PGE_2 (b) 16,16 dimethyl PGE_2 for relaxation/contraction of visceral arteries from the pig, human and rabbit (mean of 2 expts.).

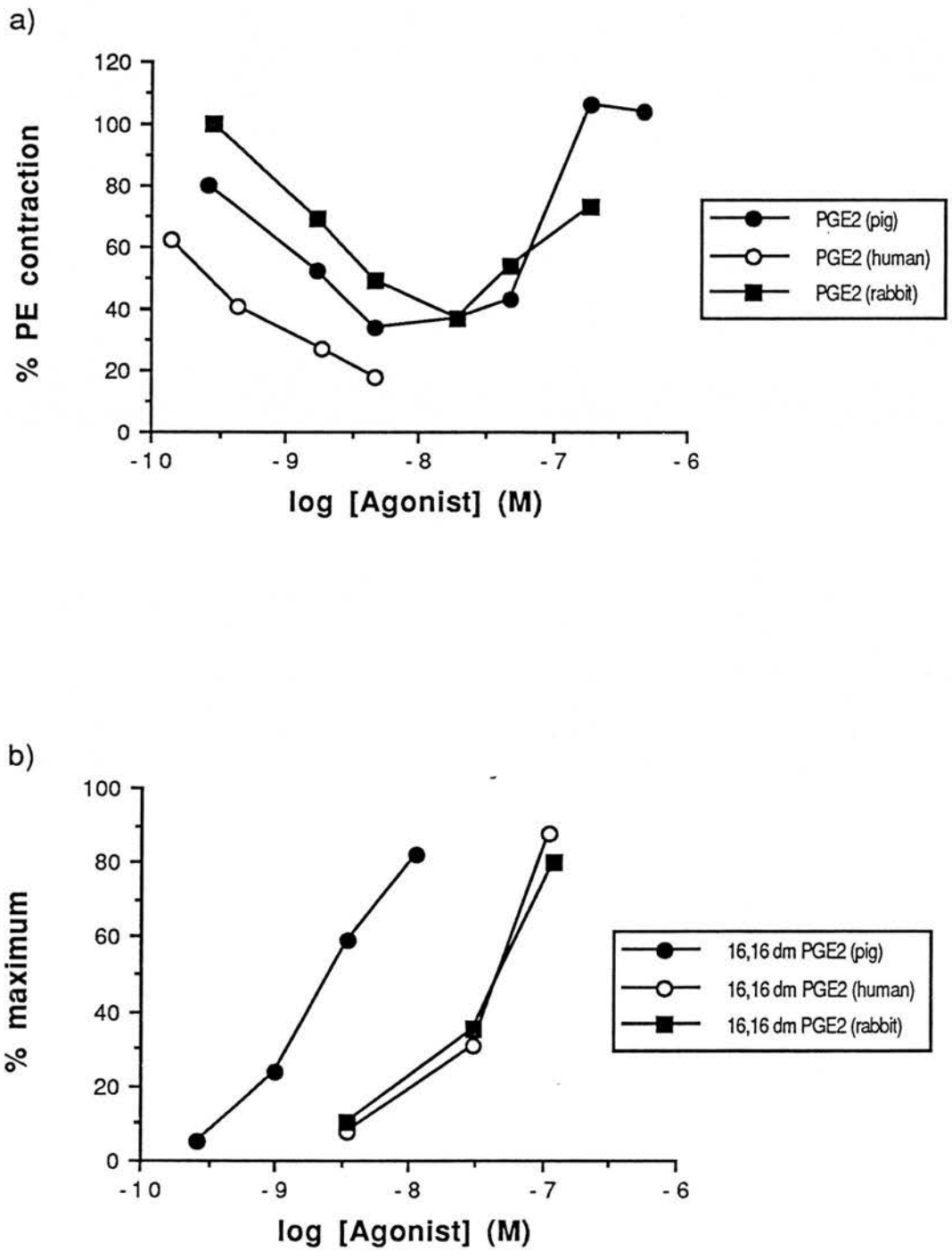


Figure 3.4: Effect of AH 6809 (10μM) on log concentration-response curves of PGE_2 in the rabbit mesenteric artery (mean of 2 expts.).

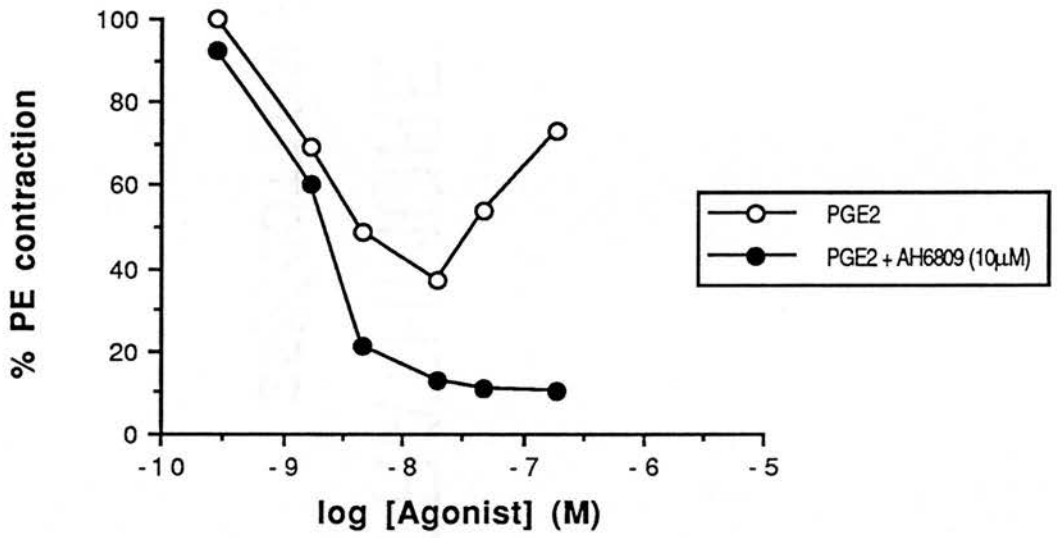
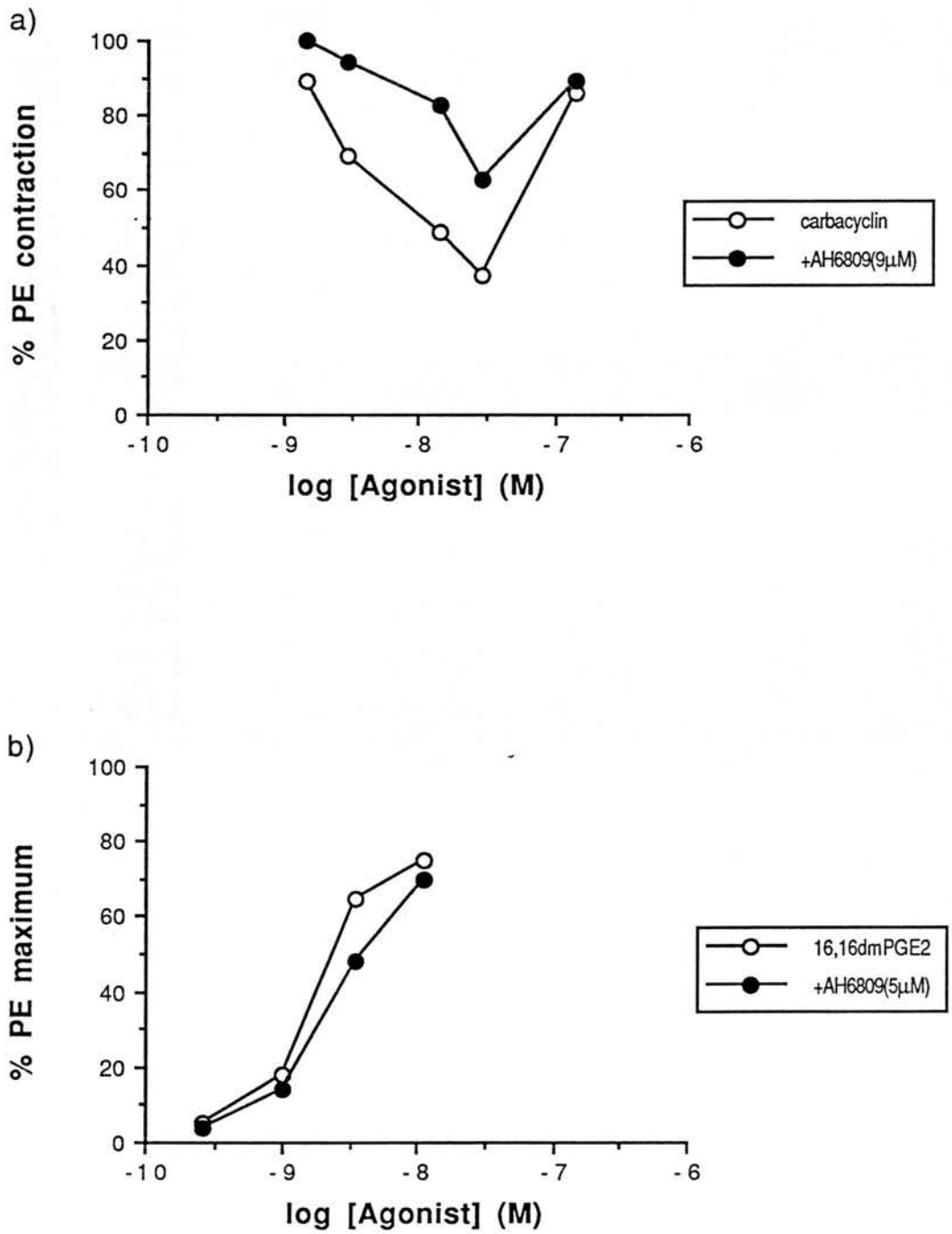
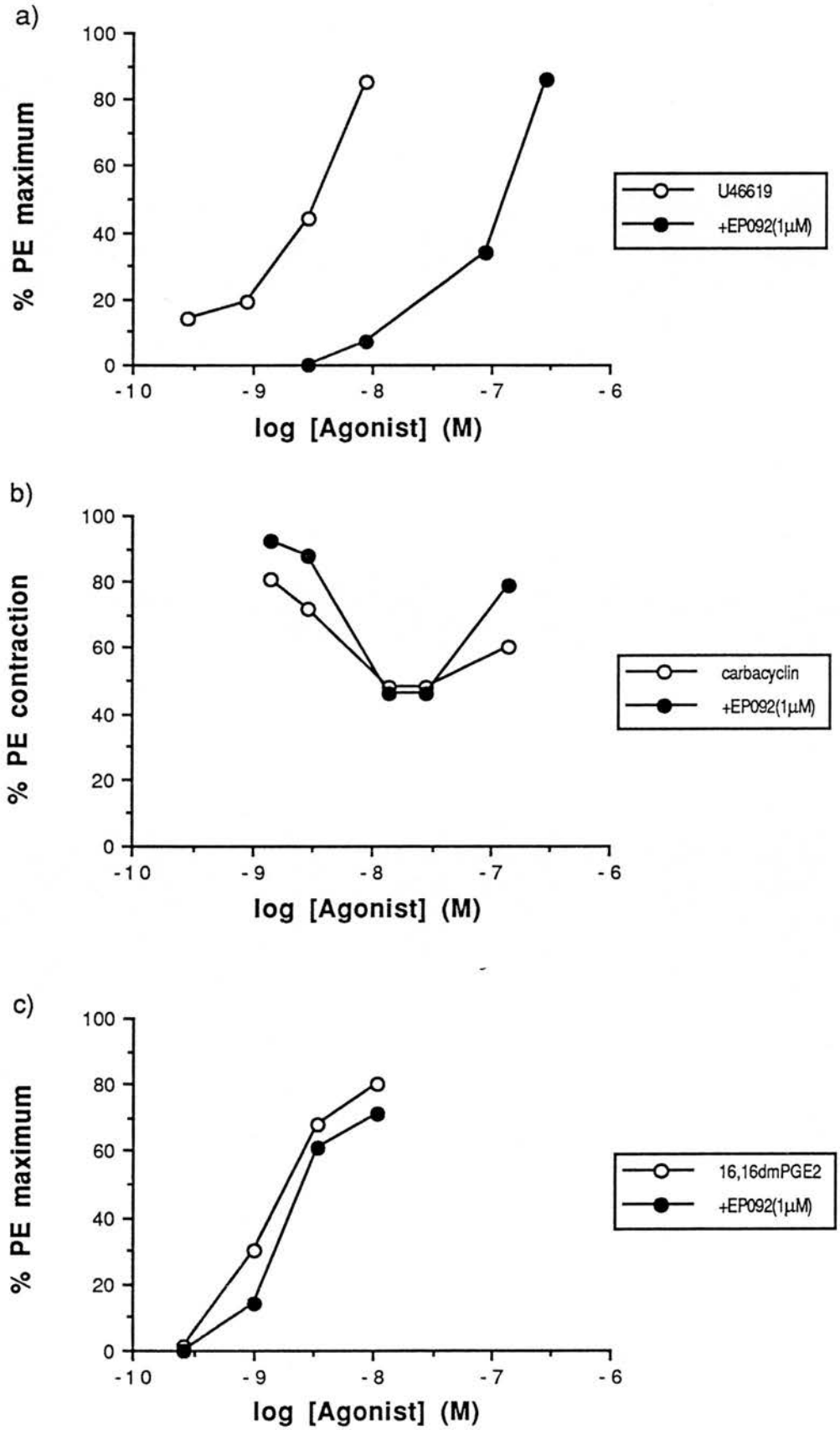


Figure 3.5: The effect of AH 6809 on the log concentration-response curves of (a) carbacyclin and (b) 16,16-dimethyl PGE_2 in the pig gastro-epiploic artery (mean 2 expts.).



concentration-response curve of carbacyclin or 16,16-dimethyl PGE_2 (Fig. 3.6)

Figure 3.6: Effect of EP 092 (1μM) on log concentration-response curves of (a) U 466219 (b) carbacyclin and (c) 16,16 dimethyl PGE_2 in contraction/relaxation of the pig gastro-epiploic artery (mean of 2 expts.).


a)
% PE maximum
100
80
60
40
20
0
-10
-9
-8
-7
-6
log [Agonist] (M)
U46619
+EP092(1μM)
b)
% PE contraction
100
80
60
40
20
0
-10
-9
-8
-7
-6
log [Agonist] (M)
carbacyclin
+EP092(1μM)
c)
% PE maximum
100
80
60
40
20
0
-10
-9
-8
-7
-6
log [Agonist] (M)
16,16dmPGE2
+EP092(1μM)

Firure 3.7A: Trace showing the relaxation of the human mesenteric artery, precontracted with phenylephrine (2μM), by (a) iloprost and (b) EP 157.

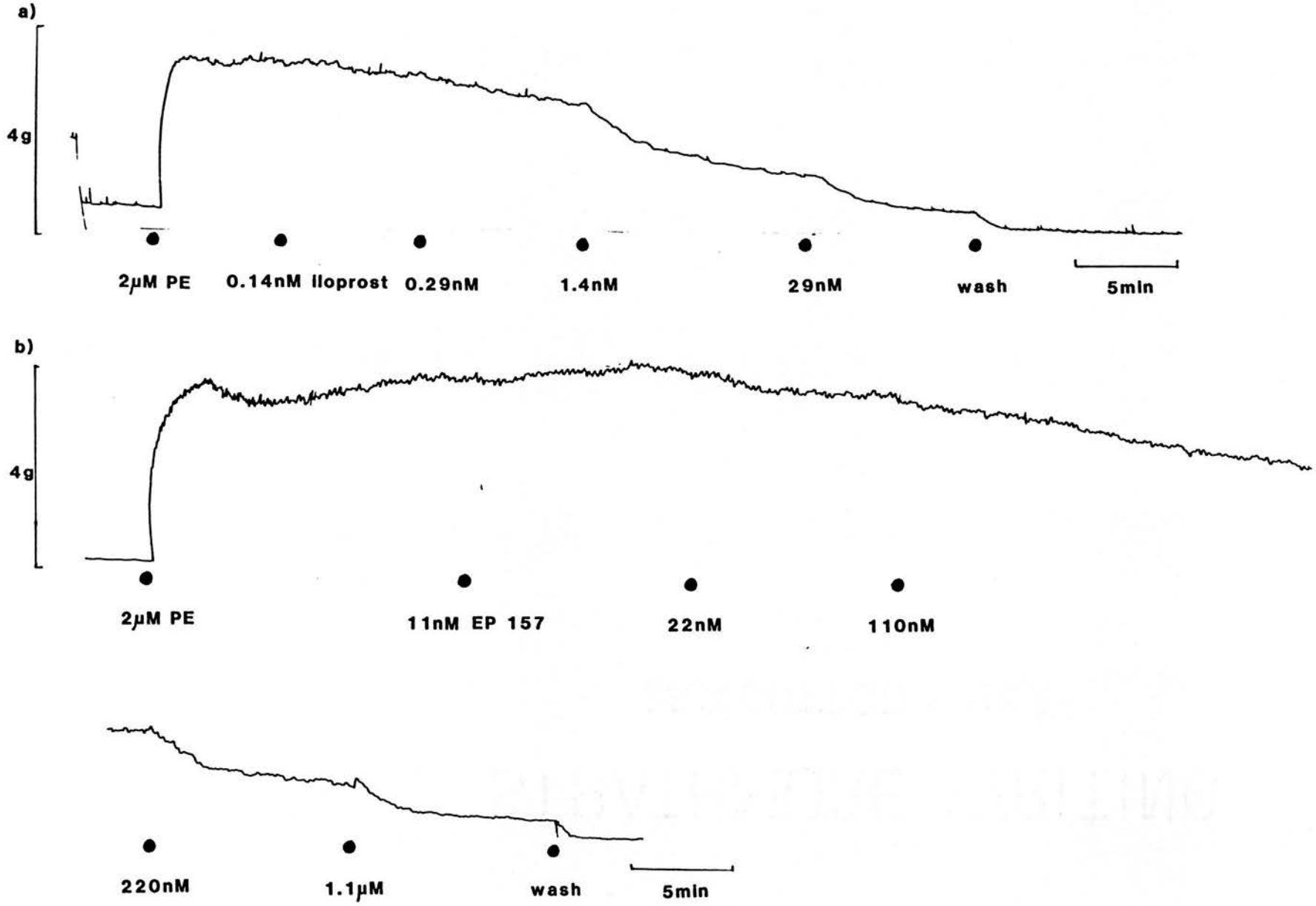

a)
4g
2μM PE
0.14nM Iloprost
0.29nM
1.4nM
29nM
wash
5min
b)
4g
2μM PE
11nM EP 157
22nM
110nM
220nM
1.1μM
wash
5min

Figure 3.7B: Trace showing therelaxation of the rabbit mesenteric artery precontracted with phenylephrine (3μM) by (a) iloprost and (b) carbacyclin.

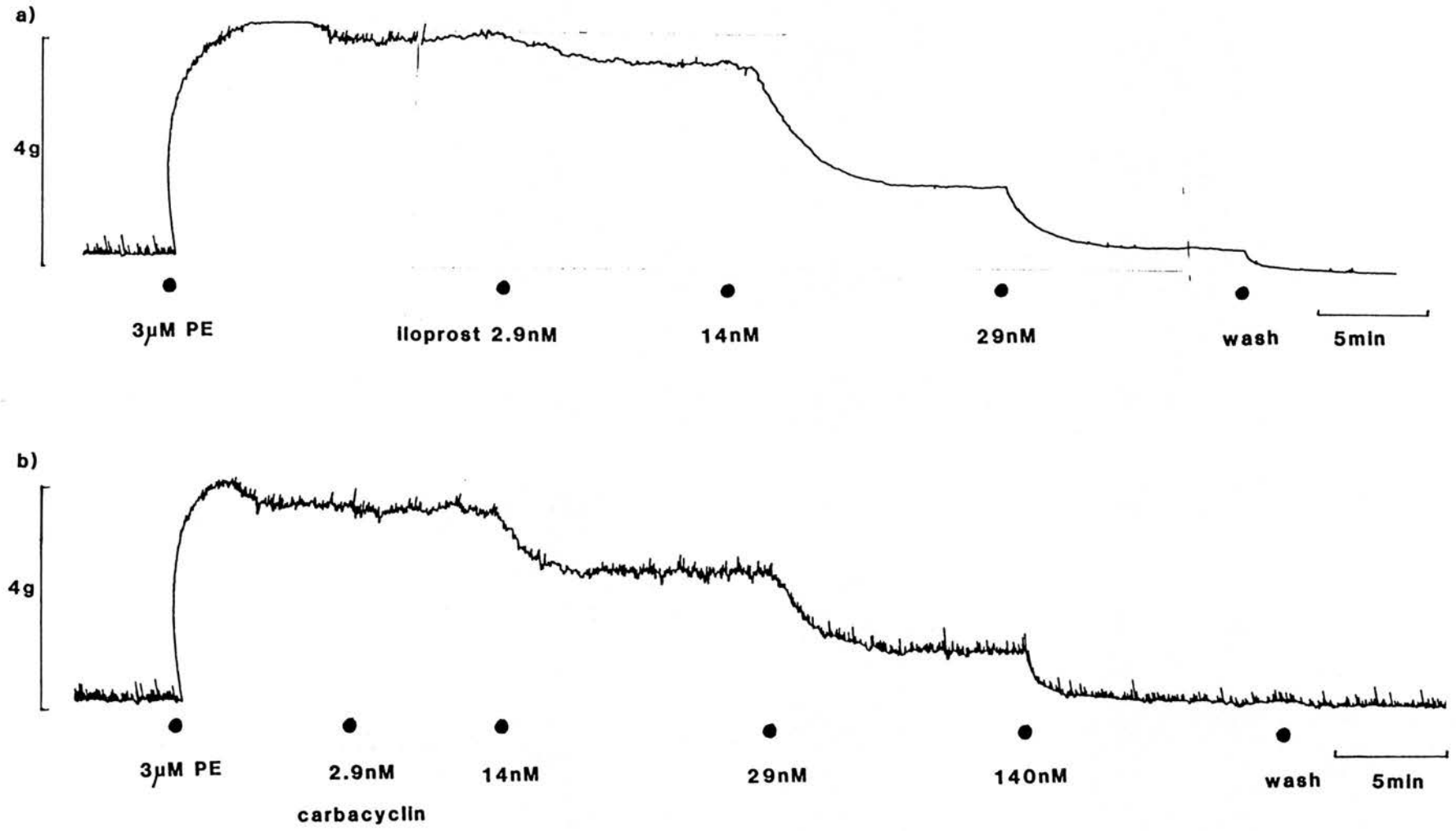
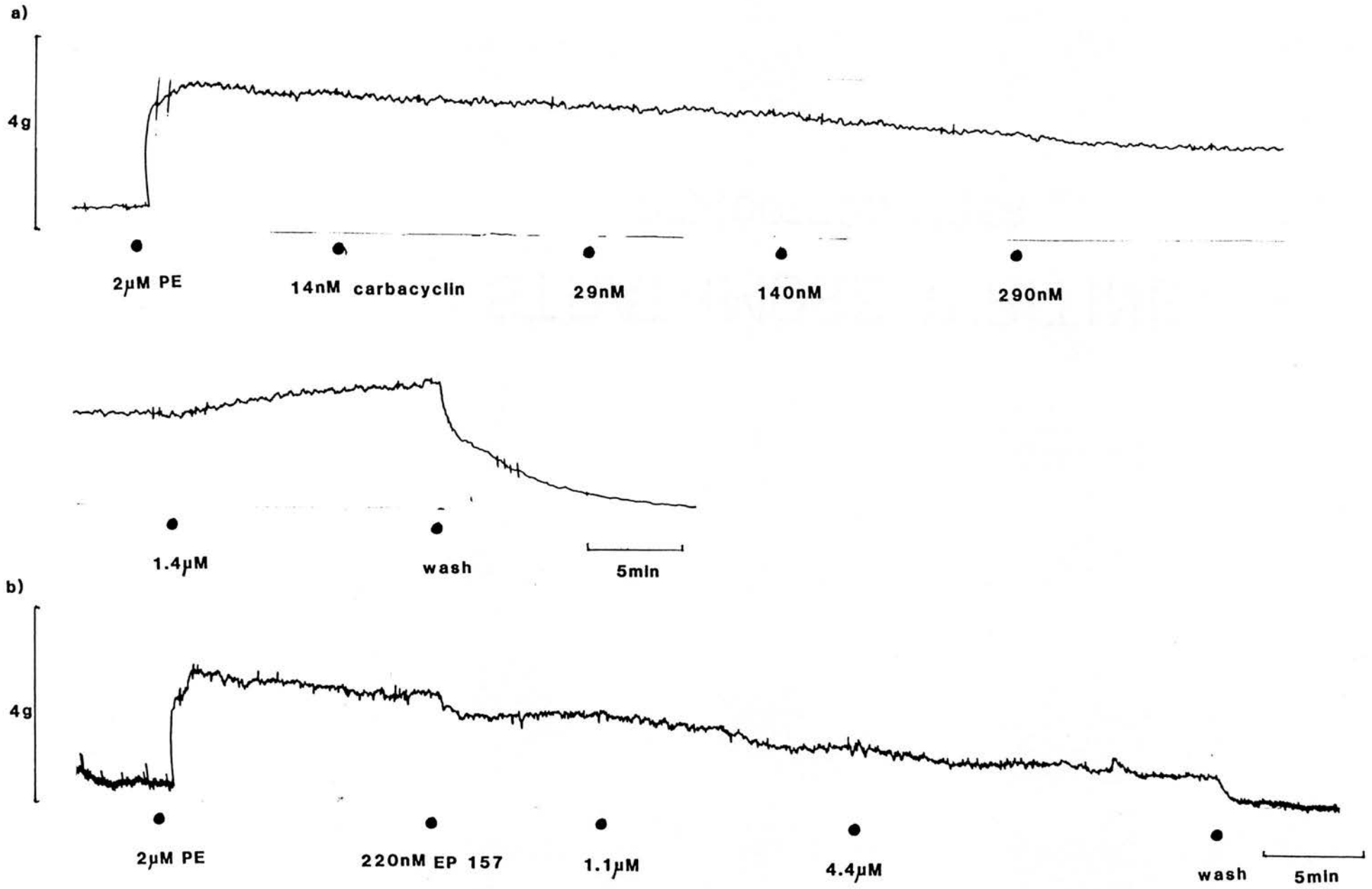

a)
4g
3μM PE
Iloprost 2.9nM
14nM
29nM
wash
5min
b)
4g
3μM PE
2.9nM
carbacyclin
14nM
29nM
140nM
wash
5min

Figure 3.7C: Trace showing the relaxation of the pig gastro-epiploic artery precontracted with phenylephrine (2μM) by (a) carbacyclin and (b) EP 157.


a)
4g
2μM PE
14nM carbacyclin
29nM
140nM
290nM
1.4μM
wash
5min
b)
4g
2μM PE
220nM EP 157
1.1μM
4.4μM
wash
5min

GUINEA-PIG ILEUM

Concentration-response curves

Log concentration-response curves on preparations untreated with antagonists/inhibitors are shown in Fig. 3.8. All the PGI analogues tested acted as contractile agents, the rank order of potency being cicaprost > iloprost > PGI_2 > MMM-I-135 > carbacyclin. The curves for cicaprost and MMM-I-135 reached a considerably lower maximum than those of PGI_2, iloprost and carbacyclin. In addition, the curve for cicaprost appears to be bell-shaped, turning back on itself at concentrations of greater than 10 nM.

Action of morphine and TTX

Single doses of the prostanoids: Morphine caused a dose-related inhibition of responses to single submaximal doses (which gave responses of 60-70% of the Ach maximum) of cicaprost, iloprost and carbacyclin (Fig. 3.9). The action of cicaprost (13 nM) was abolished by morphine (IC_{50} = 13 nM). The contractile action of iloprost (14 nM) and carbacyclin (150 nM) could only be partially blocked by morphine (60% and 30% respectively). At 1 μM TTX exhibited an identical profile of inhibitory action to that of 1 μM morphine: the response to cicaprost was abolished and that to iloprost and carbacyclin partially inhibited (Table 3.1). The effect of 100nM on a single dose of cicaprost is shown in Figure 3.15A.

Concentration-response curves: Figure 3.10 shows the effect of morphine on the log concentration-response curves of the prostanoids. The curves of PGI_2 and iloprost were subject to a significant shift in the presence of 1 μM morphine. In addition, the log concentration-response curve of iloprost appears to plateau at a maximum of 55-60%. In contrast, the curve for carbacyclin was subject to a small shift which was not statistically significant. In the presence of 1 μM morphine cicaprost (see Fig. 3.15B) and MMM-I-135 were devoid of activity at concentrations of up to 1 μM.

Figure 3.8: Log concentration-response curves of (a) cicaprost and MMM-I-135 and (b) iloprost, PGI_2 and carbacyclin in the guinea-pig ileum (means of 4-6 expts.).

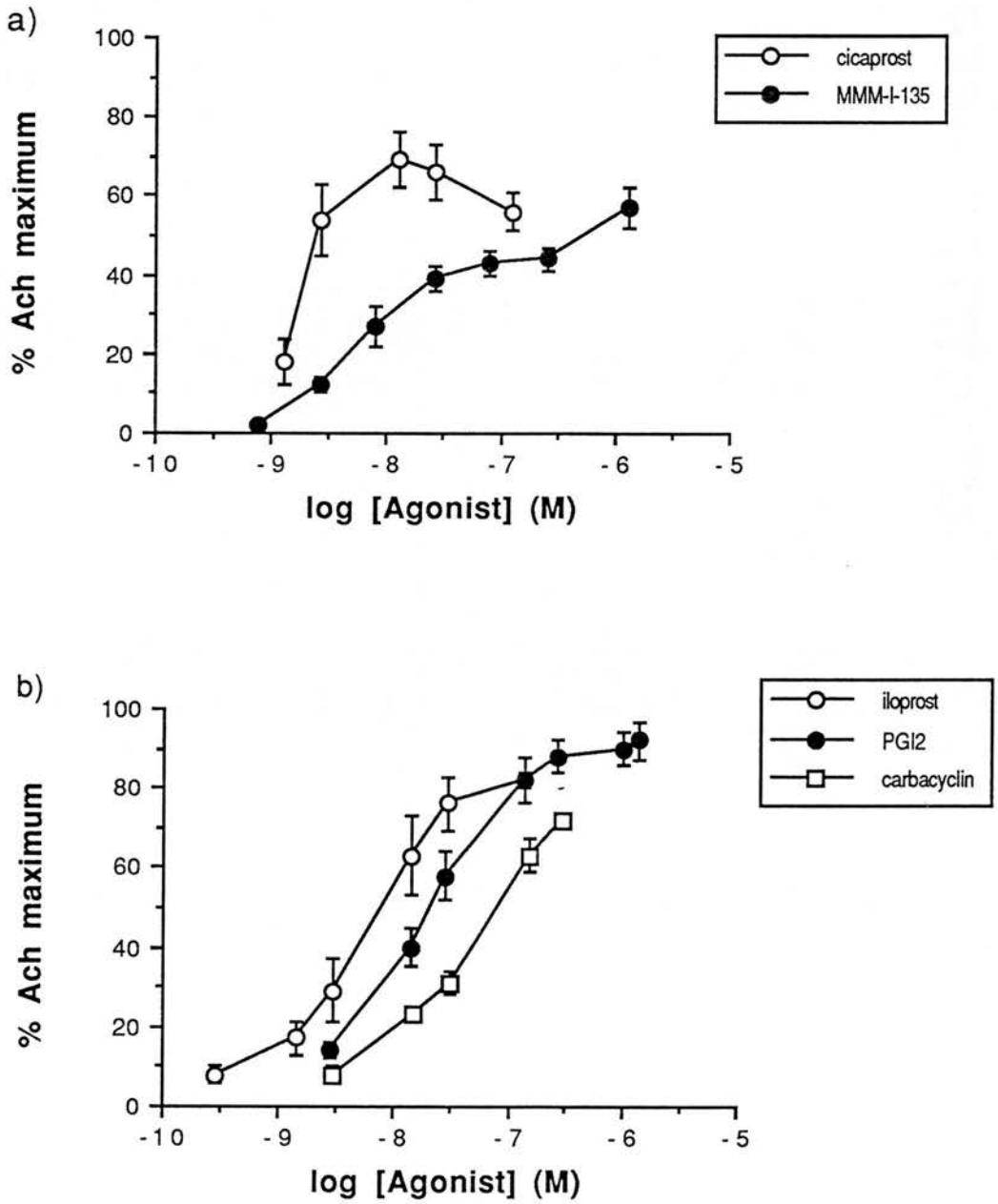


Figure 3.9: Log concentration-response curves for morphine *vs* single doses of PGE_2 (14nM), cicaprost (13nM), iloprost (14nM) and carbacyclin (150nM) (mean 4-6 expts.).

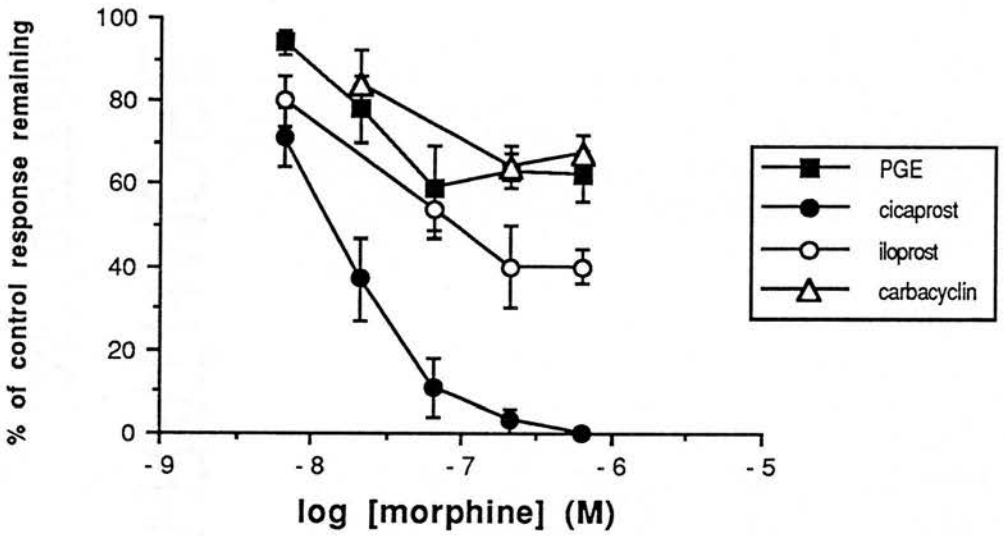


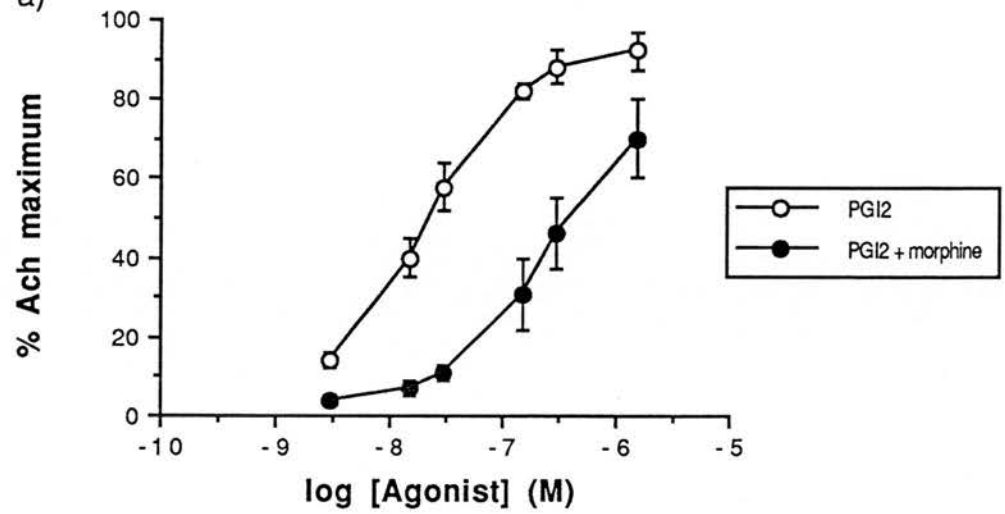
Table 3.1 : The effect of TTX (1μM), Morphine (1μM) and AH 6809 (1μM) on the response to single doses of PGE_2, cicaprost, iloprost, carbacyclin and PGI_2 in the guinea-pig ileum (mean of 4-6 expts.).(± s.e.m)

TREATMENT	% CONTROL RESPONSE REMAINING				
	PGE (14nM)	cicaprost (13nM)	iloprost (14nM)	carbacyclin (150nM)	PGI_2 (1μM)
TTX (1μM)	63 ± 3**	0***	37 ± 7**	61 ± 5**	_
morphine (1μM)	62 ± 6**	0***	40 ± 3***	67 ± 5**	_
AH6809 (1μM)	33 ± 4***	87 ± 5	45 ± 6***	54 ± 8**	58 ± 5**
AH6809 (1μM) +morphine (1μM)	7 ± 4***	0	_	_	27 ± 10**

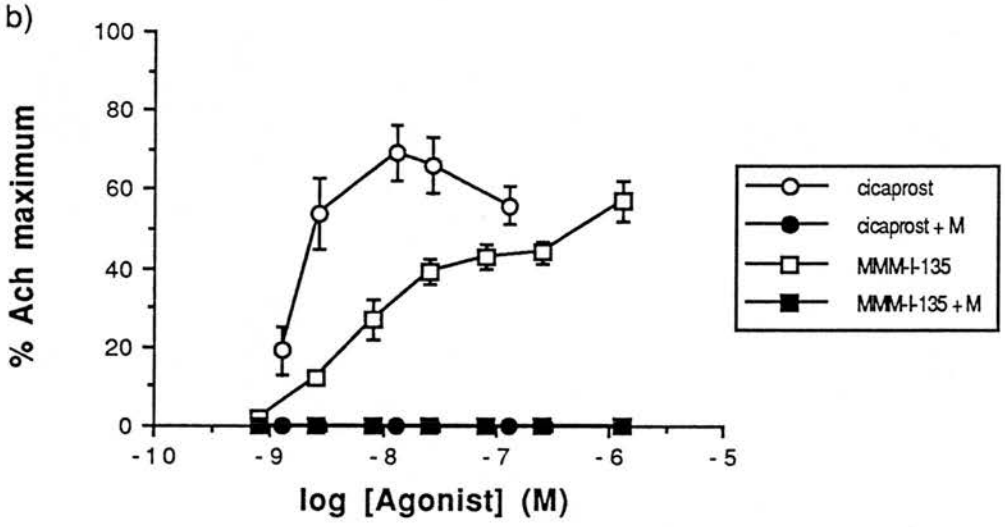
*** P>0.001
** P>0.01

Figure 3.10: Effect of morphine (M) (1μM) on the log concentration-response curves of (a) PGI_2 (b) cicaprost and MMM-I-135 and (c) iloprost and carbacyclin in the guinea-pig ileum (mean 4-6 expts.).

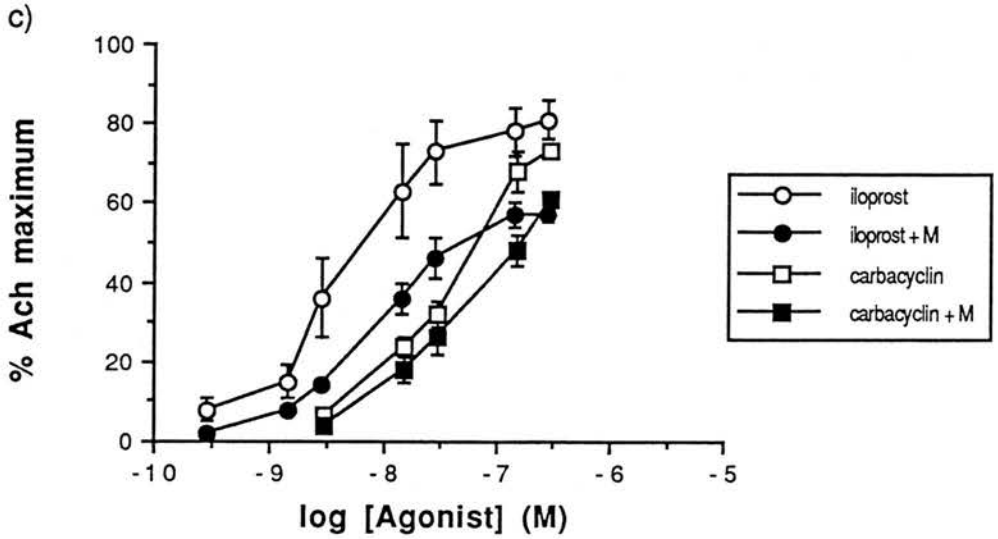
a)



b)



c)



Action of AH 6809

Single doses of the prostanoids: The response to a single dose of cicaprost (13 nM) was unaffected by 1 μM AH 6809, however that to iloprost (14 nM), carbacyclin (150 nM) and PGI_2 (1 μM) were significantly inhibited (Table 3.1).

Concentration-response curves: The presence of 2 μM AH 6809 in the Krebs solution was found to greatly increase the spontaneous activity of the preparation, making response measurements impossible, however, the presence of morphine (1 μM) significantly reduced this effect. Hence, concentration-response curves were obtained in the presence of morphine (1 μM) and in the presence of morphine (1 μM) plus AH 6809 (2 μM), thus eliminating any neuronally-mediated action of the prostanoids. AH 6809 caused a highly significant shift in the curves for both iloprost and carbacyclin (Fig. 3.11). However, the log concentration-response curves of iloprost did not appear to be parallel and the dose-ratio obtained at the EC_{25} level (245) is ~10 times greater that found for carbacyclin.

Action of atropine and SP 150

At a concentration of 200 nM atropine produced a dose ratio of 427 against Ach, consistent with a pA_2 of 9.3 (Fig. 3.12). In the presence of this concentration of atropine the concentration-response curve of cicaprost appears to have a lower maximum, however, the EC_{50} values (as calculated from the maximum of each curve) are similar (see Fig. 3.15C). The concentration-response curves of iloprost and carbacyclin were shifted to a similar extent as in the presence of 1 μM morphine (Fig. 3.13).

Table 3.2 shows results demonstrating that at concentrations of 5 and 10 μM of the SP blocker appeared to be non-specific, inhibiting responses to both SP (3 nM) and Ach (37 nM). However, at 1 μM SP 150 reduced the response to a submaximal dose of SP by 91%, but had no effect on the response to Ach. In addition, this dose of SP 150 significantly inhibited (37%) the response to 13 nM cicaprost (Table 3.3).

Figure 3.11: Effect of AH 6809 (2μM) on the log concentration-response curves of (a) iloprost and (b) carbacyclin in the guinea-pig ileum (mean of 4-6 expts.).

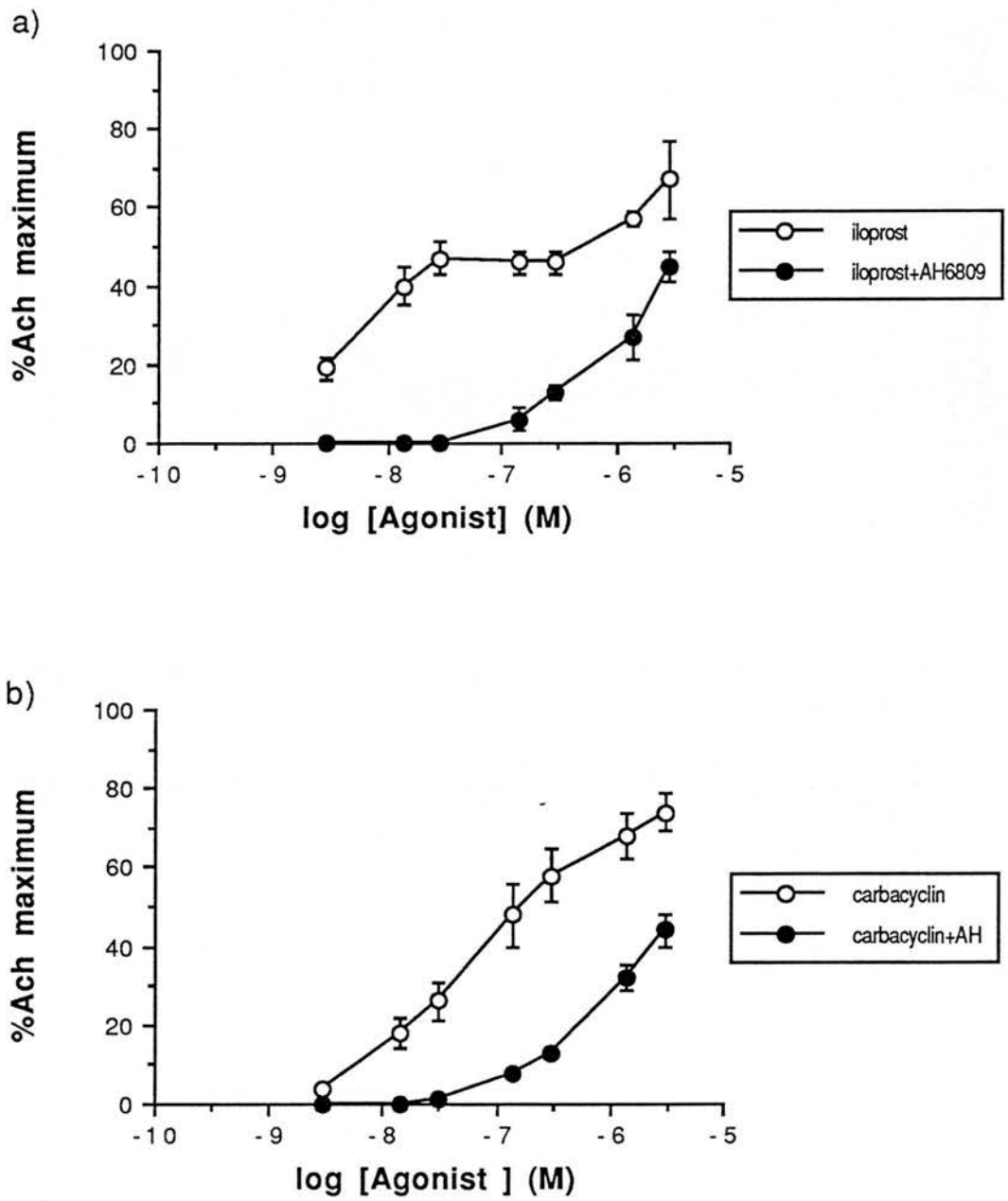


Figure 3.12: Effect of atropine (200nM) on the log concentration-response curves of Ach in the guinea-pig ileum (mean of 2 expts.).

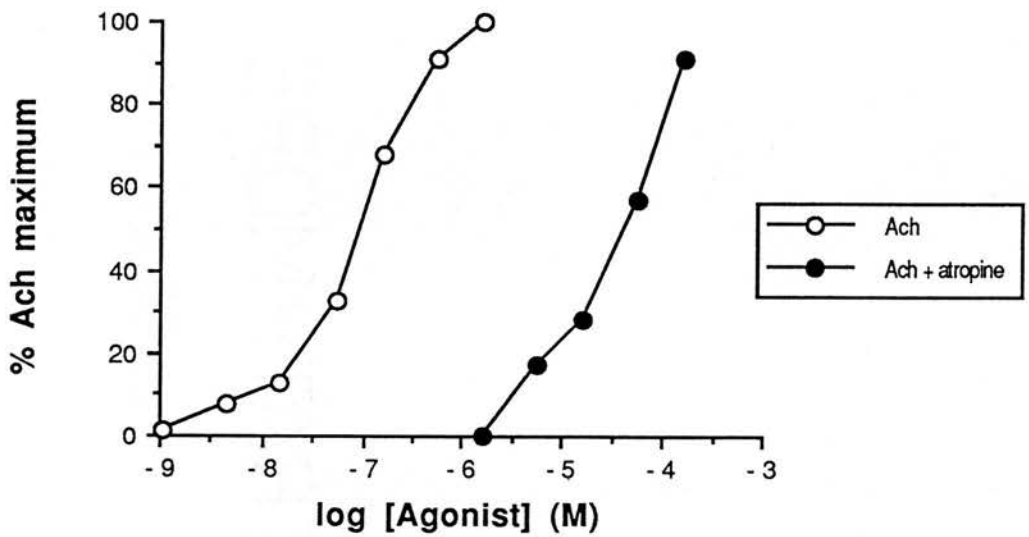
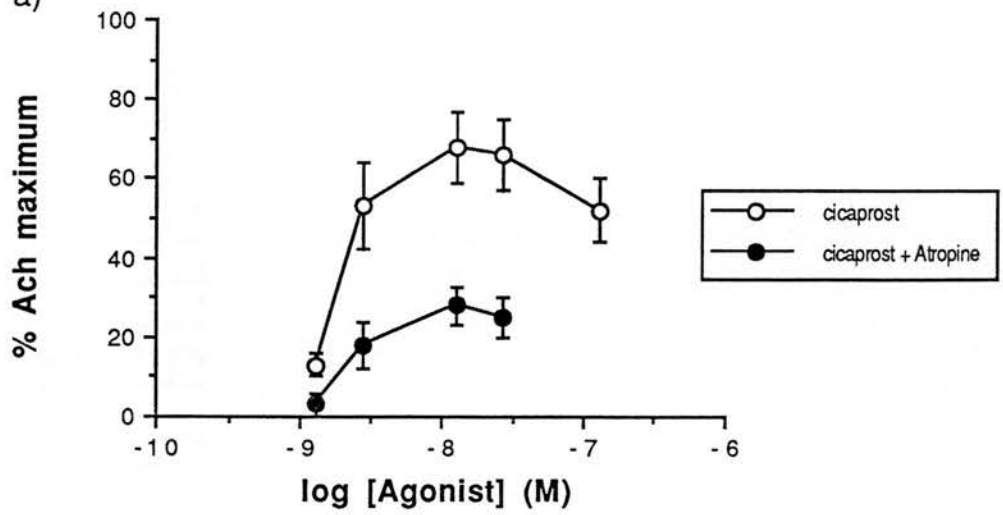
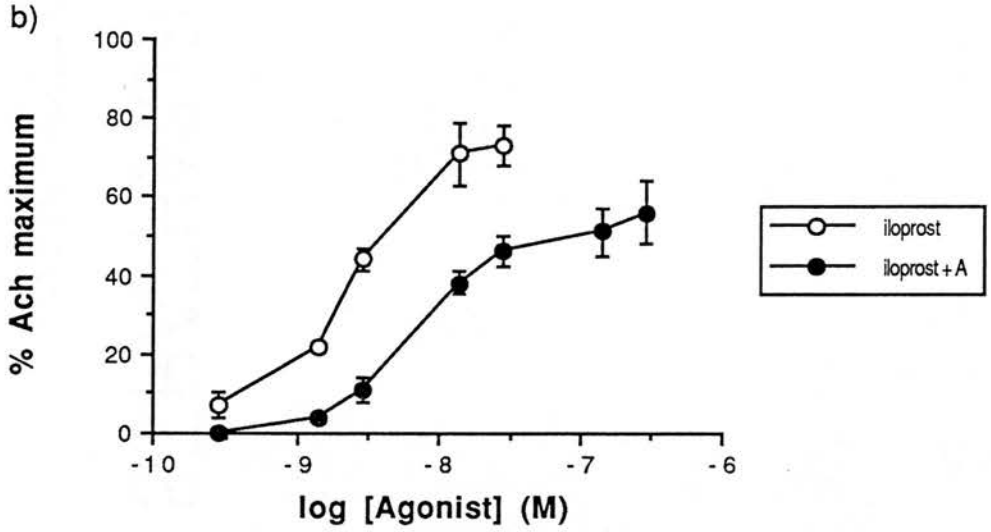


Figure 3.13: Effect of atropine (200nM) on log concentration-response curves of (a) cicaprost (b) iloprost and (c) carbacyclin (mean 4-6 expts.).

a)


100
80
60
40
20
0
% Ach maximum
-10
-9
-8
-7
-6
log [Agonist] (M)
cicaprost
cicaprost + Atropine

b)


100
80
60
40
20
0
% Ach maximum
-10
-9
-8
-7
-6
log [Agonist] (M)
iloprost
iloprost + A

c)

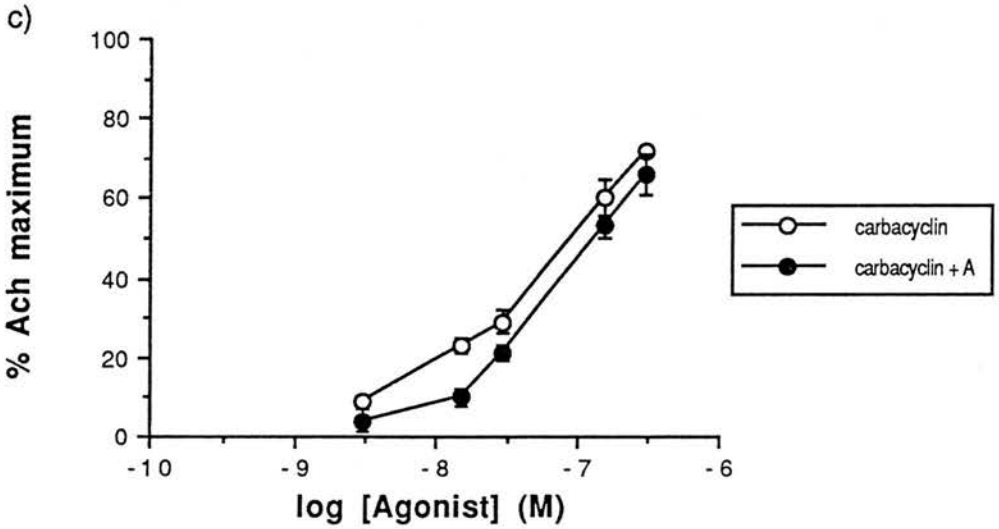

100
80
60
40
20
0
% Ach maximum
-10
-9
-8
-7
-6
log [Agonist] (M)
carbacyclin
carbacyclin + A

Table 3.2: The effect of various concentrations of SP 150 on the response to single doses of Ach, cicaprost, PGE_2 and Substance P in the guinea-pig ileum.

	% INHIBITION			
SP 150 concentration	ACh (37nM)	Cicaprost (13nM)	PGE_2 (14nM)	Substance P (3nM)
10 μM (n = 2)	50	100	60	100
5 μM (n = 2)	35	81	42	100
1 μM (n = 5)	0 ± 0	37 ± 6	22 ± 5	98 ± 2

Table 3.3: The effect of morphine (1 μM), Atropine (200 nM) and SP 150 (1 μM) on the response to single doses of PGE_2, cicaprost, Ach and Substance P in the guinea-pig ileum (mean of 4-6 expts.).

TREATMENT	% CONTROL RESPONSE REMAINING			
	PGE_2 (14nM)	cicaprost (13nM)	Ach (37nM)	substance P (3nM)
atropine (200nM)	73 ± 1*	34 ± 11*	0	80 ± 4*
SP150 (1μM)	78 ± 5*	63 ± 6**	98 ± 2	9 ± 2***
atropine (200nM) + SP150 (1μM)	53 ± 5**	0	–	–
morphine (1μM)	62 ± 5**	0	–	–
morphine (1μM) + SP150 (1μM))	63 ± 6	–	–	–

*** P<0.001
** P<0.01
* P<0.05

Inhibition of Histamine-induced contractions

Cicaprost consistently caused a dose-related inhibition of contractions of the ileum induced by sub-maximal doses of histamine (Fig. 3.14 and 3.15D), the IC_{25} obtained was 22 ± 3 nM. PGI_2 and iloprost (see Figure 3.15D) also inhibited Histamine contractions. However, they caused an initial contraction of the tissue prior to inhibition and the inhibitory effect did not reach a maximum until several subsequent histamine doses had been added. In a number of tissues (2/6 for iloprost and 2/5 for PGI_2) no inhibition was observed. In contrast, carbacyclin did not inhibit histamine concentrations but instead potentiated these responses.

Figure 3.14: Log concentration-response curve of cicapost for the inhibition of histamine -induced contraction of the guinea-pig ileum (mean of 5 expts.).

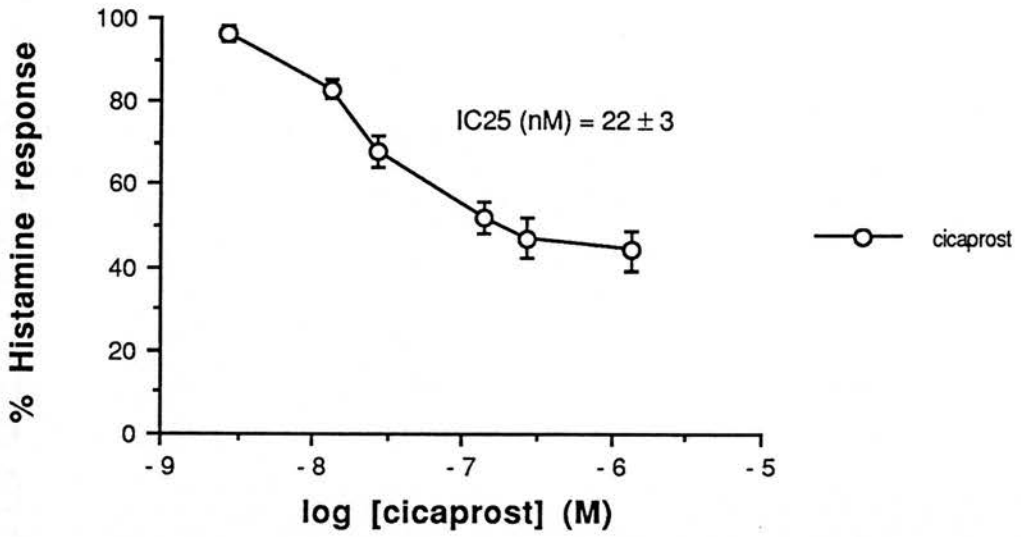
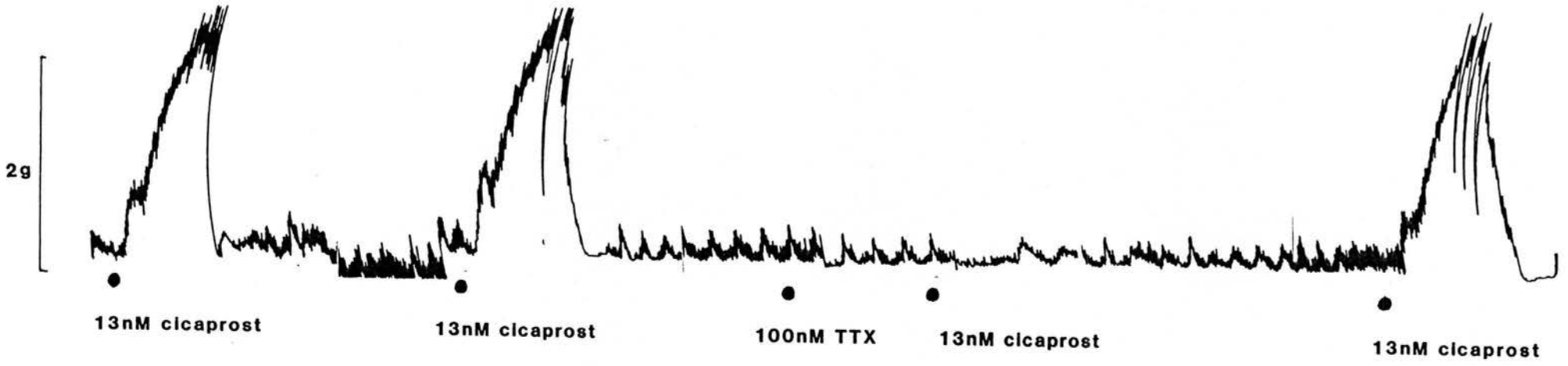


Figure 3.15A: Trace showing contraction of the guinea-pig ileum by cicaprost (13nM) and inhibition of this effect by TTX (100nM).


2g
13nM cicaprost
13nM cicaprost
100nM TTX
13nM cicaprost
13nM cicaprost

Firure 3.15B: Trace showing the effect of morphine (1μM) on the contractile action of increasing concentrations of cicaprost in the guinea-pig ileum.

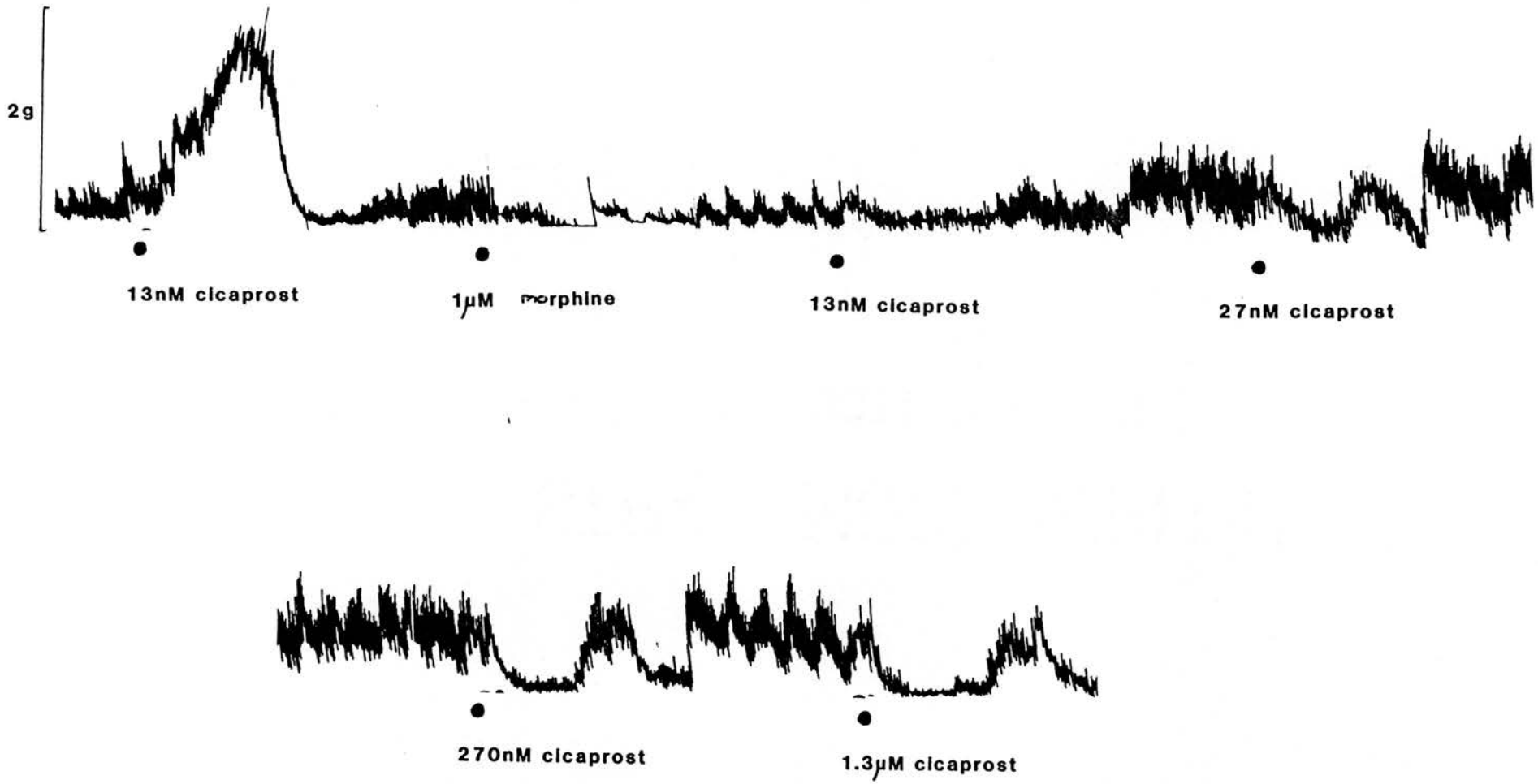

2g
13nM cicaprost
1μM morphine
13nM cicaprost
27nM cicaprost
270nM cicaprost
1.3μM cicaprost

Figure 3.15C: Trace showing the effect of (a) atropine (200nM) and SP 150 (1μM) and (b) SP 150 (1μM) on the contractile action of cicaprost (13nM) in the guinea-pig ileum.

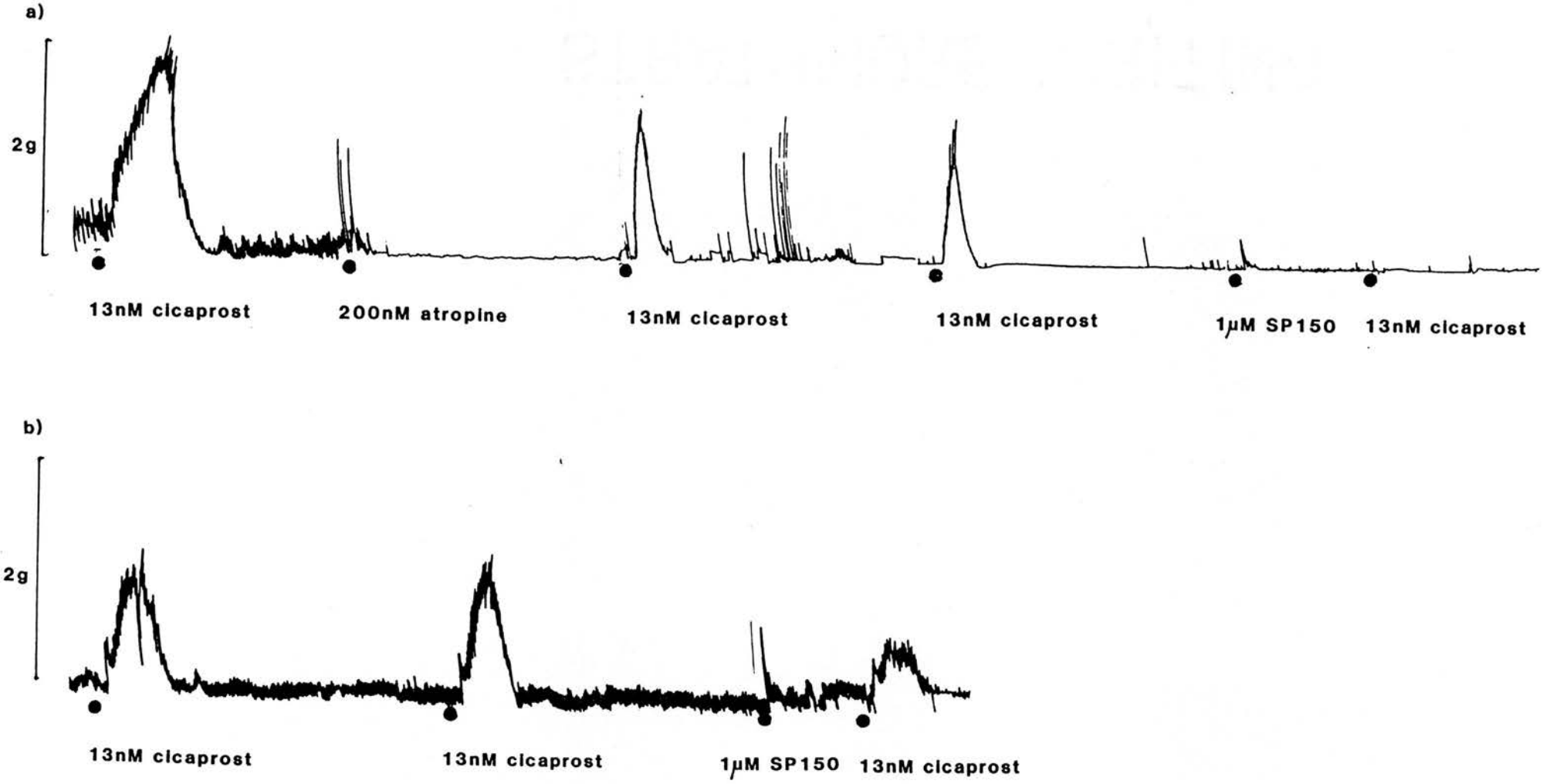
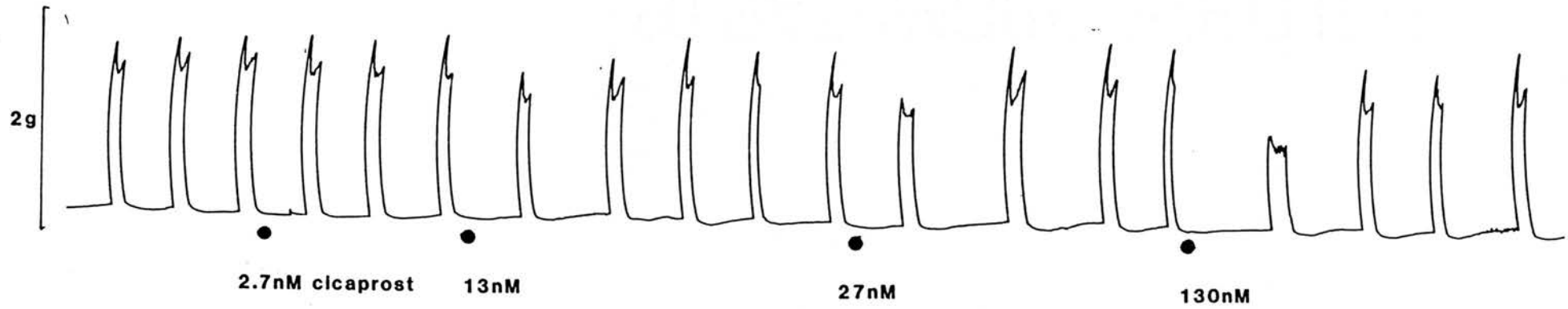

a)
2g
13nM cicaprost
200nM atropine
13nM cicaprost
13nM cicaprost
1μM SP150
13nM cicaprost
b)
2g
13nM cicaprost
13nM cicaprost
1μM SP150
13nM cicaprost

Figure 3.15D: Trace showing the inhibitory action of increasing concentrations of (a) cicaprost and (b) iloprost on contractions of the guinea-pig ileum induced by histamine. The tissues were bathed in Tyrodes solution containing AH 6809 (2μM) and morphine (1μM).

a)


2g
2.7nM cicaprost
13nM
27nM
130nM

b)


2g
14nM Iloprost
29nM
140nM

DISCUSSION

Vascular Actions

The results obtained with iloprost and cicaprost (Table 3.4) closely parallel those obtained by Hadhzay (1986) who found that PGI_2 relaxed the human mesenteric artery with an IC_{50} of 7 nM and the sensitivity of the mesenteric vasculature decreased in the order man, dog, rabbit, pig. The data presented show that the human mesenteric artery is the most sensitive preparation, with an IC_{50} of 1.2 nM for iloprost. Investigations of the pig vasculature showed that the pulmonary, intralobular and coronary arteries were not relaxed by PGI_2, and the gastro-epiploic artery was found to be the most sensitive vessel (iloprost IC_{50} = 24 nM).

It is possible that the true activity of the compounds at the IP-receptor may be obscured/accentuated by agonist actions at additional prostanoid receptor sites present on the smooth muscle. Table 3.5 shows the vascular relaxant and contractile activity of PGE_2 and 16,16-dimethyl PGE_2. In visceral vessels from the pig, man and dog PGE_2 is highly active as a dilator, the vessel from man being the most sensitive (IC_{50} = 0.27 nM). In general smooth muscle relaxation is thought to be mediated by an EP_2 receptor (Dong et al, 1986; Coleman et al, 1989), this is supported by the finding that PGE_2-mediated relaxation in the rabbit mesenteric artery is not inhibited by the EP_1 receptor blocker, AH 6809. However, previous work has shown that iloprost has little activity at EP_2-receptors in the guinea-pig and cat trachea (Dong et al, 1986; Sheldrick et al, 1988). In addition, our studies suggest that iloprost and carbacyclin have little activity at the EP_2-receptor mediating relaxation of the rabbit jugular vein (see Chapter 4). Thus, an agonist action of the PGI_2 analogues at the EP_2 receptors of the arteries is unlikely.

There is, however, evidence for the presence of an EP-receptor mediating contractile responses in these vessels: (1) In the pig and rabbit the log concentration-response curve for PGE_2-mediated relaxation reverses at higher concentrations, and (2) 16,16-dimethyl PGE_2, which is highly active at EP_1/EP_3 receptors (Dong et al, 1986; see Chapter 4) contracts all three preparations. In vessels from man and

TABLE 3.4 : Relaxant activity of prostacyclin mimetics on visceral arterial vessels.

COMPOUND	Equi-effective molar ratio			
	Human mesenteric artery	Pig gastro-epiploic artery	Rabbit mesenteric artery	Dog mesenteric artery
iloprost	1.0 (IC_{50} = 1.2 ± 0.3nM)	1.0 (24 ± 3nM)	1.0 (10 ± 1nM)	1.0 (2.4 ± 0.6nM)
cicaprost	0.36 ± 0.08	0.87 ± 0.17	0.53 ± 0.03	0.46 ± 0.03
carbacyclin	9.6 ± 1.5	(see text)	2.1 ± 0.2	3.6 ± 0.04
EP157	220 ± 46	81 ± 26	230 ± 32	898 ± 134

Each value is the mean of 6-9 observations in the human, pig and rabbit vessels and 4-6 in the dog vessel.

Table 3.5: Relaxant and contractile activity of PGE_2 and 16,16 dimethyl PGE_2 in arterial vessels from the human, pig and rabbit.

PROSTANOID	IC_{50} / EC_{50} (nM)		
	Human	Pig	Rabbit
PGE_2	↓ 0.27	↓ 2	↓ 4
	–	↑ >100	↑ >100
16,16 dm PGE_2	*↑ 40	*↑ 5	*↑ 30

↓ Relaxation of Phenylephrine-induced contraction

↑ Contraction in the presence/*absence of Phenylephrine

rabbit, 16,16-dimethyl PGE_2 contracts the tissue at relatively high concentrations (EC_{50} >30 nM) and there is no evidence that the PGI-mimetics are acting on EP-contractile receptors. However, in the pig gastro-epiploic artery, in which the EC_{50} for 16,16-dimethyl PGE_2 is 5 nM, carbacyclin has a considerably lower maximum for relaxation than iloprost and at concentrations above 100 nM it causes discrete contractions. An opposing action of carbacyclin at contractile EP-receptors may explain these results. In this preparation, the concentration-response curves of carbacyclin and 16,16-dimethyl PGE_2 are unaffected by AH 6809 indicating that the contractile receptor is not EP_1. It is notable that Ahluwalia et al (1988) have demonstrated EP_3 mediated vascular smooth muscle contraction in the rabbit renal artery. Experiments (Table 3.6 Jones, unpublished data) have shown that carbacylin has EP_3 agonist activity in the guinea-pig vas deferens, although it is considerably less active than PGE_2 (EMR, 70). The low sensitivity of the pig vessel to relaxation induced by IP receptor agonists may allow this contractile action to become apparent. An action of carbacyclin at contractile thromboxane (TP)-receptors is excluded by the failure of the TP-receptor blocker, EP 092 to affect the concentration-response curve (Fig. 3.6).

PGI_2 has previously been shown to have a weak contractile action in vessels from the pig, human and rat (Dusting et al, 1977; Levy, 1978; Davis et al, 1980); these effects are probably mediated by EP_1/EP_3 receptors. It is notable that when Rucker and Schror (1983) investigated the binding of [^{3}H]-iloprost to smooth muscle cells of the pig aorta they found that the Hill plot was linear with a slope of 1.9. The authors presented two possible explanations for this: (1) positive cooperativity with two agonists binding to the same site and (2) the ligand binding to a component other than the IP-receptor. The data presented here would suggest that the PGI_2 analogue may have bound to an EP_1/EP_3 receptor.

Invoking the above explanation for the low maximum of carbacyclin in the pig, the data suggest that the IP-receptors present in vessels from man, pig and rabbit are the same. In these tissues the order of potency is cicaprost, iloprost, carbacyclin, EP 157. EP 157 relaxed at concentrations 100-200 times that of iloprost. However, in the dog

Table 3.6: pD_{50} values for PGE_2, cicaprost, iloprost and carbacyclin for contraction of the guinea-pig trachea and for inhibition of the twitch response of the guinea-pig vas deferens (mean of 4 expts.).

PROSTANOID	Guinea-pig vas deferens		Guinea-pig trachea
	pD_{50}	EMR	pD_{50}
PGE_2	8.76 ± 0.03	(1)	_
cicaprost	≤ 6.0	(>500)	< 6.0
iloprost	6.79 ± 0.09	(88)	8.08 ± 0.13
carbacyclin	6.96 ± 0.12	(70)	7.57 ± 0.10

TABLE 3.7: Comparison of the abilities of prostacyclin mimetics to inhibit aggregation and to raise cyclicAMP levels (bracketed values) in platelets from different species (Armstrong et al, 1989).

	Equi-effective molar concentrations				
Prostanoid	Human	Pig	Horse	Rabbit	Rat
iloprost	1.0 IC_{50}= 0.15 ± 0.04	1.0 2.3 ± 0.8nM	1.0 23nM	1.0 17.1 ± 2.6nM	1.0 4.7 ± 1.5nM
cicaprost	0.88 ± 0.06 (1.0)	1.1 ± 0.26 (1.20)	0.4 (1.0)	1.3 ± 0.21 (0.91)	0.6 ± 0.09 (0.96)
carbacyclin	31 ± 6 (23)	13.5 ± 6.7 (27)	15 (5.8)	3.3 ± 0.4 (2.9)	3.5 ± 0.5 (2.7)
EP157	54 ± 9 (72)	101 ± 23* (>1100)	98 (57)	990 ± 210* (>890)	3500 ± 1500* (>1300)

mesenteric artery, although the order of potency is the same as that found in the other vessels, EP 157 was 900 times less active than iloprost. Similar results were found in initial studies by Armstrong and Jones (unpublished observations) on anaesthetized dogs in which changes in arterial blood flow were recorded in response to close intra-arterial infusion of different prostanoids: iloprost was a highly active dilator but EP 157 had no effect. Taken together these results suggest that the IP receptor present in the dog vasculature may differ from that present in the other species. However, no work has been done to investigate the vasodilator activity *in vivo* of EP 157 in the other species.

As discussed in the introduction the emphasis of recent chemistry/pharmacology has been on synthesis of a PGI_2 analogue which retains antiaggregatory actions but has reduced vascular activity. However, in this study the order of activity of EP 157 in the vasculature closely parallels that found on the platelet (Table 3.7) of the same species (man, pig and rabbit) (Armstrong et al, 1989), suggesting that the IP- receptors present in the two systems are the same. This finding seems to leave little hope for separation of the two effects in man. However, the possibility of partial agonism at the platelet IP receptor has been demonstrated (Armstrong et al, 1989). It may be that a compound with lower efficacy and higher affinity than EP 157 could suppress platelet aggregation while having little effect on the vasculature.

Guinea-pig ileum

We have confirmed the findings of Gaion and Trento (1983) indicating the presence of a neuronal PGI_2 receptor in the guinea-pig ileum: the contractile action of PGI_2 is inhibited by morphine and appears to be mediated by activation of the enteric neurones. The results obtained are summarised in Table 3.8. All the PGI_2 analogues tested were active as contractile agents, and the responses to single submaximal doses of cicaprost, iloprost and MMM-I-135 were significantly reduced by both morphine and TTX, indicating that they are agonists at the neuronal IP-receptor site. However, only the responses to cicaprost and MMM-I-135 were abolished by morphine; in the presence of 1 μM morphine

Table 3.8: pD_{25} values for PGI_2 analogues in the guinea-pig ileum in the absence and presence of morphine (1 μM) and AH 6809 (2 μM) (mean of 4-6 expts.).

PROSTANOID	MAX (%Ach)	pD_{25}	pD_{25} MORPHINE (1μM)	pD_{25} AH6809 (2μM)
cicaprost	70 ± 8	8.89 ± 0.11	<6	_
iloprost	85 ± 7	8.62 ± 0.12	8.15 ± 0.06	5.96 ± 0.12
carbacyclin	80 ± 5	7.72 ± 0.08	7.59 ± 0.14	6.05 ± 0.11
PGI_2	95 ± 2	8.12 ± 0.08	6.99 ± 0.17	_
MMM-I-135	~60	8.05 ± 0.18	<5.89	_

concentration-response curves to PGI_2, iloprost and carbacyclin could be obtained.

The longitudinal muscle of the guinea-pig ileum contains both EP_1 and EP_3 receptors mediating contraction via a direct action on the smooth muscle cells (see Chapter 4). Investigation of the effect of AH 6809 on the action of the PGI_2 analogues suggests that the morphine-resistant component of action of the PGI_2 analogues is mediated by an EP receptor: (1) the responses to single submaximal doses of PGI_2, iloprost and carbacyclin are significantly inhibited by 1 μM AH 6809 and (2) the concentration-response curves of these compounds are subject to a large shift in the presence of 2 μM AH 6809. We have found PGI_2, iloprost and carbacyclin to contract the guinea-pig trachea, a preparation known to contain EP_1 receptors. Although they appear to be more selective for the EP_1-receptor, the compounds also have some activity on the EP_3 receptors of the chick ileum and the guinea-pig vas deferens (Table 3.6). These results parallel earlier findings on the actions of PGI_2 and iloprost on EP_1 and EP_3 receptors (Dong et al, 1986; Sheldrick et al, 1988). As mentioned in the introduction Gaion and Gambaratto (1987) found that at 1 μM PGI_2 retains a contractile action in the presence of TTX. They suggest that this is mediated by an IP-receptor present on the smooth muscle. However, our data indicates that the TTX/morphine - resistant component of action of PGI_2 is more likely to be due to activation of EP_1 (and perhaps EP_3) receptors. The complete inhibition of the contractile action of cicaprost by 1 μM morphine confirms the work of Dong et al (1986) demonstrating that it is a highly selective IP-receptor agonist. MMM-I-135 would appear to be less active at, but equally selective for the IP receptor.

Further investigations of the nature of the excitatory transmitters released in the response to neuronal IP receptor activation revealed that the action of cicaprost is only partially inhibited by atropine (Table 3.9). It may be that it is easier for atropine to block the effects of exogenous Ach than that released from the myenteric plexus. For example, it has been shown that considerably higher doses of atropine were required to inhibit the response to cholinergic transmission in response to nerve stimulation than those needed to inhibit the response to exogenously

Table 3.9 : The effect of Morphine (1 μM) and Atropine (200 nM) on the concentration-response curves of PGI_2 analogues in the guinea-pig ileum (mean of 4-6 expts.)..

PROSTANOID	DOSE RATIO IN THE PRESENCE OF:	
	Morphine (1 μM)	Atropine (200 nM)
PGI_2	18.9 ± 5 ***	_
cicaprost	>1000	4.2 ± 2 *
iloprost	3.5 ± 1.2 *	4.4 ± 0.73 *
carbacyclin	1.6 ± 0.5	1.9 ± 0.32 *
MMM-I-135	>200	_

*** $P < 0.001$

* $P < 0.05$

added Ach (Bowman et al, 1968). Alternatively, cicaprost may be mediating the release of an additional transmitter whose action is not blocked by atropine but whose release is blocked by morphine. The data would suggest that the residual action of cicaprost is mediated by release of substance P: the substance P blocker, SP 150 abolished the response to cicaprost in the presence of 200 nM atropine. Pharmacological (Franco et al, 1979a and b) and immunological evidence (Donnerer et al, 1987) suggest that Ach or the ganglionic stimulant DMPP are capable of stimulating the intrinsic SP neurones of the intestine. While there is also evidence for the ability of SP to release Ach from the myenteric plexus (Yau et al, 1982 ; Bartho and Holzer, 1985). However, our data show that both atropine and SP 150 inhibit the response to cicaprost, and their action appears to be additive, thus it would appear that IP- receptor activation mediates the release of both SP and Ach.

It has been shown by a number of groups (see *'Action of morphine'*) that the release of both Ach and SP is inhibited by morphine. This appears to be true in this system where neither transmitter is released in the presence of 1 μM morphine. In fact, Bartho and Holzer (1985) stated that "among the drugs and procedures that influence SP release there is not one which would not influence the release of Ach in the same direction". It has been proposed that the similarities in the pharmacological profiles of enteric cholinergic and SP neurones is due to their co-localization in the same enteric neurones (Furness et al, 1984). It is notable that it has previously been suggested that SP release from nerve endings could be implicated in the algesic response to both PGE_1 and PGI_2. Nakamura and Smith (1988) reported that the inflammatory hyperalgesic response to both prostanoids was mimicked by SP and inhibited by a SP antagonist.

The fact that the log concentration-response curve for cicaprost is bell shaped (Fig 3.8) prompted us to investigate the activity of the compound as an inhibitory agent. Fig.3.15D shows that cicaprost causes a dose-related inhibition of histamine-induced responses, occurring at doses corresponding to those at which the log concentration-response curve bells. These results suggest that the preparation contains an additional

IP-receptor present on the smooth muscle (any neuronal activity is inhibited by the presence of 1μM morphine), which mediates inhibitory responses.

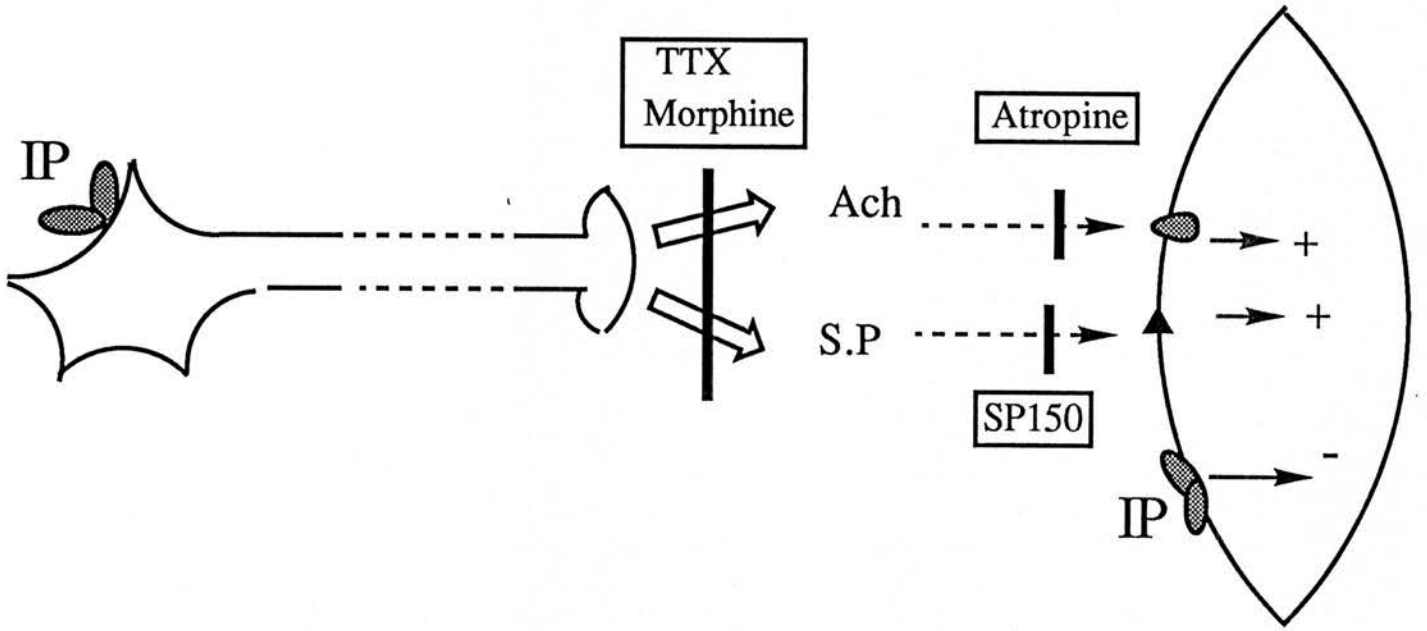
PGI_2 and iloprost also inhibit histamine responses, however, the results are inconsistent. Although these experiments were carried out in the presence of 1 μM morphine and 2 μM AH 6809, in some preparations there is evidence of an increase in tone prior to inhibition. This is probably due to an EP_1 (overcoming the block) and/or and EP_3 action of these compounds and makes it difficult to assess the level of inhibition obtained with these compounds. It is notable that the shift in the concentration-response curve of iloprost in the presence of 2 μM AH 6809 (Dose ratio = 249) is considerably larger than that of any other PGE (Dose ratio against PGE_2 is 34, see following Chapter) or PGI analogue tested. This may reflect a high EP_1-selectivity of this compound, however, it seems more likely that the shift is accentuated by the agonist action of high concentrations of iloprost at IP-relaxant receptors. In contrast, carbacyclin does not display any inhibitory actions but rather contracts the tissue thus potentiating histamine-induced responses. As found in the pig gastro-epiploic artery this is probably due to an agonist action of this compound at contractile EP_1/EP_3 receptors opposing any inhibitory effects mediated by IP-receptors.

A schematic representation of the IP receptors found in the longitudinal muscle of the guinea-pig ileum is shown in Figure 3.16. Taking into account the EP receptor activity of the analogues, the data presented shows that the EMR values obtained are similar at both the neuronal and the smooth muscle IP-receptor sites. Thus, there is no evidence to suggest that these sites differ from one another or from those found on the vasculature of the human, pig or rabbit.

Structure-Activity Relationships

The results presented confirm the finding that the introduction of a CH_3 group at the C16 position of the carbacyclin molecule enhances the potency of the compound at all the IP-receptor sites investigated. As mentioned in the introduction iloprost used in this study contains both

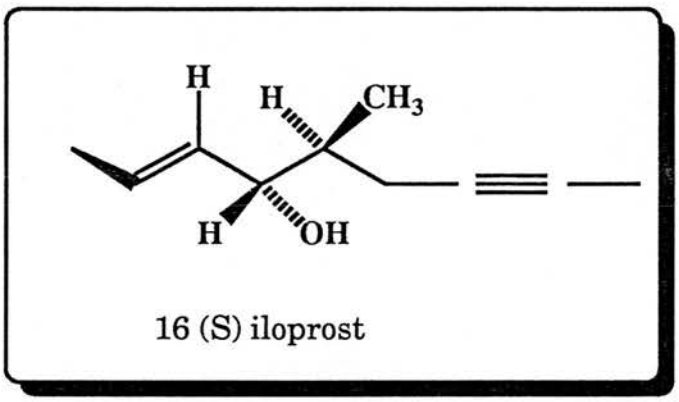
Figure 3.16: Schematic representation of IP receptor sites in the guinea-pig ileum



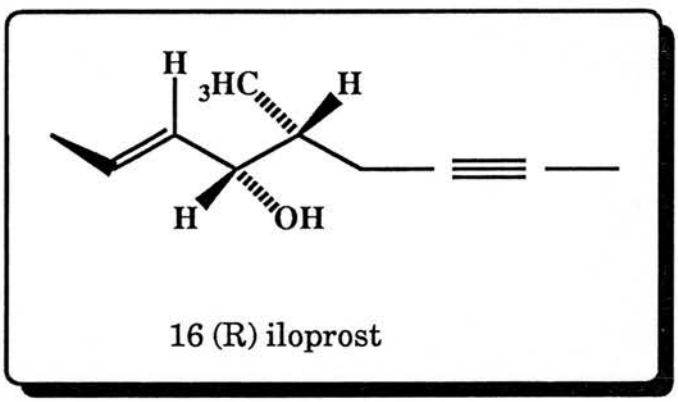
the 16(R) and the 16(S) isomers (Figure 3.17). However, Tsai et al (1988) have recently demonstrated that the 16(S) isomer was 20-times more potent than the 16(R) isomer in inhibiting collagen-induced platelet aggregation. In addition, study of the binding of the isomers to platelet membranes demonstrated that the 16(S) isomer had a significantly higher affinity for the site than the 16(R) isomer (K_d = 13 nM and 288 nM respectively). The authors suggest that the (S) methyl group limits the flexibility of the molecule which facilitates the binding and allows close interaction of the functional groups of iloprost with the IP receptor site. These results have recently been confirmed in experiments by Imaki et al (1989). They synthesised carbacyclin and 6-keto PGE_1 (an IP receptor agonist - Hoult and Moore, 1986) with an alkyl cyclopentane ring on the ω chain in which there are two new chiral centres at C16 and C18 and found that the 16(S) isomers of both compounds were considerably more potent than the 16(R) isomer in both platelet and blood vessel. In view of these findings it would be of interest to investigate whether the IP activity of these compounds is confined to the 16(S) and the EP_1/EP_3 to the 16(R) isomer.

The potency and specificity of cicaprost for the IP receptor may therefore be due to: (a) the introduction of a pure 16(S) methyl group (Figure 3.17) and (b) further restriction of side chain movement conferred by the 13,14-acetylenic bond. While MMM-I-135 appears to retain the specificity of cicaprost due to the 13,14 acetylenic bond, the absence of the CH_3 group at C16 may account for the lower potency of the compound.

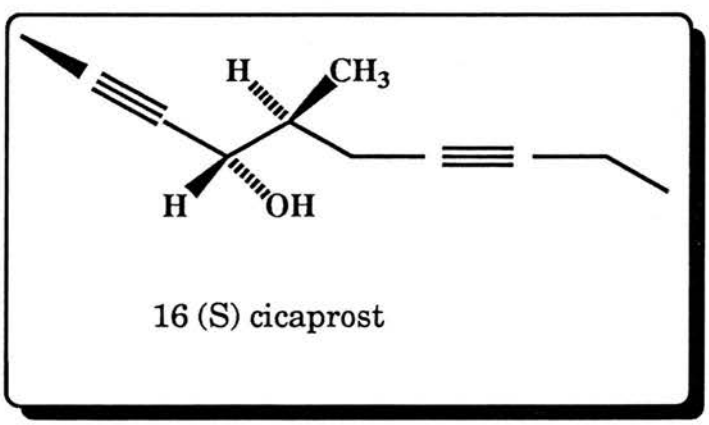
Figure 3.17: Structures of the 16 (S) and 16 (R) isomers of iloprost and cicaprost.



16 (S) iloprost



16 (R) iloprost



16 (S) cicaprost

Chapter 4

EP-Receptor Studies

Prostaglandin E (PGE) was first isolated by Bergstrom (1966) in a pure crystalline form from sheep prostatic glands. Since their discovery, prostanoids of the E series have been shown to have activity in a range of smooth muscle systems. They are known to be potent vasodilators in most vascular beds (Holmes et al, 1963; Horton, 1963 and 1965; Bergstrom et al, 1965; Euler, 1966; Nakano and McCurdy, 1967; Hatano et al, 1980), and more recently PGE_2 has been shown to constrict vascular smooth muscle (Hatano et al, 1980 and 1981; Hayashi et al, 1986; Ahluwalia et al, 1988; Baxter et al, 1989). PGE also mediates both relaxation and constriction of respiratory and uterine smooth muscle (Horton, 1969; Apperley et al, 1979; Gardiner and Collier, 1980; Coleman and Kennedy, 1985; Coleman et al, 1989). Finally, PGE has potent actions in the gastrointestinal tract: in general it relaxes circular and contracts longitudinal smooth muscle (Horton, 1965; Bennet et al, 1968; Bennet et al, 1975).

In addition to their actions on smooth muscle, PGE_1 and PGE_2 can both inhibit and promote neurotransmitter release in a number of tissues (Ehrenpreis et al, 1973). They are potent inhibitors of gastric acid secretion in many species (Karim et al, 1973; Konturek et al, 1976; Frame and Main, 1980; Broughton-Smith and Whittle, 1981; Reeves et al, 1988) and have a range of actions on both the eye and the kidney (Pederson, 1975; Bentley and McGahen, 1982; Remuzzi et al, 1987).

Prostaglandins of the E series have also been implicated in the pathophysiology of a range of conditions including: inflammation (Lewis, 1983); rheumatism (Moilanen et al, 1987); pain (Deraedt et al, 1980; Ferreira, 1983); cancer (Bennet, 1983) and pyrexia (Milton and Wendlandt, 1971).

PGE receptors (see Table 4.1a and 4.1b)

Bennet and Posner (1971) first suggested that subtypes of the PGE_2 receptor may exist. They showed that the dibenzoxazepine SC 19220 (Figure 4.1C) blocked the contractile action of PGE_2 on the longitudinal muscle of the guinea-pig ileum, while the relaxant action on the

Table 4.1a: Summary table of the EP-receptor work prior to this thesis.

Receptor	Tissue	Response	Agonists	Antagonists
EP_1	Guinea-pig ileum longitudinal muscle	contraction	ICI 80205	SC 19220
	Guinea-pig/dog stomach fundus	"		AH 6809
	Guinea-pig trachea	"		
	Bullock iris sphincter	"		
EP_2	Cat/guinea-pig trachea	relaxation	11-deoxy PGE_1	none
	Guinea-pig ileum circular muscle	"		
EP_3	Chick ileum	contraction	sulprostone	none
	Guinea-pig vas deferens	inhibition of neurotransmitter release		
	Rat gastric mucosa	Inhibition of acid secretion		

TABLE 4.1b: Previous work on EP receptor sites in various tissues. GPT= guinea-pig trachea (* 16,16 dimethyl PGE_2 = standard agonist), RSF=rat stomach fundus, CT= cat trachea,GPVD= guinea-pig vas deferens, RGAS= rat gastric acid secretion, ChI= Chick ileum, GPF= Guinea-pig fundus.
NA = not active and p.a = partial agonist.

References (Bracketed):

(1) Dong et al (1986)
(2) Coleman et al (1988)
(3) Reeves et al (1988)
(4) Coleman et al (1987a)
(5) Coleman et al (1987b)
(6) Eglen and Whiting (1988)
(7) Gardiner (1986)
(8) Sheldrick et al (1986)
(9) Kennedy et al (1982)

PROSTANOID	EQUI-EFFECTIVE MOLAR RATIO (PGE_2=1)															
	EP_1				EP_2						EP_3					
	GPT (contraction)		RSF		GPF		CT		GPT (relaxation)		GPVD		RGAS		ChI	
PGE analogues																
16,16dimethyl PGE_2	1*	(1)	0.27	(1)	0.08	(2)	9.4	(1)	14	(1)	0.12	(3)	0.13	(3)	_	
							27	(2)								
ICI80205	0.23*	(1)	0.048	(1)	_		70	(1)	NA	(1)	_		_		_	
sulprostone	5.6	(4)	_		3.6	(4)	>10,000	(4)	>100	(4)	0.16	(3)	0.05	(3)	0.7	(5)
misoprostol	21	(7)	_		43	(2)	3.7	(2)	NA	(6)	1.0	(3)	0.24	(3)	_	
11-deoxy PGE_1	NA*	(1)	65	(1)	_		13	(1)	12	(1)	_		_		_	
butaprost	NA	(7)	>10,000	(7)	_		26	(7)	<1	(7)	_		_		>1000	(7)
PGI analogues																
PGI_2	~250	(1)	~47	(1)	20	(9)	~200	(1)	>680	(1)	_		_		13	(9)
iloprost	p.a*	(1)	p.a	(1)	0.8	(8)	1-10	(8)	>70	(8)	<70	(8)	_		_	
						>270	(1)									
cicaprost	>1000*	(1)	530	(1)			>300	(1)	>660	(1)	_		_		_	

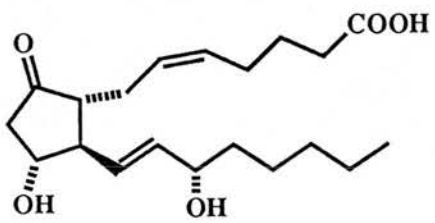
circular muscle was unaffected. More recently, it has been shown that SC 19220 blocks PGE_2-induced contractions of the guinea-pig ileum, guinea-pig and dog fundus and the guinea-pig trachea in a competitive manner (Kennedy et al, 1982; Coleman and Kennedy, 1985), whereas PGE_2-induced contraction of the chick ileum and relaxation of the cat trachea are not inhibited. These results are the basis for the classification of PGE_2 receptors into EP_1 (blocked by SC 19220) and EP_2 (unaffected by SC 19220).

In 1985, Coleman et al reported on a compound with an identical profile to SC 19220 although 10-40 fold more potent as an EP_1 antagonist. They found that AH 6809 (Figure 4.1C) was a specific competitive prostanoid antagonist (pA_2 6.8) on those preparations thought to contain EP_1 receptors (guinea-pig ileum longitudinal muscle, guinea-pig fundus and dog fundus), but weak or inactive (pA_2 <5) on those containing EP_2, FP or TP receptors (cat trachea chick ileum, dog iris and guinea-pig lung respectively).

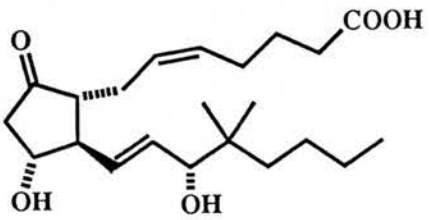
Using agonists, Coleman et al (1987a) produced further evidence for this subdivision: the PGE_2 analogue, sulprostone was found to be highly active as a contractile agent in the guinea-pig longitudinal muscle but inactive as a relaxant in the cat trachea. This pattern was reversed for the PGE_1 analogue, AY 23626. Similarly, Dong et al (1986) compared the contractile and relaxant activity of a number of PGE analogues on a range of preparations. They found that in the bullock iris sphincter, rat stomach fundus, and the guinea-pig trachea ICI 80205 and 16,16-dimethyl PGE_2 where more active as contractile agents than PGE_2, whereas, for relaxant action on the cat trachea, guinea-pig trachea and dog hind limb arterial vessels *in vivo* the order of potency was reversed. In contrast, 11-deoxy PGE_1 exhibited greater relaxant than contractile potency when compared to PGE_2.

Studies by Coleman et al (1987b) on sulprostone and AY 23626 lead to a further subdivision of PGE_2 receptors. On studying their action on the chick ileum and the cat trachea (both contain receptors resistant to block by AH 6809) they found that AY 23626 was active in both preparations (EMR of 5.5 and 1.2 respectively). However, sulprostone

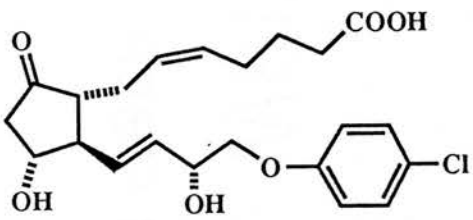
Figure 4.1A: Structures of PGE_2 analogues.



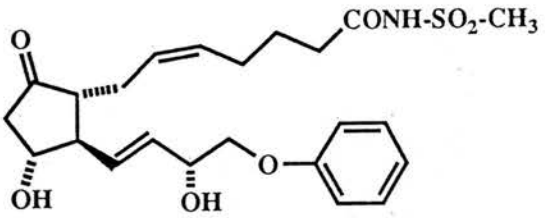
PGE_2



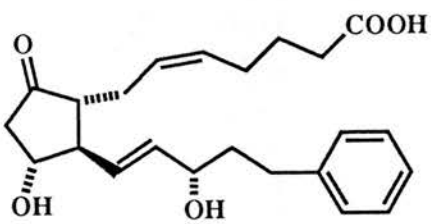
16,16 dimethyl PGE_2



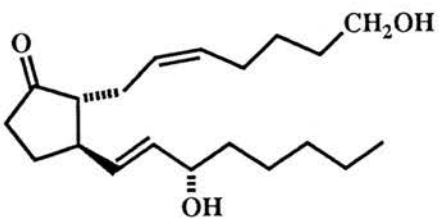
ICI 80205



Sulprostone

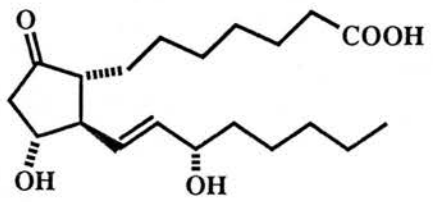


17-phenyl PGE_2-ω-trinor

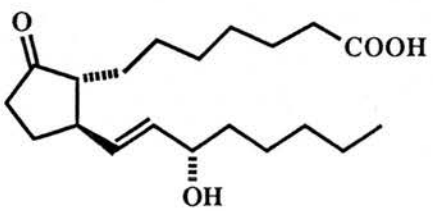


11-deoxy PGE_2 1-alcohol

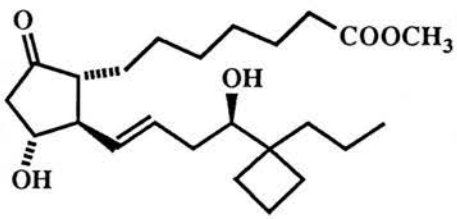
Figure 4.1B: Structures of PGE_1 analogues.



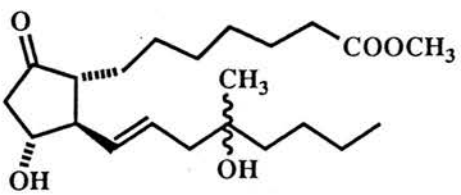
PGE_1



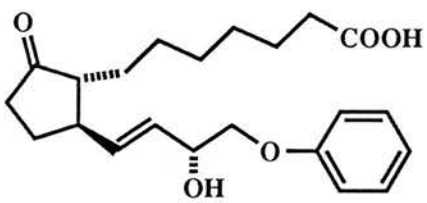
11-deoxy PGE_1



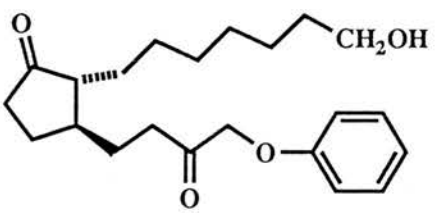
Butaprost



Misoprostol

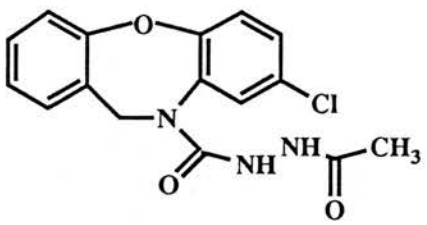


MB 28767

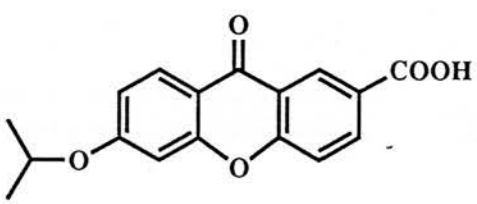


Oxoprostol

Figure 4.1C: Structures of PGE receptor antagonists



SC 19220



AH 6809

was >9000 times less active than PGE_2 in the cat trachea. They proposed the existence of a subgroup of EP_2 receptors to be known as EP_3, upon which sulprostone is highly active. Similar results were reported by the group on comparing the action of sulprostone on the guinea-pig vas deferens and the guinea-pig ileum circular muscle (Coleman et al 1987c). Again, sulprostone was found to be highly active on one preparation (as an inhibitor of twitch in field stimulated guinea-pig vas deferens) and inactive on the other (relaxation of the guinea-pig ileum circular muscle). The agonist activity of PGE_2 on both preparations was unaffected by SC 19220 (300 μM) or AH 6809 (10 μM). More recently, Reeves et al (1988) reported investigations of the activity of various prostanoids as inhibitors of histamine-induced acid secretion in rat isolated gastric mucosa. The rank order of agonist potency was very similar to that found on the guinea-pig vas deferens and the action of sulprostone was unaffected by 300 μM SC 19220. The authors proposed that the effects were mediated by an EP_3 receptor.

In this thesis the actions of PGE_2 and a range of PGE analogues have been investigated in three smooth muscle preparations. There are two primary aims of the study: (1) to further characterise the receptors present in these preparations and assess the usefulness of the tissues in future study of EP receptor subtypes and (2) to study the selectivity/specificity of a range of analogues and comment on their use in the classification of EP receptors.

Tissues investigated

Chick ileum

As discussed earlier the chick ileum and the cat trachea were initially thought to contain EP_2 receptors as PGE_2-induced relaxation was not blocked by SC 19220 (Kennedy et al, 1982). However, subsequent research has shown that sulprostone is active in this preparation but devoid of relaxant activity in the cat trachea (Coleman et al, 1987b). Thus, the receptor mediating contraction of the chick ileum has been defined as EP_3. Initial results in the guinea-pig vas deferens indicate that this tissue has a similar profile to the chick ileum (Reeves et al, 1988).

Rabbit jugular vein

Previous work has shown that the rabbit jugular vein (RJV) contains a number of receptors for non-prostanoids: adrenaline, bradykinin and histamine (Kenakin, 1984).

More recently, the existence of prostanoid receptors in this tissue has been investigated. Giles et al (1989) found that the thromboxane mimetic, U 46619 contracted the preparation with an EC_{50} of ~6 nM. The concentration-response curve of the compound was subject to a parallel rightward shift in the presence of the thromboxane blocker, BM 13.177, consistent with a pA_2 of 6.01 ± 0.01.

This group also investigated the relaxant actions of PGD_2 against histamine-induced contractions. In the absence of BM 13.177, the concentration-relaxation curve for PGD_2 was bell shaped - contractions being produced at concentrations of >100 nM. In the presence of 30 μM BM 13.177, PGD_2 displayed only inhibitory responses with a IC_{50} of ~80 nM. The relaxations induced by PGD_2 and the PGD_2-mimetic, BW 245C, were blocked by the potent DP-receptor antagonist, BW A868C. However, both compounds displayed a phase of relaxation which was resistant to the antagonist, and this effect was not blocked by the EP_1-receptor blocker, AH 6809. On the basis of these results the authors suggested that the BW A868C-resistant relaxation may be mediated by an EP_2 receptor.

We have further investigated the possibility that the rabbit jugular vein contains EP_2 receptors, investigating the relaxant activity of PGE_2, PGE_1, PGI_2 and a range of their analogues.

Guinea-pig ileum - longitudinal muscle

Prostaglandins have been found in the gastrointestinal wall of man and many other animals including the guinea-pig (Ambache et al, 1966). It has been shown that both PGE_1 and PGE_2 (2-500 nM) contract the longitudinal muscle of the guinea-pig ileum in a concentration dependent manner. These contractions are unaffected by hexamethonium but partially blocked by tetrodotoxoin (TTX), hyoscine and atropine (Horton, 1965; Bennet et al, 1968.). In view of these results it

was suggested that prostanoids stimulate two sites in the longitudinal muscle: intrinsic cholinergic nerves and smooth muscle cells (Bennet et al, 1968). Further evidence for a neural component of the action of PGE_2 was found in experiments involving the use of morphine (see Chapter 3 - Introduction). Sanner (1971) demonstrated that 0.26 μM morphine produced an inhibition of the contractile response to PGE_1 (60%) and PGE_2 (41%). He noted that "prostaglandins have the potential for producing part of their stimulatory effect by way of the intrinsic nervous system. Whether this action is seen under physiological conditions is open to conjecture".

A number of attempts have been made to measure the effect of PGE_2 on Ach release in the isolated guinea-pig ileum longitudinal muscle. Hadhzay et al (1973) and Illes et al (1974) found that PGE_2 in concentrations up to 50 nM had no effect on Ach release. However, Kadlec et al (1978) reported data showing that PGE_2 caused a significant increase in Ach release at low concentrations (6 nM). The discrepancy may be explained by the finding that the ability of PGE_2 to increase Ach was inversely proportional to the initial output (Kadlec, 1978). Yagasaki et al (1981) demonstrated the release of Ach from Auerbach's plexus by PGE_1, this action was inhibited by TTX.

A physiological role for PGE_2 in cholinergic transmission has been postulated on the basis of experiments investigating the effect of the cycloxygenase inhibitor, indomethacin on electrically-induced contractions of the tissue. Ehrenpreis et al (1973) demonstrated that indomethacin inhibited electrically-induced contractions of the ileum, although the very high concentration used (111 μM) has been subsequently shown to have non-specific inhibitory effects on Ach and histamine contractions (Bennet et al, 1975). However, using a modified Krebs solution a number of groups have demonstrated an inhibitory action of lower concentrations (1-3 μM) indomethacin on electrically-induced contractions, which could be reversed by PGE_2 (Kadlec et al, 1974; Bennet et al, 1975; Poll et al, 1988 a and b).

In order to show unequivocally that PGE has a physiological role in mediating cholinergic transmission in the guinea-pig ileum one should

measure the degree of synthesis inhibition and the degree of twitch inhibition simultaneously in each tissue. Also, the action of a number of structurally distinct cyclo-oxygenase inhibitors should be compared. Botting and Salzmann (1974) found that 2.8 μM indomethacin caused a significant inhibition of PGE output both at rest and during field stimulation, however it had no effect on Ach output during field stimulation. In addition, Hedqvist et al (1980) demonstrated that PGE_2 enhanced the contractile responses of the ileum to nerve stimulation, Ach and direct muscle stimulation. However, the author concluded that this was due to a postsynaptic potentiating action as PGE_2 had no effect on the release of Ach during field stimulation.

EP-receptors present: Kennedy et al (1982) investigated the action of various prostanoids in the guinea-pig ileum in the presence of atropine, indomethacin and phenoxybenzamine, thus restricting their action to a direct one on smooth muscle cells. They found that SC 19220 (30-300 μM) caused a dose-related parallel rightward shift in the concentration-response curves of PGE_1, PGE_2 and $PGF_{2\alpha}$. In addition, PGI_2 was almost 100 times less active than PGE_2, while PGD_2 and the thromboxane mimetic, U 46619 were found to be inactive. On the basis of these results they defined the guinea-pig ileum as an EP_1-selective preparation. More recently, AH 6809 has been shown to antagonise the contractile action of PGE_2 in this tissue (Coleman et al, 1987a). The neuronal EP receptor has never been fully characterised. Recent work by Poll et al (1988a and b) (see discussion) suggests that this receptor may be a subtype other than EP_1. Finally, Gardiner (1986) found that high concentrations (3-10 μM) of butaprost (which the authors have proposed to be EP_2-selective agonist) caused a non-competitive inhibition of histamine-induced contractions of this preparation. They proposed the existence of an additional EP_2 receptor mediating smooth muscle relaxation.

The guinea-pig longitudinal muscle is at present widely accepted to be an EP_1 selective preparation, however, in view of the above results there is a need for further study of the nature of the EP receptors present. In this thesis the EP receptors on both the smooth muscle and neuronal

elements have been characterised using a range of PGE analogues and the EP_1 receptor antagonist, AH 6809.

Analogues investigated (see Figure 4.1 and Table 4.1)

PGE_2 analogues (Figure 4.1A)

16,16-dimethyl PGE_2: This prostanoid has been shown to contract the rabbit aortic strip and induce irreversible aggregation of human platelets: these effects are abolished by thromboxane receptor blockade (Jones et al, 1979; 1982). It is a potent EP_1 receptor agonist, being more active than PGE_2 in contraction of the bullock iris sphincter, rat stomach fundus strip, guinea-pig trachea, fundus and ileum (Dong et al, 1986; Coleman et al, 1987; Coleman et al, 1988). It is also highly active at the EP_3 receptors of the guinea-pig vas deferens and the rat isolated gastric mucosa (Reeves and Stables, 1985; Reeves et al, 1988). However, Dong et al (1986) found it to be less active than PGE_2 at EP_2 receptors mediating relaxation of the guinea-pig trachea, cat trachea and dog hind limb blood flow *in vivo* . A recent report indicated that 16,16-dimethyl PGE_2 was 27 times less active than PGE_2 in the cat trachea (Coleman and Sheldrick, 1988).

ICI 80205: Similarly to 16,16-dimethyl PGE_2, ICI 80205 has thromboxane mimetic properties (Dong et al 1986). Jones et al (1979) and Dong and Jones (1982) also found this compound to have a similar profile to that of 16,16-dimethyl PGE_2 at EP receptors, although it appears to be more selective for EP_1 receptors. Their studies showed it to be highly active at the contractile receptors of the bullock iris sphincter, the rat stomach fundus and the guinea-pig trachea, but inactive as a relaxant agent in the guinea-pig trachea, and only weakly active in the cat trachea and the dog hind limb. ICI 80205 has not yet been studied on any EP_3 systems.

17-phenyl PGE_2-ω-trinor: No biological data has been reported in the literature.

Sulprostone: Coleman et al (1987a) have shown sulprostone to be active at EP_1 receptors in the guinea-pig ileum and the guinea-pig and dog

fundus, where it is only 2-4 fold less active than PGE_2. It is, however, highly potent in all tissues containing EP_3 receptors: chick ileum (Coleman et al, 1987b), guinea-pig vas deferens (Coleman et al 1987c) and rat isolated gastric mucosa (Reeves et al, 1988). Sulprostone appears to be devoid of any EP_2 agonist activity, being inactive or weakly active on the cat trachea, guinea-pig ileum circular muscle, and dog saphenous vein (Coleman et al 1987a).

11-deoxy PGE_2 1-alcohol: No biological data is available in the literature.

PGE_1 analogues (Figure 4.1B)

MB28767: Banerjee et al (1981a and b) found that this compound caused prolonged inhibition of pentagastrin-stimulated gastric acid secretion by oral dosing in conscious rats, and was 17 times more potent than their control compound: 16,16-dimethyl PGE_2 methyl ester. Although not reported as such this may have been due to a potent action on EP_3 receptors, now known to be present in this system (Reeves et al, 1988). However, more recently (Banerjee et al,1985) MB28767 has been shown to have potent contractile action on the rabbit aorta (EC_{50}, 2 μM) and mesenteric artery (EC_{50}, 200 nM), the EMR vs U 46619 being ~2.5 and 1 respectively. It also induced irreversible aggregation of rat and human platelets *in vitro* (Banerjee et al, 1985; Halushka et al, 1987). These actions are indicative of thromboxane agonist activity.

Oxoprostol: No biological data as yet available in the literature, for chemistry -see Facchini and Jones (1988).

Misoprostol: Misoprostol (Collins et al, 1985) has been shown to be an effective anti-secretory agent against a number of stimuli and provides protection of the gastric mucosa of a range of species (Bauer, 1985; Hanson et al, 1988). These types of activity have since been associated with EP_3 receptors (see Coleman et al, 1990). In addition, the compound is highly active on the guinea-pig vas deferens and the rat isolated gastric mucosa (Reeves et al, 1988) - both known to contain EP_3 receptors. Conflicting results have been reported on the activity of misoprostol on EP_1 and EP_2 receptors. Coleman et al (1988) reported results showing a high potency for this compound at EP_2 receptors of

the cat trachea, but low activity in the guinea-pig fundus (EP_1). While Eglen and Whiting (1988) found misoprostol to be equi-active with PGE_2 in two EP_1 contractile preparations (guinea-pig ileum, and oesophageal muscularis muscle) and 20 times less active in another (guinea-pig trachea), but inactive as an EP_2 agonist (relaxation in guinea-pig trachea).

11-deoxy PGE_1: Dong et al (1986) reported a low activity of this compound at EP_1 contractile receptors in the bullock iris sphincter, rat stomach fundus and the guinea-pig trachea. However, it was found to have greater activity at EP_2 receptors in the guinea-pig trachea, the cat trachea and the dog hind limb. 11-Deoxy PGE_1 has not to date been investigated in any preparations containing EP_3 receptors.

Butaprost: Gardiner (1986) has shown that on preparations containing both EP_1 and EP_2 receptors (guinea-pig trachea and lung, cat lung strip and human bronchial tissue) the compound acted as a potent relaxant, in comparison to PGE_2 which displayed considerable contractile effects. In the cat trachea, butaprost was found to be 26 times less potent than PGE_2. However, butaprost was shown to be devoid of activity on the chick ileum and was only weakly active in the guinea-pig ileum , the maximal response being <20% of that of histamine.

RESULTS

CHICK ILEUM

Log Concentration-response curves

Results are shown in Figure 4.2. All the PGE analogues tested contracted the chick ileum. At the EC_{25} level the rank order of potency was: 16,16-dimethyl PGE_2 > ICI80205 > PGE_2 > MB 28767 > sulprostone > butaprost > misoprostol > oxoprostol > 17-phenyl PGE_2-ω-trinor > 11-deoxy PGE_2 1-alcohol.

The log concentration-response curve of PGE_2 reached ~80% of the Ach maximum. However, the maxima of some of the analogues tested were significantly lower: sulprostone (40% ± 4), butaprost (47% ± 3), MB 28767 (56% ± 4) and oxoprostol (~56 %).

Action of AH 6809

2 μM AH 6809 caused a rightward shift in the log concentration-response curve of PGE_2 (Fig. 4.3). The dose-ratio of 2.7 ± 0.8 was significant at the $P < 0.05$. This is consistent with a pA_2 of 5.9 for AH 6809.

Interaction of PGE_2 and sulprostone

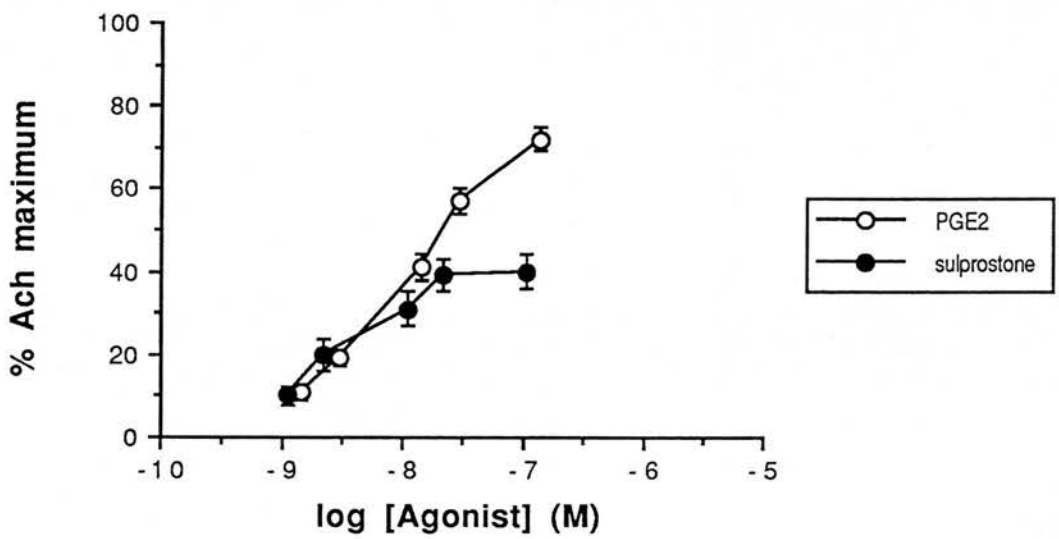
When doses of PGE_2 were added to a preparation which had been precontracted with a supramaximal (220 nM) concentration of sulprostone, the response levels corresponded closely to those predicted for an additive interaction between the two agonists (see Fig 4.4 and Fig. 4.5).

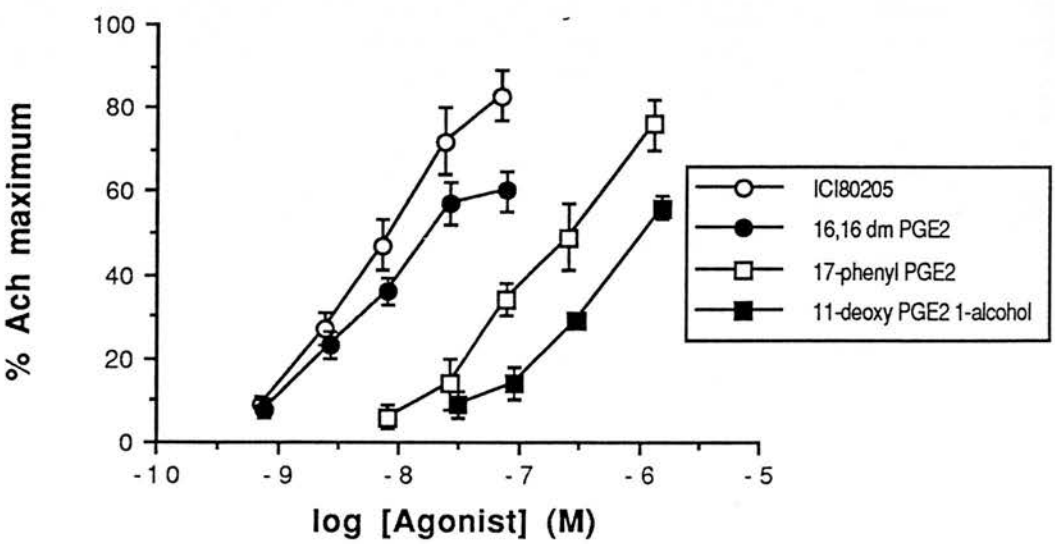
The effect of the 'cocktail' of antagonists on PGE_2 and Butaprost

The cocktail of antagonists (see discussion) used by Gardiner (1986) had no effect on the log concentration-resposne curves of PGE_2 and butaprost (Fig. 4.18)

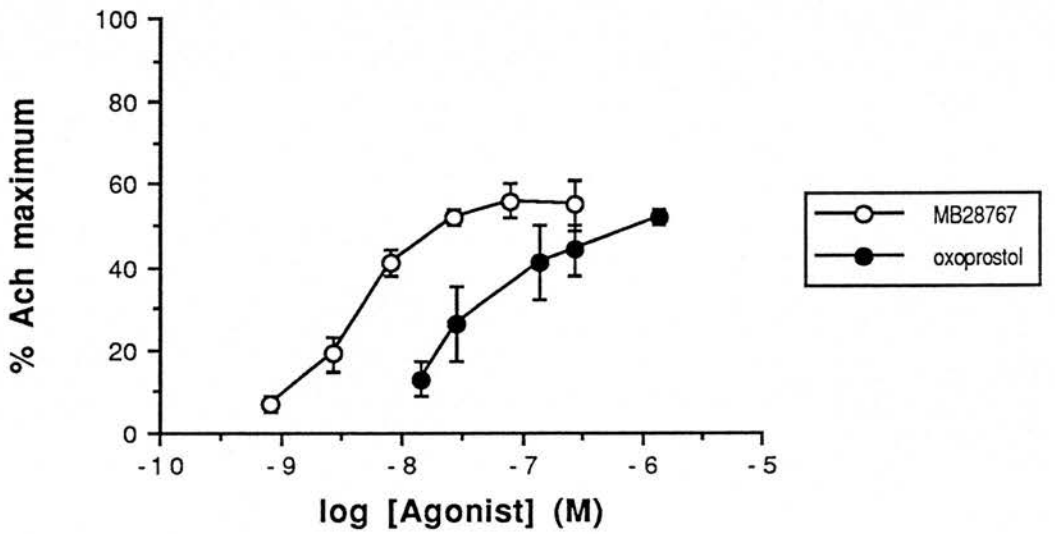
Figure 4.2: Log concentration-response curves of (a) PGE_2 analogues and (b) PGE_1 analogues for contraction of the chick ileum (mean of 4-6 expts.). (bars represent the s.e.m)

a)





b)


100
80
60
40
20
0
% Ach maximum
-10
-9
-8
-7
-6
-5
log [Agonist] (M)
MB28767
oxoprostol

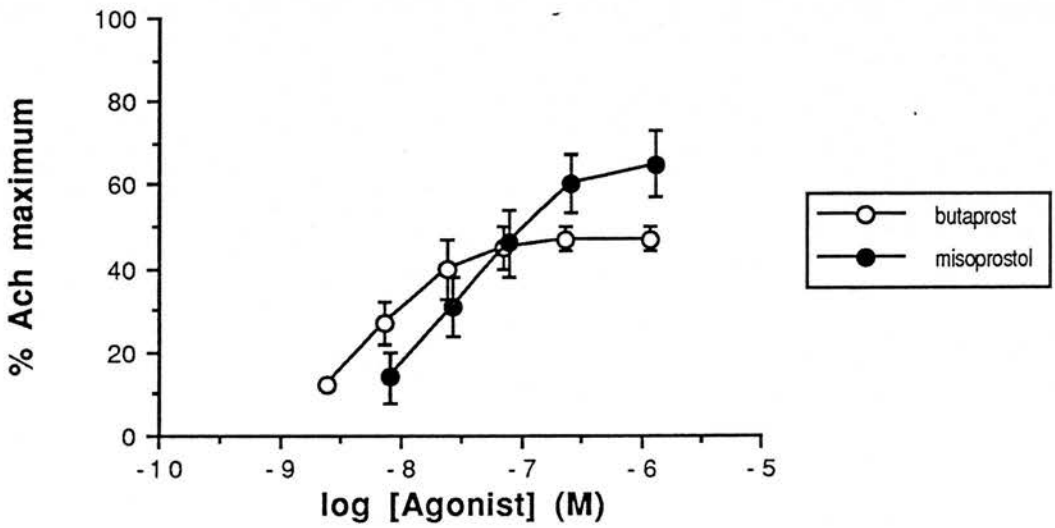

100
80
60
40
20
0
% Ach maximum
-10
-9
-8
-7
-6
-5
log [Agonist] (M)
butaprost
misoprostol

Figure 4.3: Effect of AH 6809 (2μM) on the log concentration-response curve of PGE_2 in the chick ileum (mean of 4 expts.).

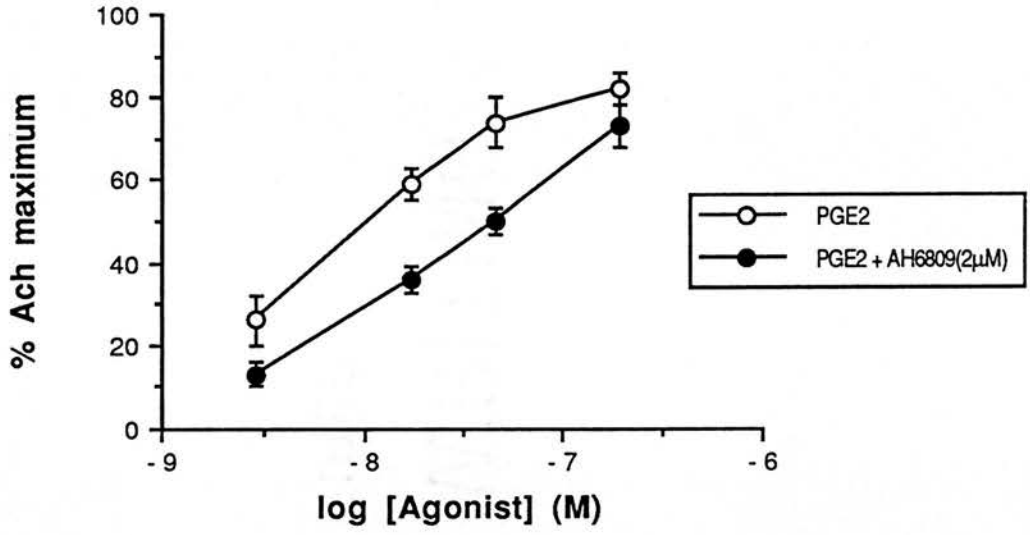


Figure 4.4: (a) Log concentration-response curve of PGE_2 in the absence and the presence of 220nM sulprostone and (b) Log concentration-response curve of PGE_2 alone and that predicted in the presence of 220nM sulprostone if the inter action of the compounds is additive in nature.

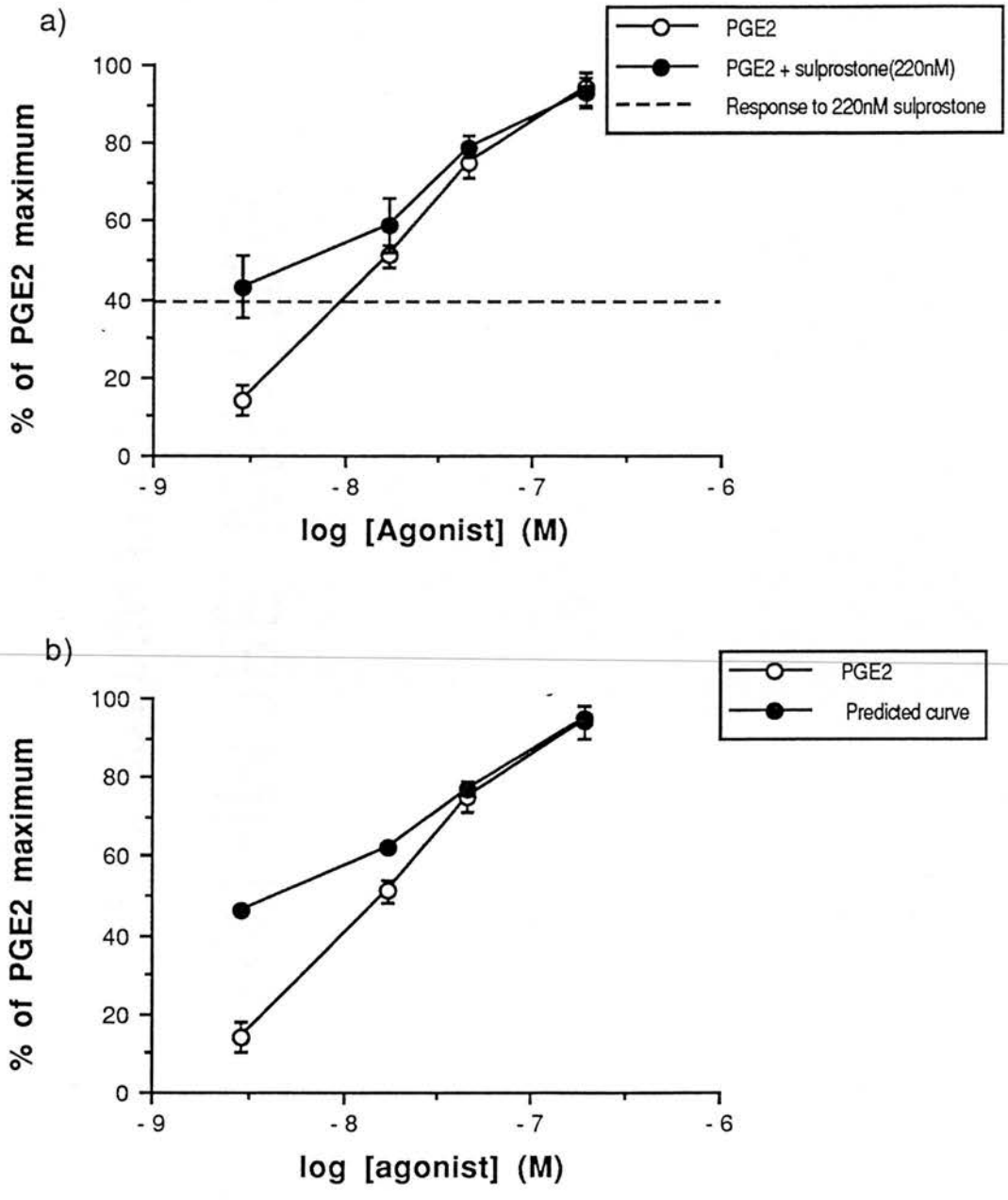
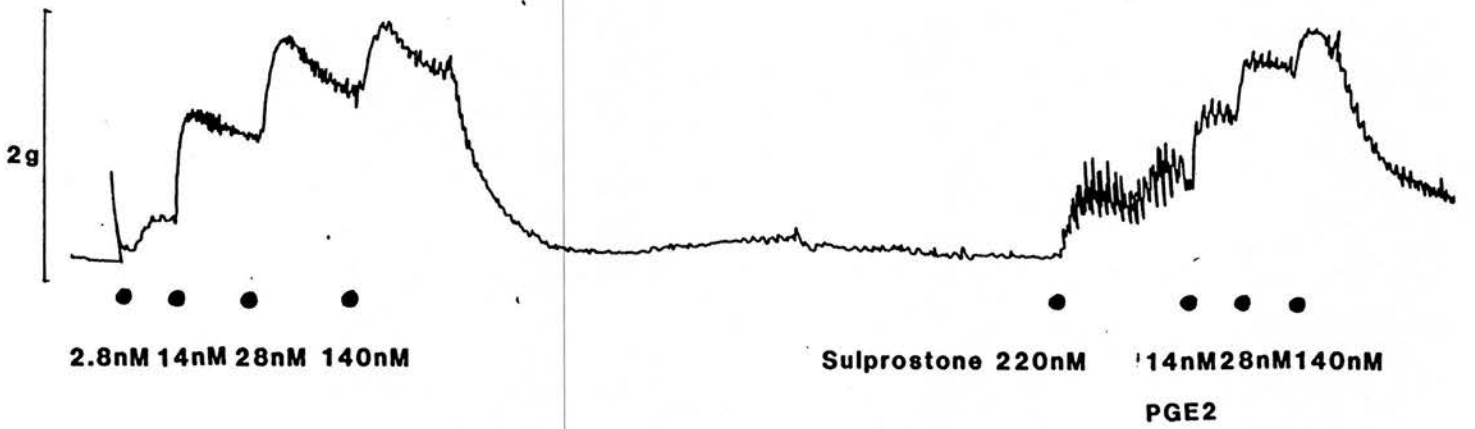


Figure 4.5: Trace showing the contraction of the chick ileum by cumulative additions of PGE_2 and the effect of sulprostone (200nM) on these responses.


2g
2.8nM 14nM 28nM 140nM
Sulprostone 220nM
14nM28nM140nM
PGE2

RABBIT JUGULAR VEIN

Relaxation of histamine-induced contractions

The log concentration-response curve of PGE_2 was unaffected by the presence of 10 μM GR 32191, the IC_{50} being 0.69 ± 0.15 and 0.61 ± 0.11 in its absence and presence respectively.

In the presence of 10 μM GR 32191 the rank order of potency of PGE analogues was as follows: PGE_2 > 16,16-dimethyl PGE_2 ≥ (nat) 11-deoxy PGE_2, (rac) 11-deoxy PGE_1 > 11-deoxy PGE_2 1-alcohol > misoprostol > ICI80205 > 17-phenyl PGE_2-ω-trinor > butaprost > MB 28767 > sulprostone > oxoprostol (see Fig 4.9). For PGI analogues: cicaprost > iloprost > carbacyclin (Fig. 4.6).

The log concentration-response curves of sulprostone (Fig. 4.9B) and oxoprostol failed to reach the IC_{25} level, the maxima being 22 ± 8 (at the highest concentration tested, 3.6 μM) and 14 ± 5 % (at the highest concentration tested, 1.4 μM) respectively. The curve for MB 28767 appears to be bell-shaped, reaching a maximum relaxation of 47 ± 9 % at 100-400 nM, with an IC_{25} of 76 ± 40 nM. Concentrations of MB 28767 in excess of 400 nM produced a contractile response (Fig 4.9B).

The effect of the TP-receptor antagonist GR 32191

The TP-receptor agonist, U 46619 produced dose-related contractions of the preparation, with an EC_{50} of 4.6 ± 1 nM. In the presence of 10 μM GR 32191 the log concentration-response curve for U 46619 was subject to a parallel rightward shift consistent with a pA_2 of 7.2 ± 0.1 (Fig. 4.7 and Fig. 4.9C).

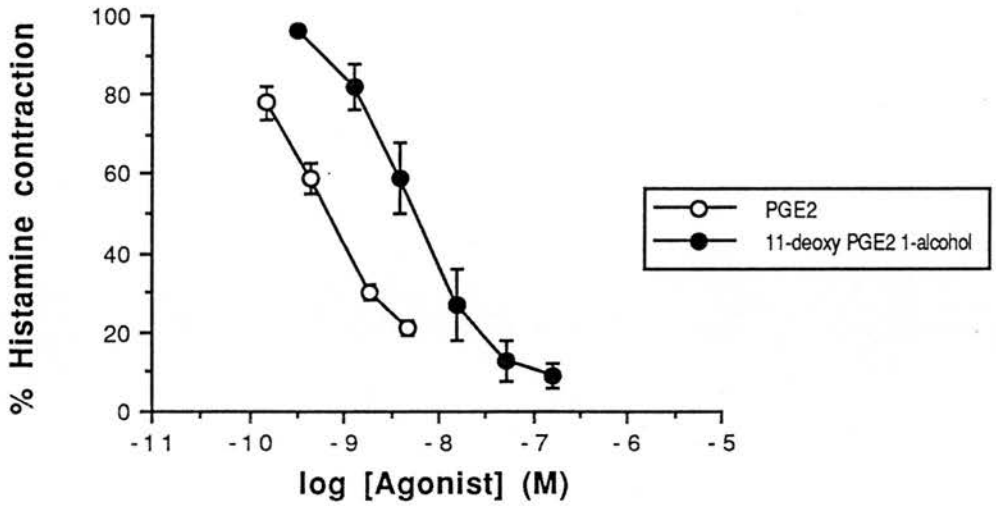
In the absence of GR 32191, MB 28767 contracted the vessel rings (EC_{50}, 26 ± 3 nM). At 10 μM the blocker caused a large rightward shift in the curve. The pA_2 could not be accurately measured as the maximum response obtained in the presence of GR 32191 was 28 ± 15 % at 4.3 μM. However, an estimate of 7.5 for the pA_2 was made from the dose ratio obtained at the IC_{25} level.

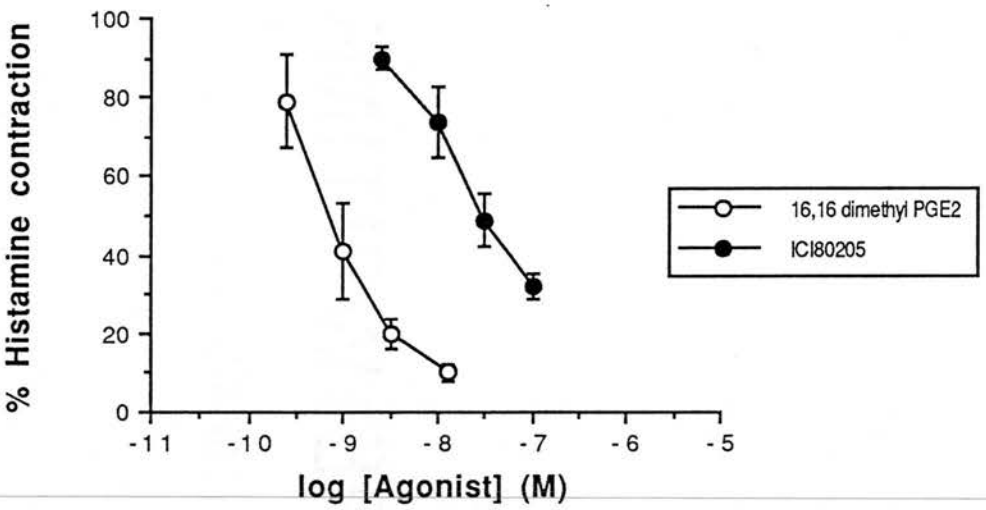
The effect of AH 6809

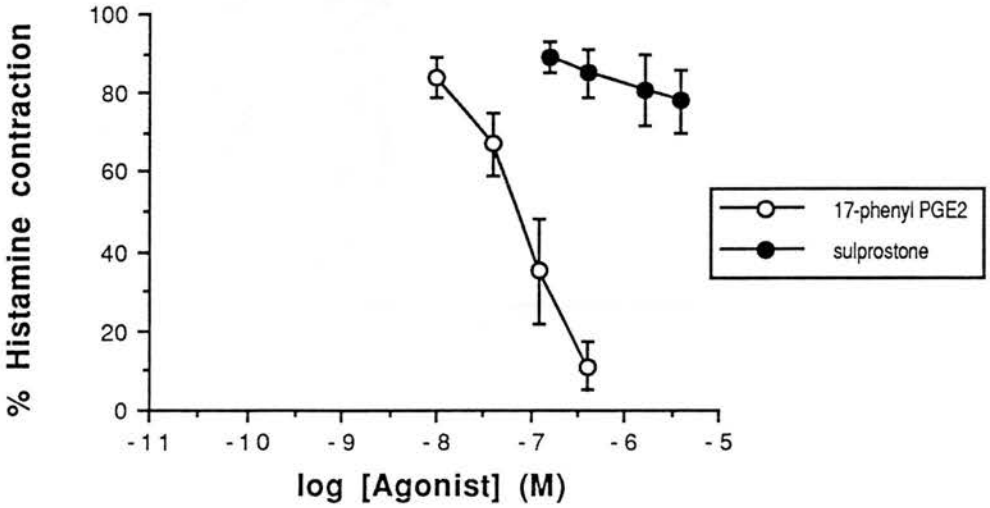
At a concentration of 2 μM AH 6809 had no significant ($P < 0.001$) effect on the log concentration-response curve of PGE_2 (Fig. 4.8), the dose ratio was 0.87 ± 0.03.

Figure 4.6: Log concentration-response curves of (a) PGE_2 analogues and (b) PGE_1 analogues for relaxation of histamine-induced contraction in the rabbit jugular vein (mean of 4-6 expts.).

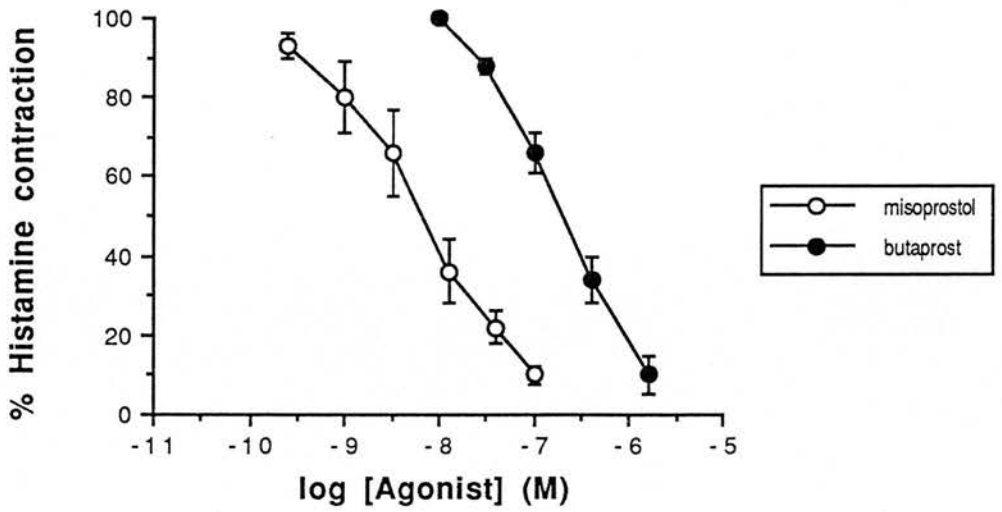
a)

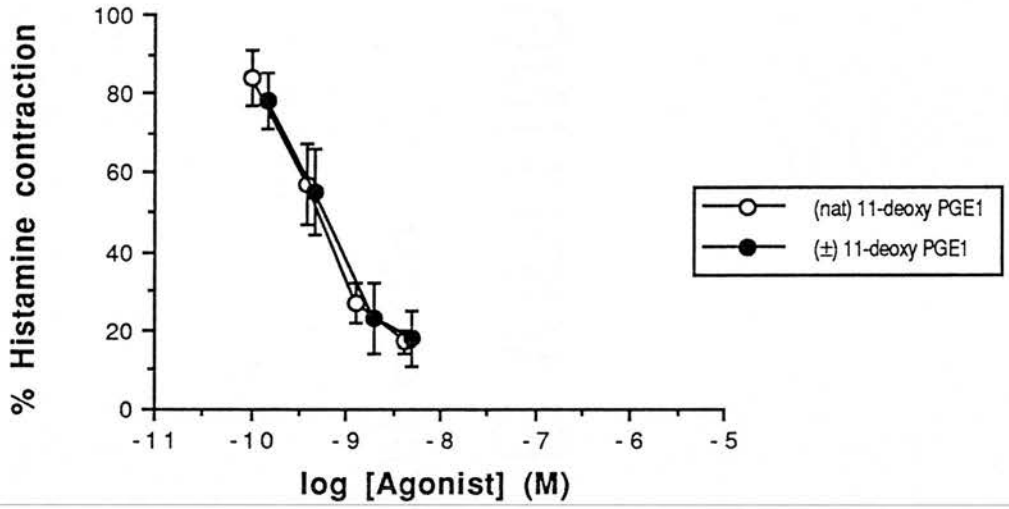

% Histamine contraction
100
80
60
40
20
0
-11
-10
-9
-8
-7
-6
-5
log [Agonist] (M)
PGE2
11-deoxy PGE2 1-alcohol


% Histamine contraction
100
80
60
40
20
0
-11
-10
-9
-8
-7
-6
-5
log [Agonist] (M)
16,16 dimethyl PGE2
ICI80205


% Histamine contraction
100
80
60
40
20
0
-11
-10
-9
-8
-7
-6
-5
log [Agonist] (M)
17-phenyl PGE2
sulprostone

b)


% Histamine contraction
100
80
60
40
20
0
-11
-10
-9
-8
-7
-6
-5
log [Agonist] (M)
misoprostol
butaprost


% Histamine contraction
100
80
60
40
20
0
-11
-10
-9
-8
-7
-6
-5
log [Agonist] (M)
(nat) 11-deoxy PGE1
(±) 11-deoxy PGE1

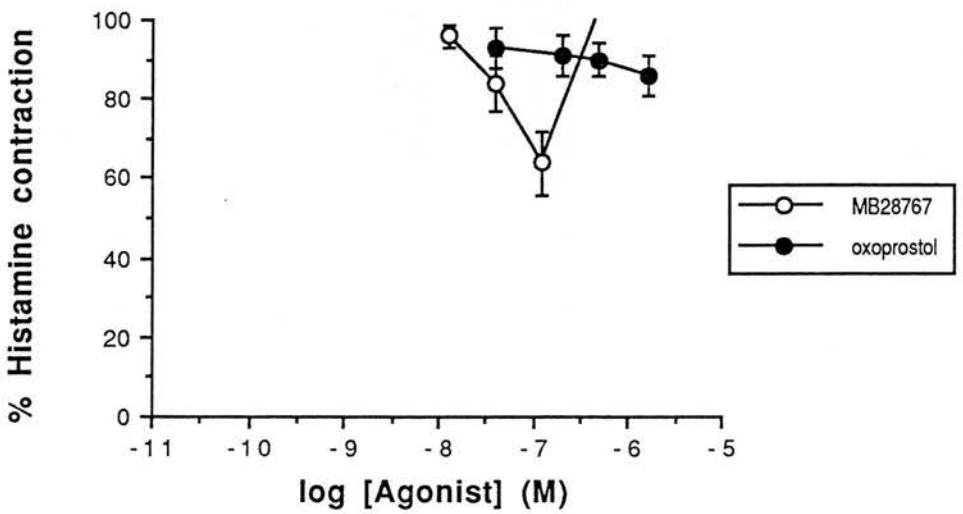

% Histamine contraction
100
80
60
40
20
0
-11
-10
-9
-8
-7
-6
-5
log [Agonist] (M)
MB28767
oxoprostol

Figure 4.7: Effect of GR 32191 (10μM) on the log concentration-response curves of U 46619 and MB 28767 for contraction of the rabbit jugular vein (mean of 4 expts.).

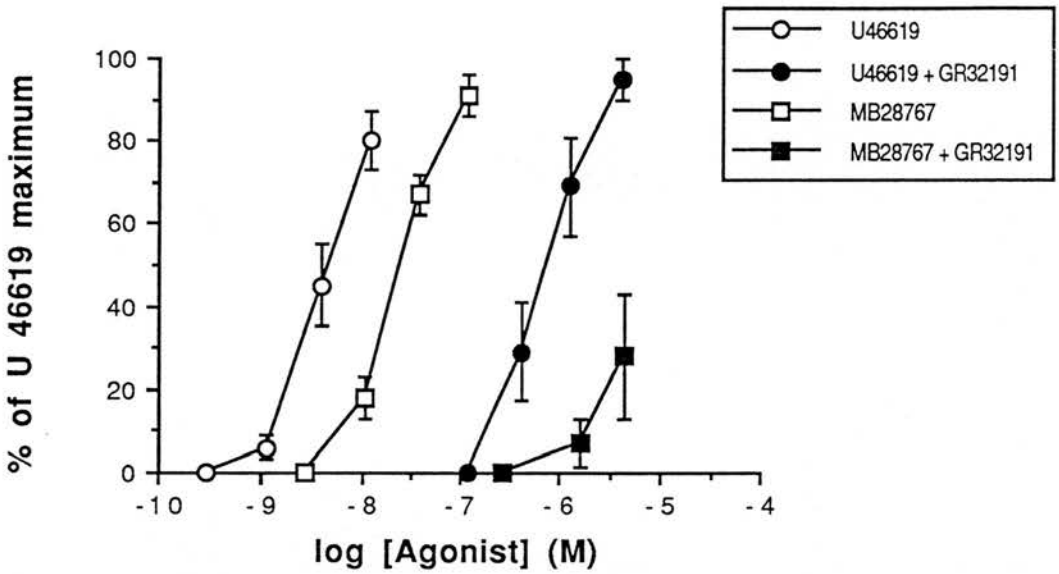


Figure 4.8: Effect of AH 6809 (2μM) on the log concentration-response curve of PGE_2 in the rabbit jugular vein (mean of 5 expts.).

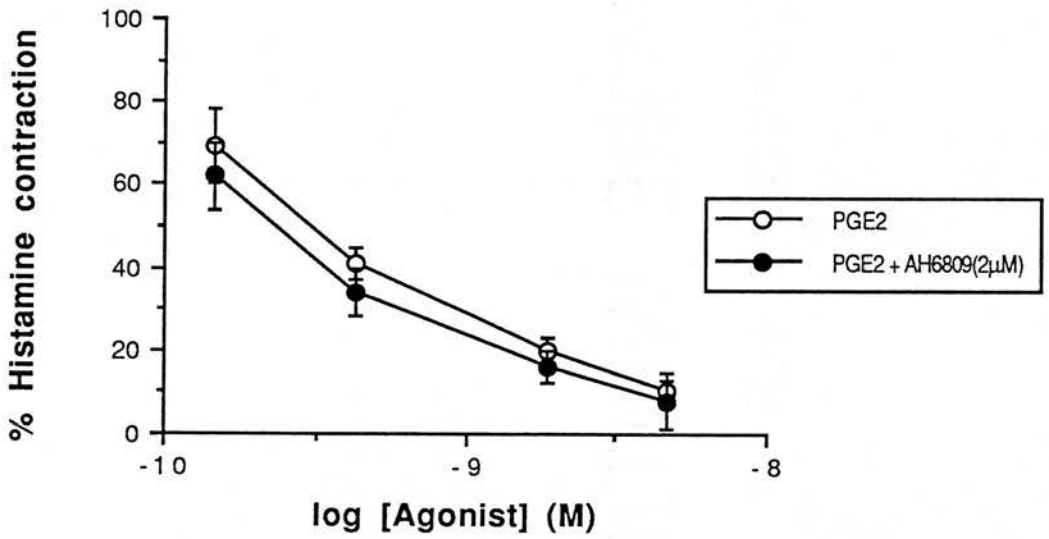


Figure 4.9A: Trace showing the relaxation of the rabbit jugular vein, precontracted with histamine (1μM), by (a) PGE_2 (b) 11-deoxy PGE_1 and (c) butaprost. The tissues were bathed in Krebs solution containing GR 32191 (10μM).

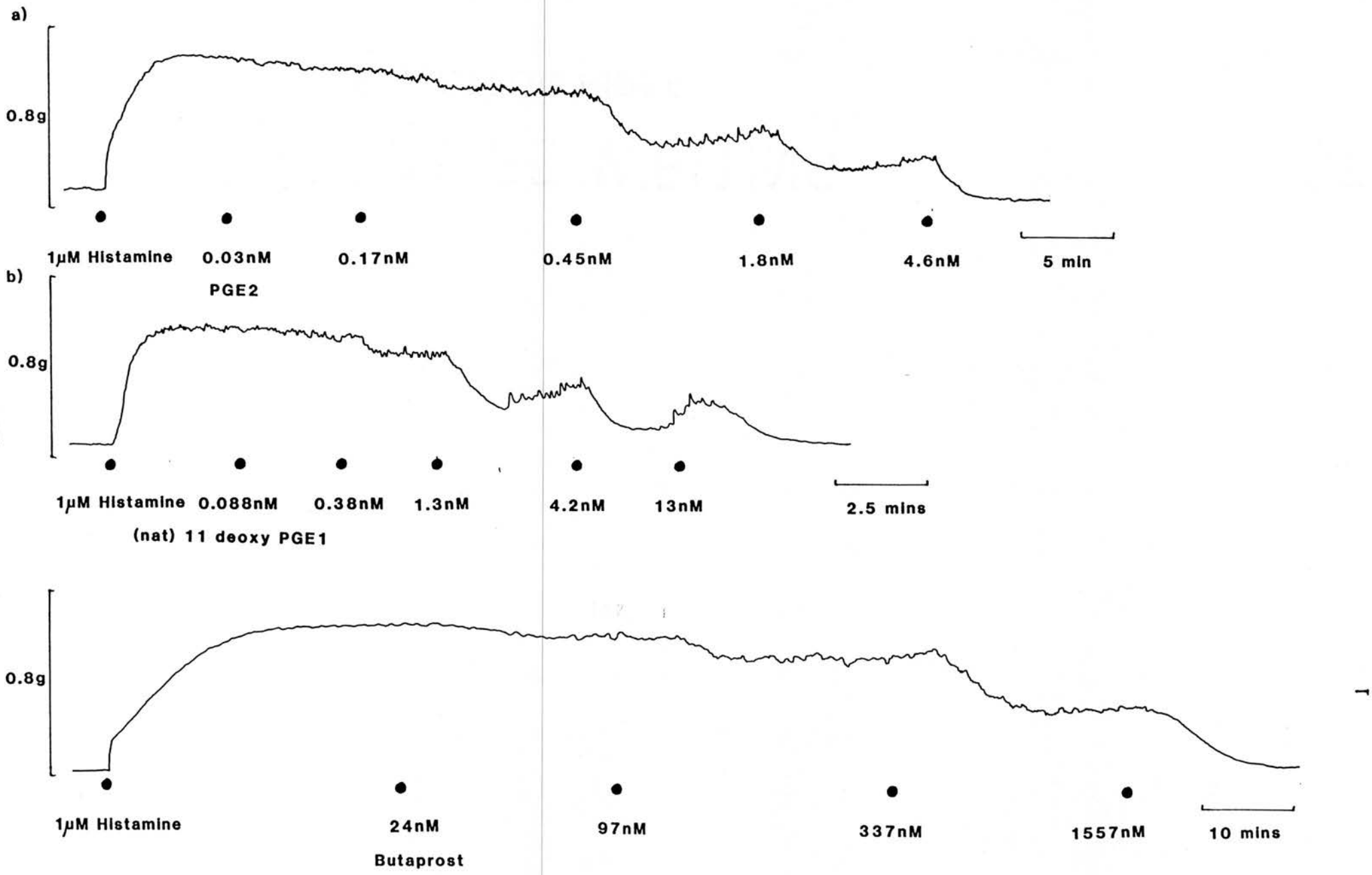

a)
0.8g
1μM Histamine
0.03nM
0.17nM
0.45nM
1.8nM
4.6nM
5 min
b)
PGE2
0.8g
1μM Histamine
0.088nM
0.38nM
1.3nM
4.2nM
13nM
2.5 mins
(nat) 11 deoxy PGE1
0.8g
1μM Histamine
24nM
Butaprost
97nM
337nM
1557nM
10 mins

Figure 4.9B: Trace showing the relaxation of the rabbit jugular vein precontracted with histamine (1μM) by (a) sulprostone and (b) MB 28767. The tissues were bathed in Krebs solution containing GR 32191 (10μM).

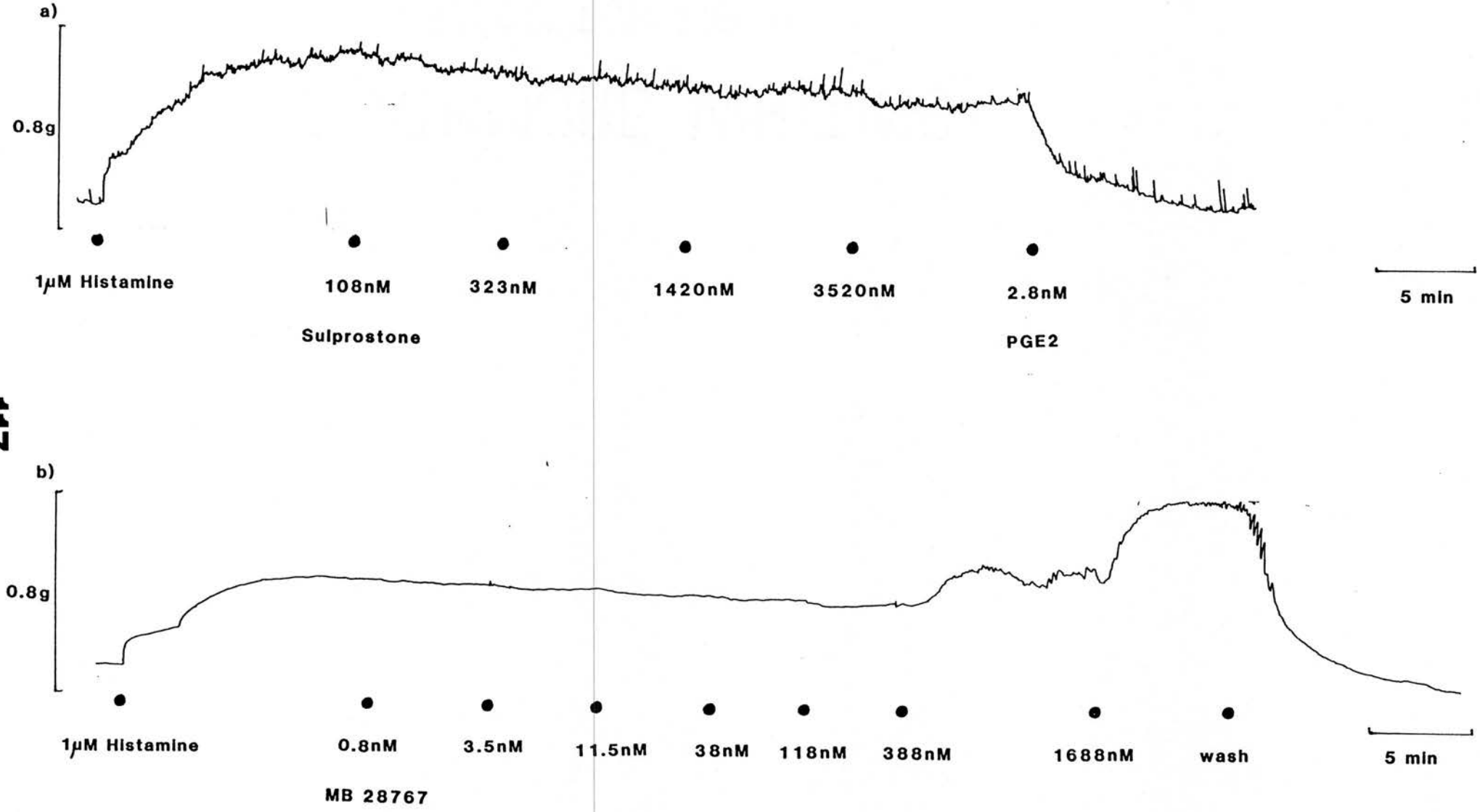
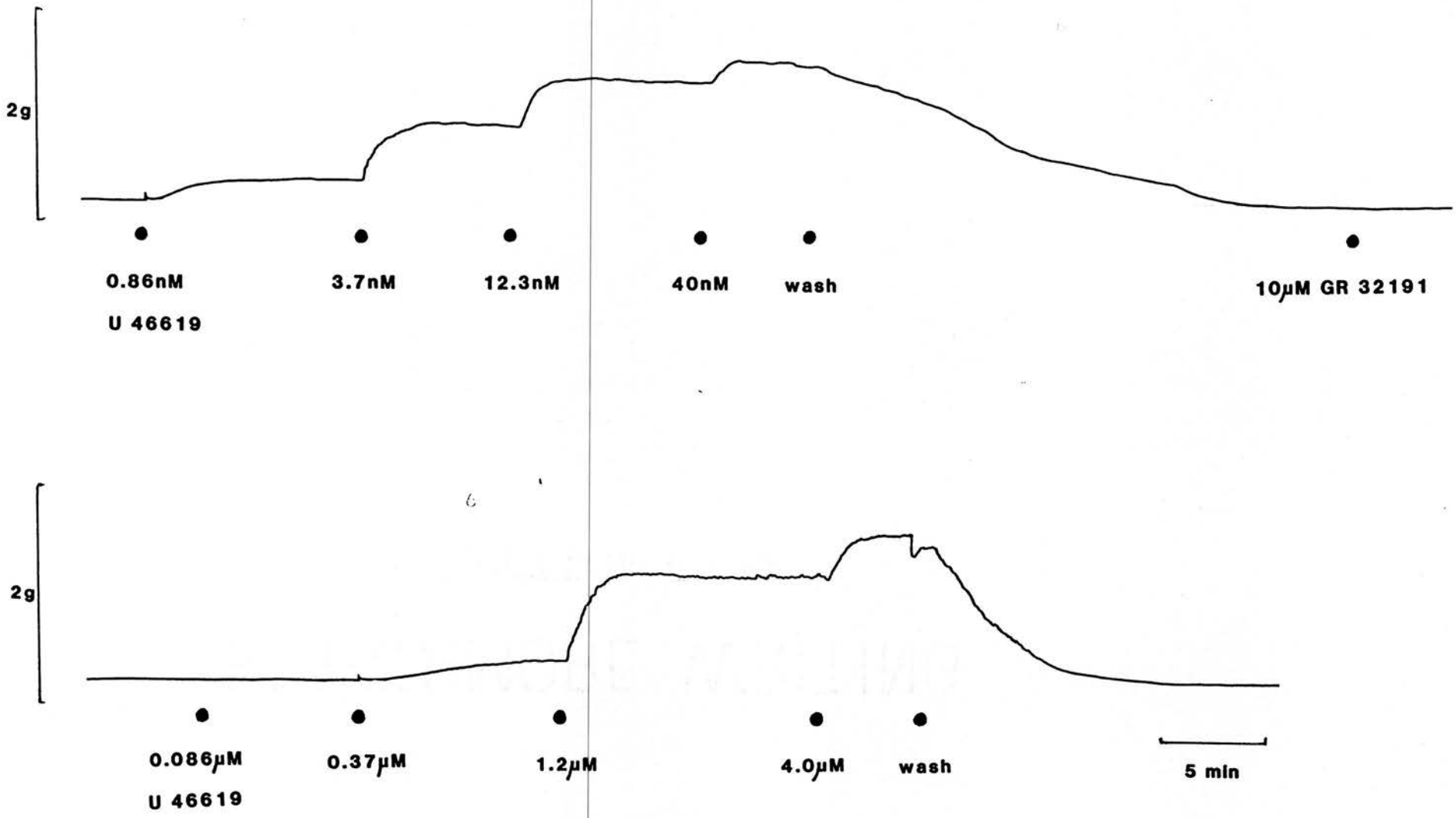

a)
0.8g
1μM Histamine
108nM
323nM
1420nM
3520nM
2.8nM
Sulprostone
PGE2
5 min
b)
0.8g
1μM Histamine
0.8nM
3.5nM
11.5nM
38nM
118nM
388nM
1688nM
wash
MB 28767
5 min

Figure 4.9C: Trace showing the contraction of the rabbit jugular vein by U 46619 and inhibition of this contractile action by GR 32191 (10μM).


2g
0.86nM
U 46619
3.7nM
12.3nM
40nM
wash
10μM GR 32191
2g
0.086μM
U 46619
0.37μM
1.2μM
4.0μM
wash
5 min

GUINEA-PIG ILEUM

Log concentration-response curves

PGE analogues: Log concentration-response curves are shown in Figure 4.10. All the PGE analogues tested were found to have some activity as contractile agents. The rank order of potency was as follows: ICI80205 > 16,16-dimethyl PGE_2 > PGE_2 > MB 28767 > 17-phenyl PGE_2-ω-trinor > misoprostol > sulprostone > 11-deoxy PGE_2 1-alcohol > oxoprostol > 11-deoxy PGE_1 > butaprost. The curves for a number of compounds reached a significantly lower maximum than PGE_2: 11-deoxy PGE_2 1-alcohol , 11-deoxy PGE_1, oxoprostol and butaprost.

Other prostanoids: The thromboxane mimetic, U 46619 was inactive at Concentrations up to 3 μM. $PGF_{2\alpha}$ contracted the preparation (EC_{25}, ~200 nM), however, the prostanoid retained little activity in the presence of 2 μM AH 6809. The $PGF_{2\alpha}$ mimetic, ICI 81008 was inactive as a contractile agent at concentrations up to 2 μM (Table 4.2).

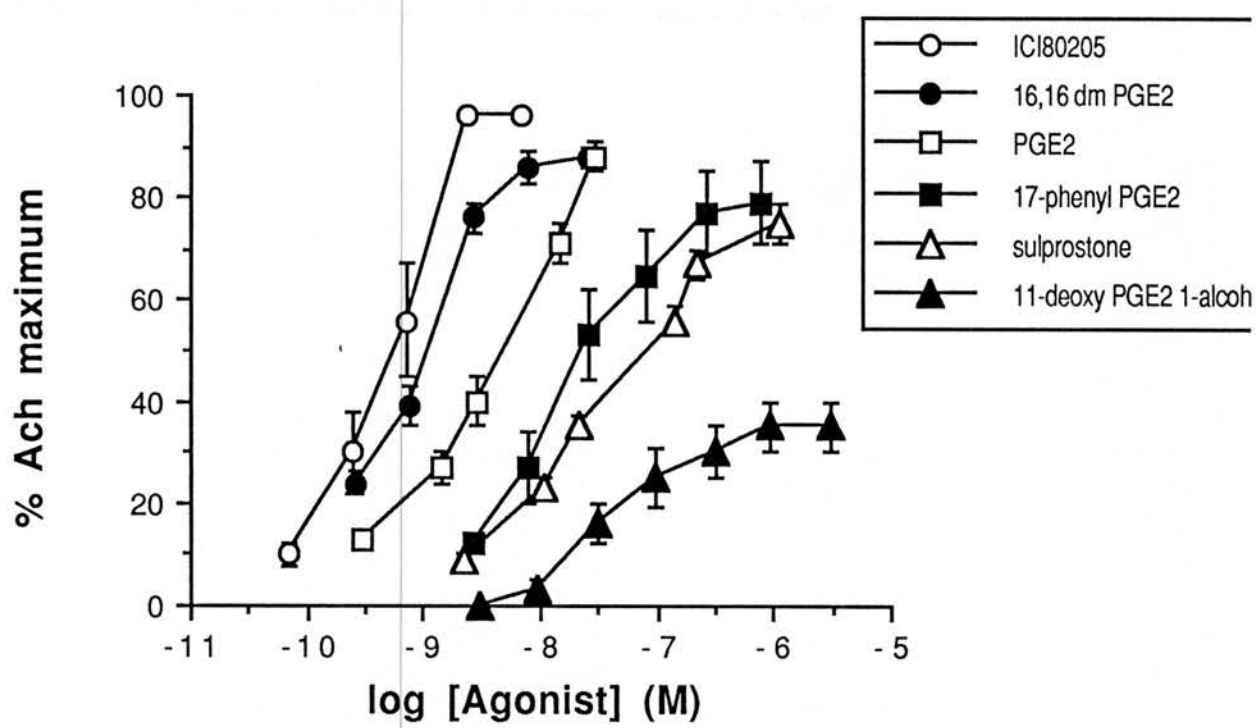
Effect of morphine and TTX

Single dose of PGE_2: At a concentration of 1 μM morphine caused a significant inhibition (38% ± 6) of the response to a single sub-maximal dose (14nM) of PGE_2 . The inhibitory action of 1 μM TTX was similar (37% ± 3) (Fig. 4.11). Inhibition of a single dose of PGE2 by 100 nM TTX is shown in Figure 4.17A.

Log concentration-response curves: Log concentration-response curves are shown in Figure 4.12. At the EC_{25} level the log concentration-response curves of sulprostone and 17-phenyl PGE_2-ω-trinor were unaffected by the presence of 1 μM morphine and the small shifts in the curves of ICI80205, 16,16-dimethyl PGE_2 and oxoprostol were not significant. However, the Concentration-response curves of PGE_2 and MB 28767 were subject to a small, but statistically significant, rightward shift in the presence of morphine.

Figure 4.10: Log concentration-response curves of (a) PGE_2 analogues and (b) PGE_1 analogues for contraction of the guinea-pig ileum (mean of 4-6 expts.).

a)



b)

% Ach maximum
100
80
60
40
20
0
-10
-9
-8
-7
-6
-5
log [Agonist] (M)
MB28767
misoprostol
11-deoxy PGE1
oxoprostol
butaprost

Table 4.2: pD_{25} values of U 46619, $PGF_{2\alpha}$, ICI 81008 and substance P in the guinea-pig ileum.

COMPOUND	PD_{25}	
	Alone	+ AH6809 (2μM)
U 46619	<5.54	_
$PGF_{2\alpha}$	6.75	<5.85
ICI 81008	<5.85	_
Substance P	9	_

Figure 4.11: Histograms showing the effect of (a) TTX (1μM) and morphine (1μM) and (b) AH 6809 (1μM) on the reponse to a single submaximal dose of PGE_2 (14nM), cicaprost (13nM), iloprost (14nM) and carbacyclin (150nM) (mean of 4-6 expts.).

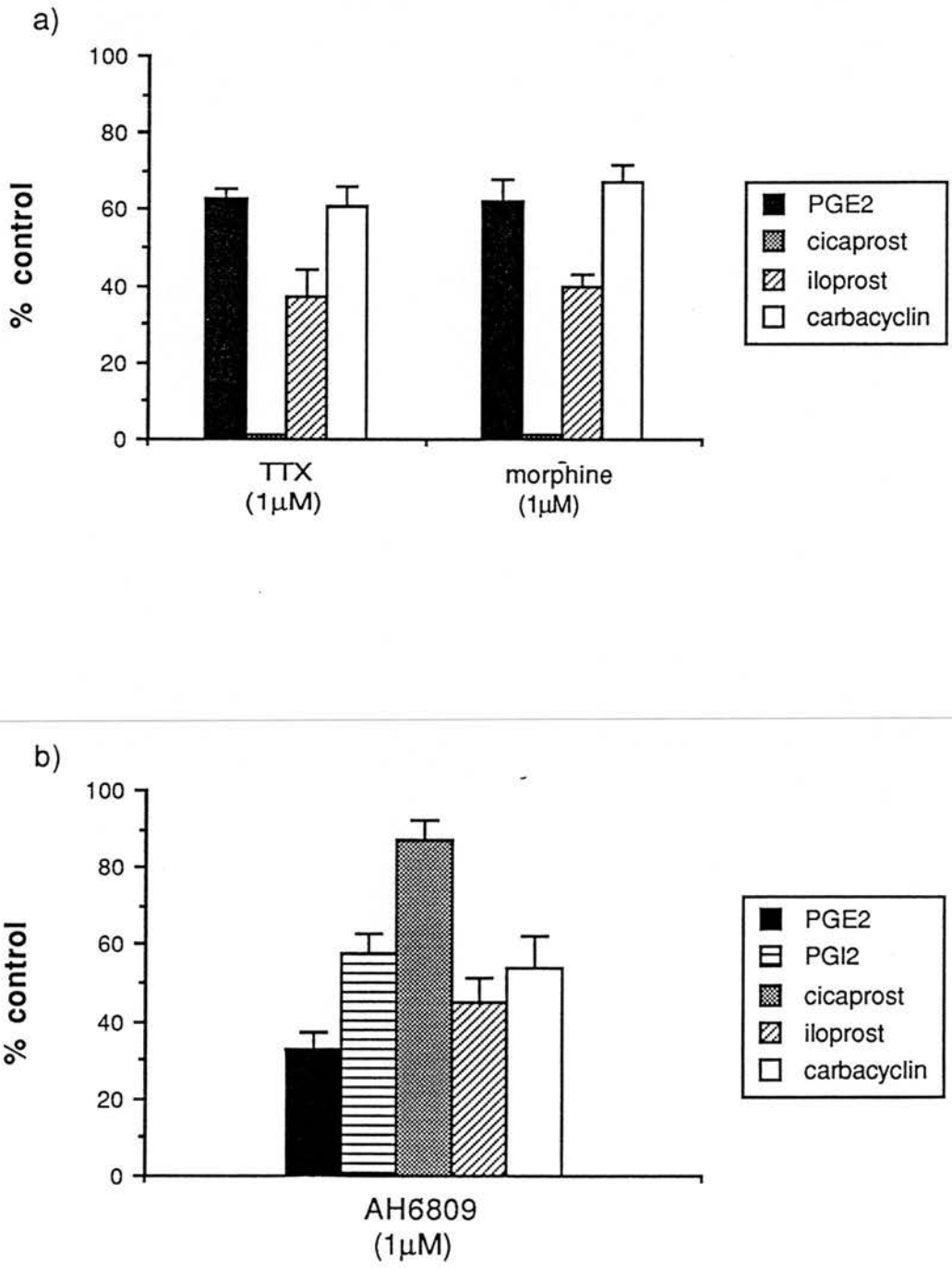
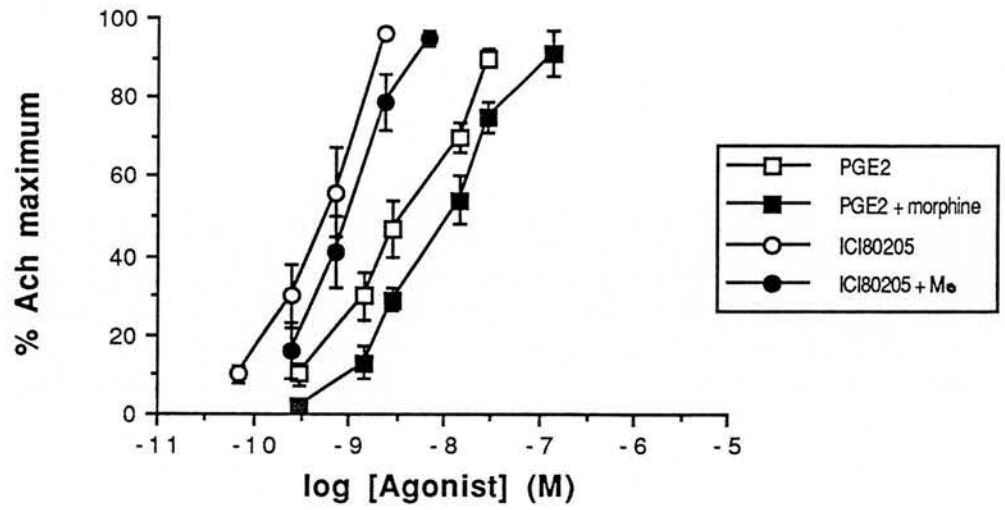
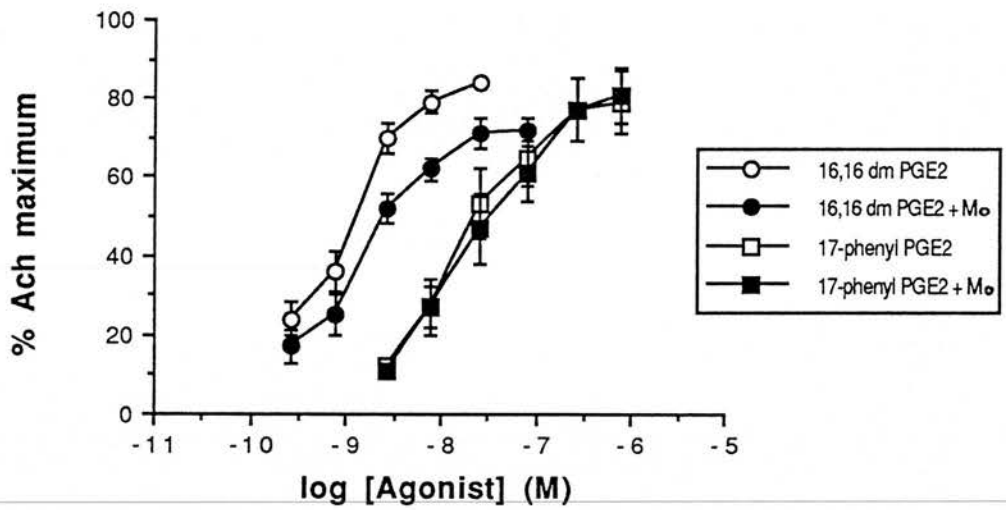
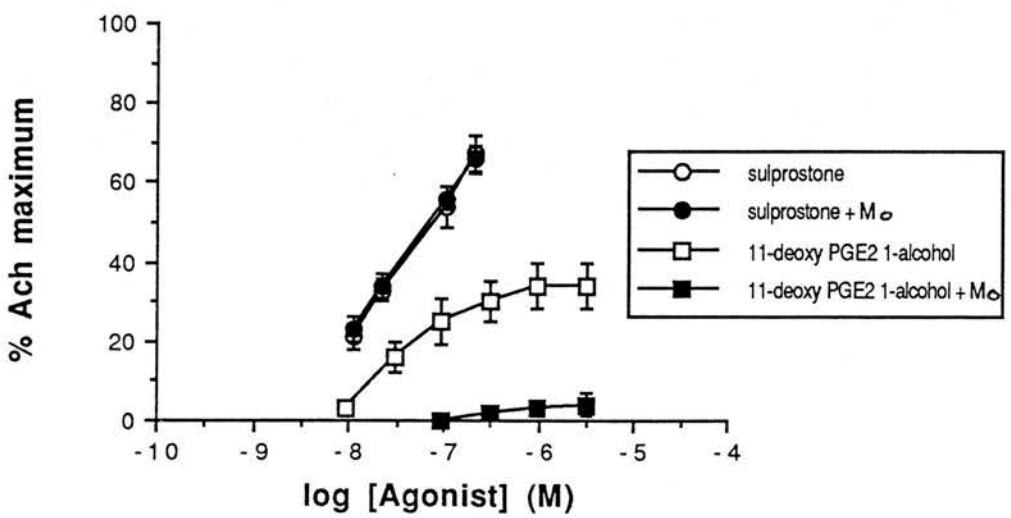


Figure 4.12: Effect of morphine (M_9) (1μM) on the log concentration-response curves of (a) PGE_2 and (b) PGE_1 analogues in the guinea-pig ileum (mean of 4-6 expts.).

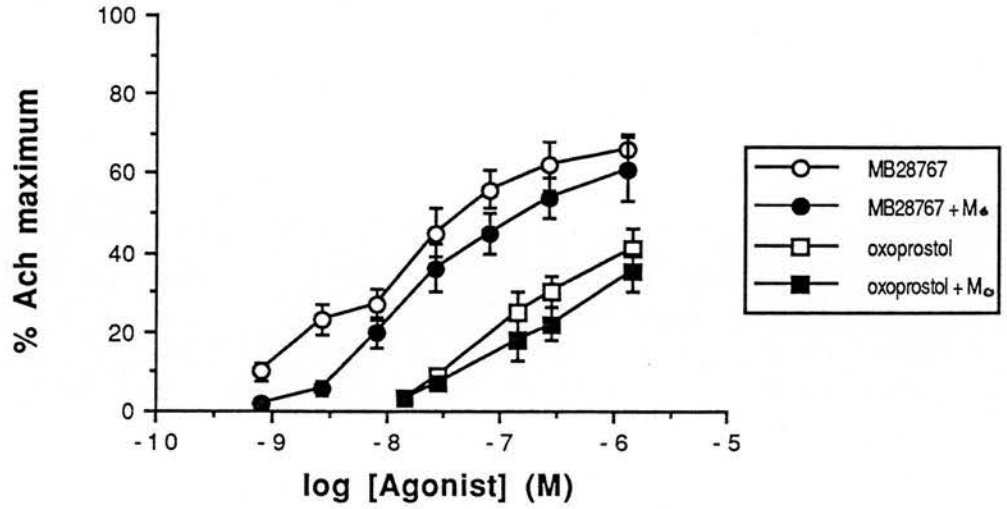
a)

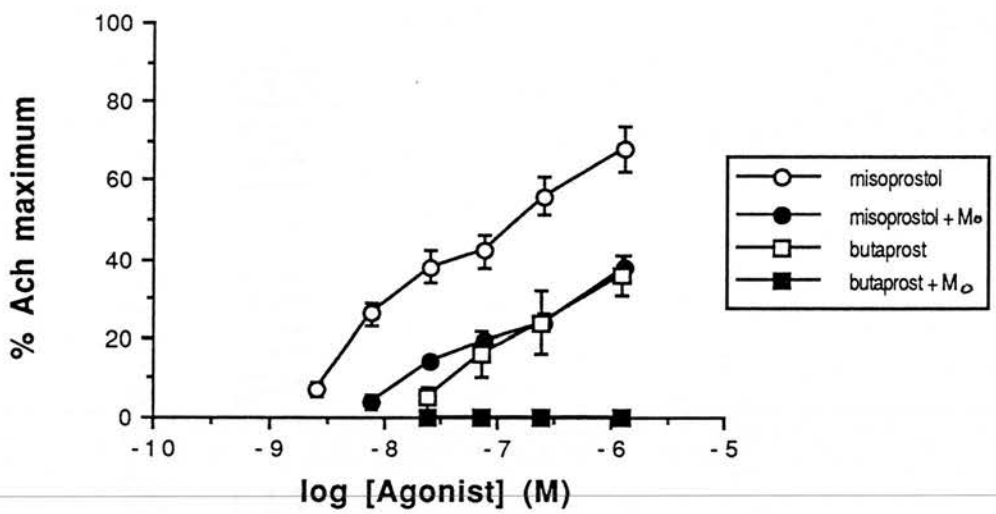

% Ach maximum
100
80
60
40
20
0
-11
-10
-9
-8
-7
-6
-5
log [Agonist] (M)
PGE2
PGE2 + morphine
ICI80205
ICI80205 + Mo

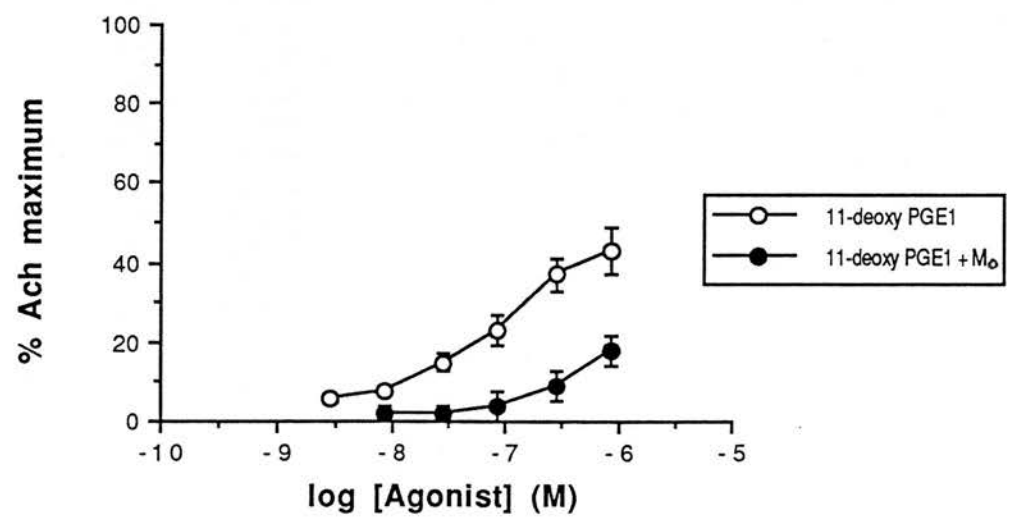

% Ach maximum
100
80
60
40
20
0
-11
-10
-9
-8
-7
-6
-5
log [Agonist] (M)
16,16 dm PGE2
16,16 dm PGE2 + Mo
17-phenyl PGE2
17-phenyl PGE2 + Mo


% Ach maximum
100
80
60
40
20
0
-10
-9
-8
-7
-6
-5
-4
log [Agonist] (M)
sulprostone
sulprostone + Mo
11-deoxy PGE2 1-alcohol
11-deoxy PGE2 1-alcohol + Mo

b)


% Ach maximum
100
80
60
40
20
0
-10
-9
-8
-7
-6
-5
log [Agonist] (M)
MB28767
MB28767 + M
oxoprostol
oxoprostol + M


% Ach maximum
100
80
60
40
20
0
-10
-9
-8
-7
-6
-5
log [Agonist] (M)
misoprostol
misoprostol + M
butaprost
butaprost + M


% Ach maximum
100
80
60
40
20
0
-10
-9
-8
-7
-6
-5
log [Agonist] (M)
11-deoxy PGE1
11-deoxy PGE1 + M

Misoprostol, 11-deoxy PGE_2 1-alcohol, 11-deoxy PGE_1 and butaprost were subject to a large highly significant rightward shift in the presence of 1 μM morphine.

Effect of atropine and SP150

At 200 nM atropine partially inhibited (27% ± 1) responses to a single submaximal dose (14 nM) of PGE_2 (Fig. 4.14).

The log concentration-response curve of PGE_2 and 16,16-dimethyl PGE_2 were also significantly shifted by 200 nM atropine, while that of sulprostone was unaffected (Fig 4.13).

The SP blocker, SP150 caused a significant inhibition (22% ± 5) of the response to 14 nM PGE_2 (Fig. 4.17B). However, in the presence of 1 μM morphine SP150 has no effect on the response to this dose of PGE_2 (Fig. 4.14 and 4.17B). In the presence of both SP150 and atropine the response to PGE_2 was inhibited by 47% ± 5. (Fig. 4.17C).

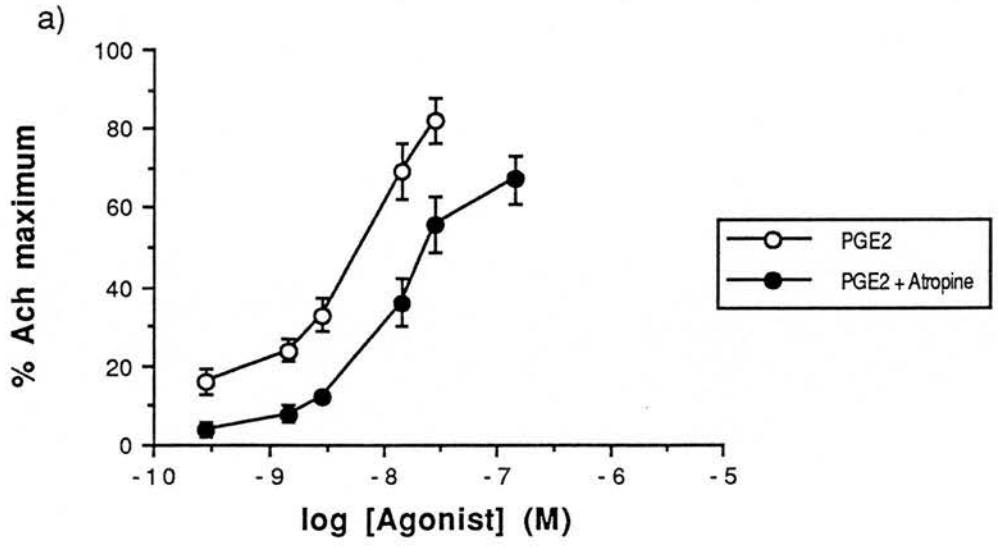
Effect of AH 6809

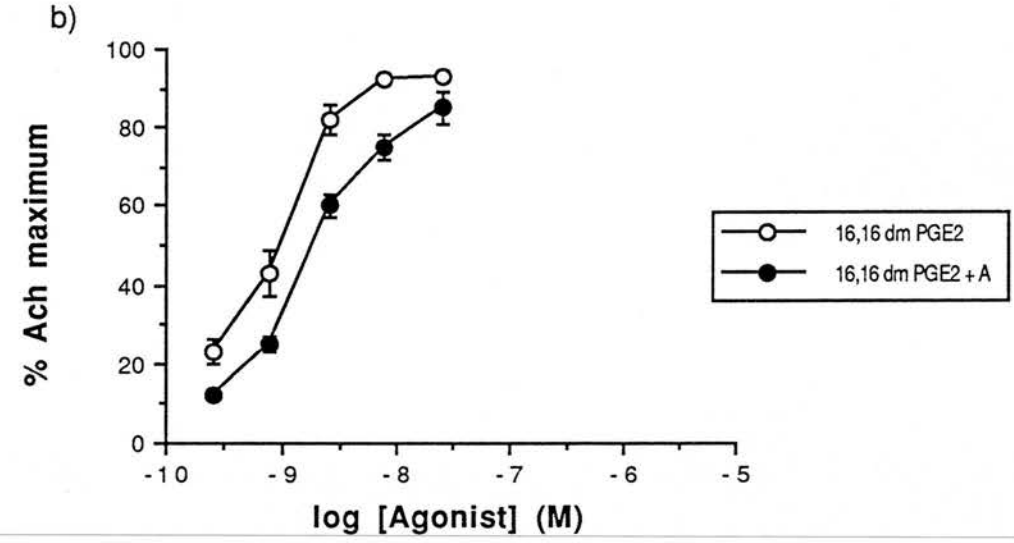
In the absence of morphine, 1 μM AH 6809 caused a 67% inhibition of the response to a single sub-maximal dose of PGE_2 (14 nM) (Fig. 4.11).

The effect of AH 6809 on log concentration-response curves is shown in Figure 4.15. With morphine present, 2 μM AH 6809 caused a parallel rightward shift in the log concentration-response curve of PGE_2 (dose ratio, 31), consistent with a pA_2 of 7.08 ± 0.14 (range = 6.3 - 7.4). The blocker caused a similar degree of shift in the curves of 16,16-dimethyl PGE_2, ICI80205, 17-phenyl PGE_2-ω-trinor and misoprostol, while that of oxoprostol was subject to a small but significant shift.

At the 10-30% response level the curve of sulprostone was unaffected by 2 μM AH 6809 but at concentrations giving responses of 40-70% the shift was similar to that produced in the PGE_2 curve. In contrast, at all the concentrations tested the responses to MB 28767 were unaffected by AH 6809.

Figure 4.13: Effect of Atropine (200nM) on the log concentration-response curves of (a) PGE_2 (b) 16,16 dimethyl PGE_2 and (c) sulprostone in the guinea-pig ileum (mean of 4 expts.).


a)
% Ach maximum
100
80
60
40
20
0
-10
-9
-8
-7
-6
-5
log [Agonist] (M)
PGE2
PGE2 + Atropine


b)
% Ach maximum
100
80
60
40
20
0
-10
-9
-8
-7
-6
-5
log [Agonist] (M)
16,16 dm PGE2
16,16 dm PGE2 + A

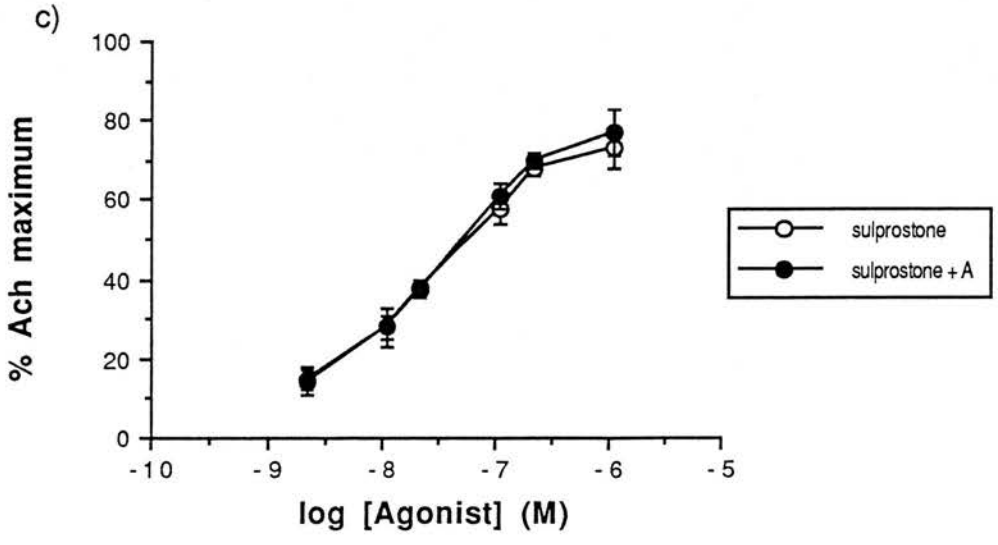

c)
% Ach maximum
100
80
60
40
20
0
-10
-9
-8
-7
-6
-5
log [Agonist] (M)
sulprostone
sulprostone + A

Figure 4.14: Histogram showing the effect of atropine (200nM), SP150 (1μM) and morphine (1μM) on the response to single submaximal doses of PGE_2 (14nM) and cicaprost (13nM) in the guinea-pig ileum (mean of 4-6 expts.).

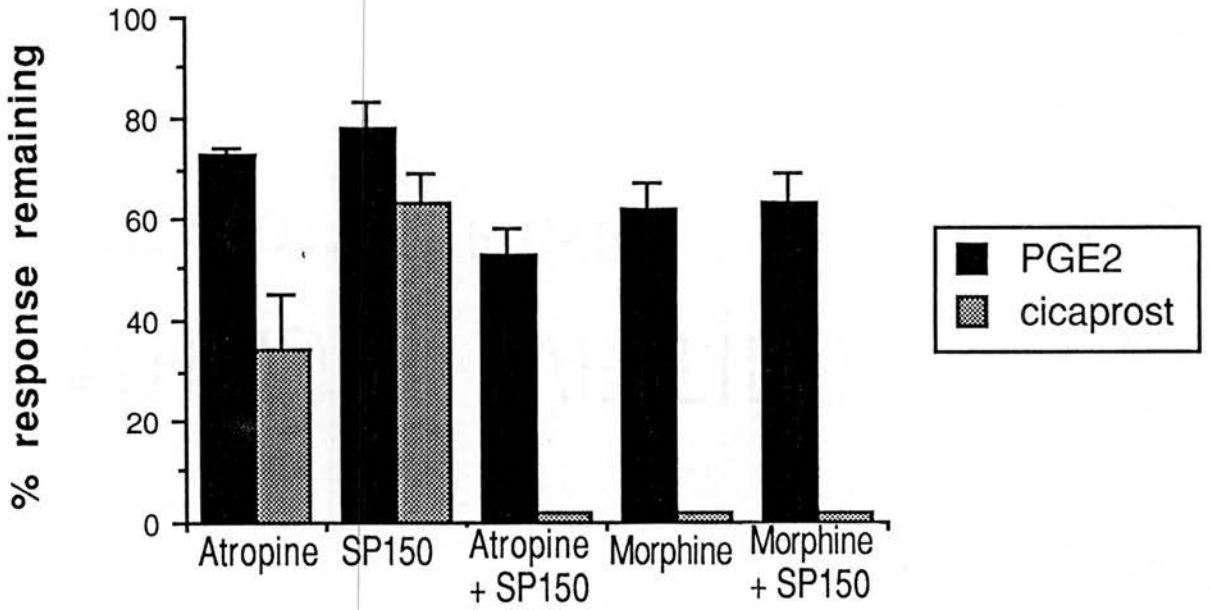
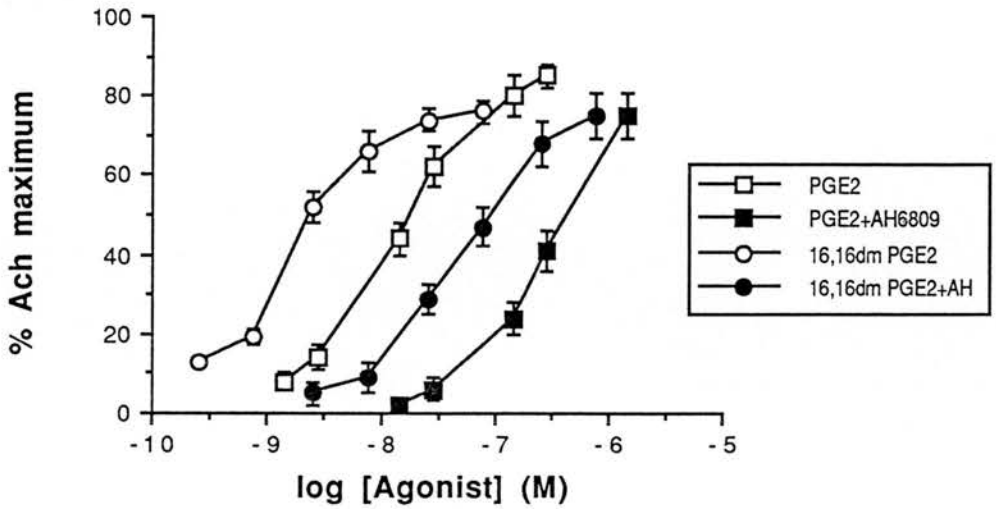
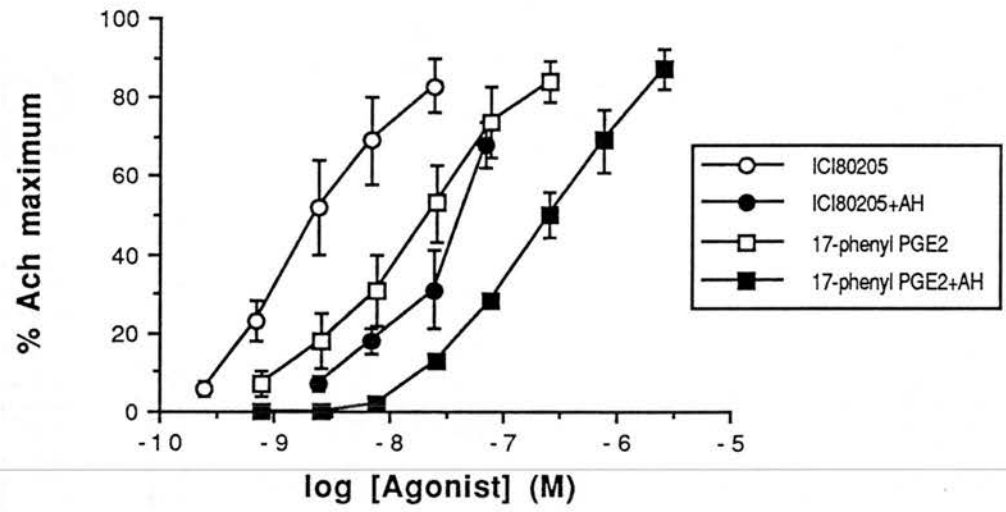
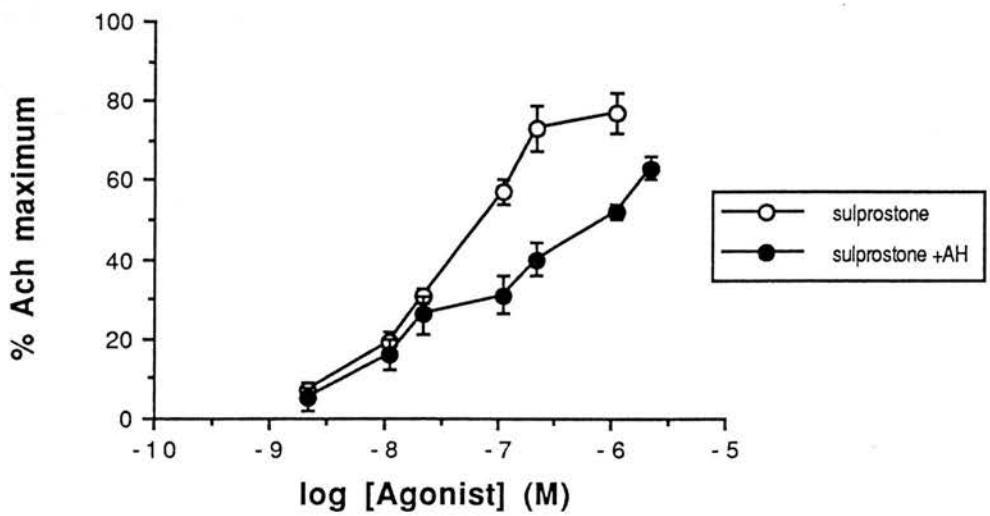


Figure 4.15: Effect of AH 6809 (2μM) on the log concentration-response curves of (a) PGE_2 analogues and (b) PGE_1 analogues in the guinea-pig ileum (mean of 4-6 expts.).

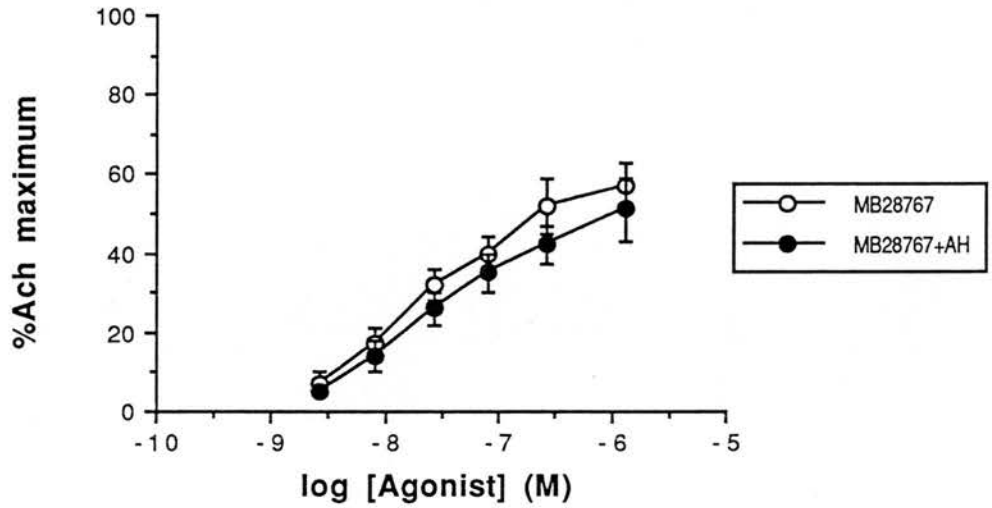
a)

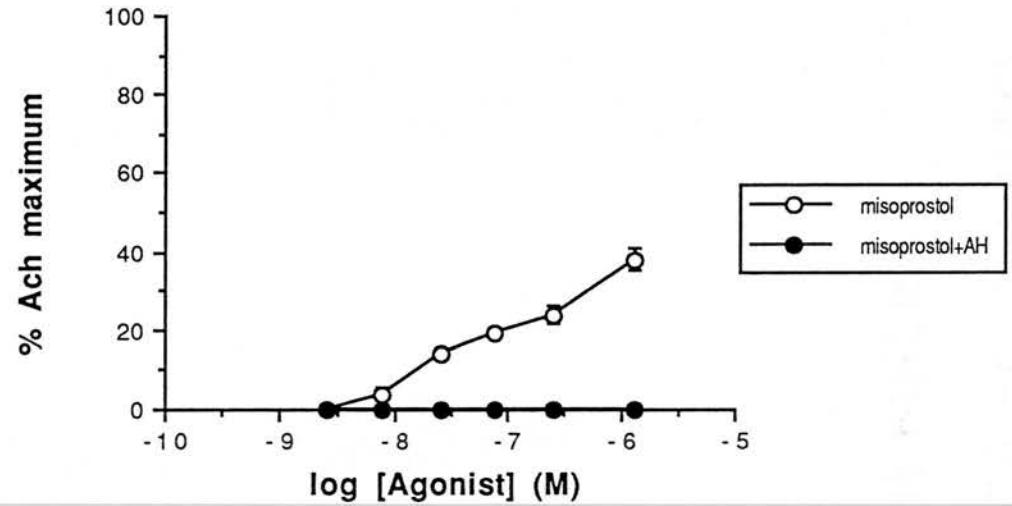

% Ach maximum
100
80
60
40
20
0
-10
-9
-8
-7
-6
-5
log [Agonist] (M)
PGE2
PGE2+AH6809
16,16dm PGE2
16,16dm PGE2+AH


% Ach maximum
100
80
60
40
20
0
-10
-9
-8
-7
-6
-5
log [Agonist] (M)
ICI80205
ICI80205+AH
17-phenyl PGE2
17-phenyl PGE2+AH


% Ach maximum
100
80
60
40
20
0
-10
-9
-8
-7
-6
-5
log [Agonist] (M)
sulprostone
sulprostone +AH

b)


100
80
60
40
20
0
%Ach maximum
-10
-9
-8
-7
-6
-5
log [Agonist] (M)
MB28767
MB28767+AH


100
80
60
40
20
0
% Ach maximum
-10
-9
-8
-7
-6
-5
log [Agonist] (M)
misoprostol
misoprostol+AH

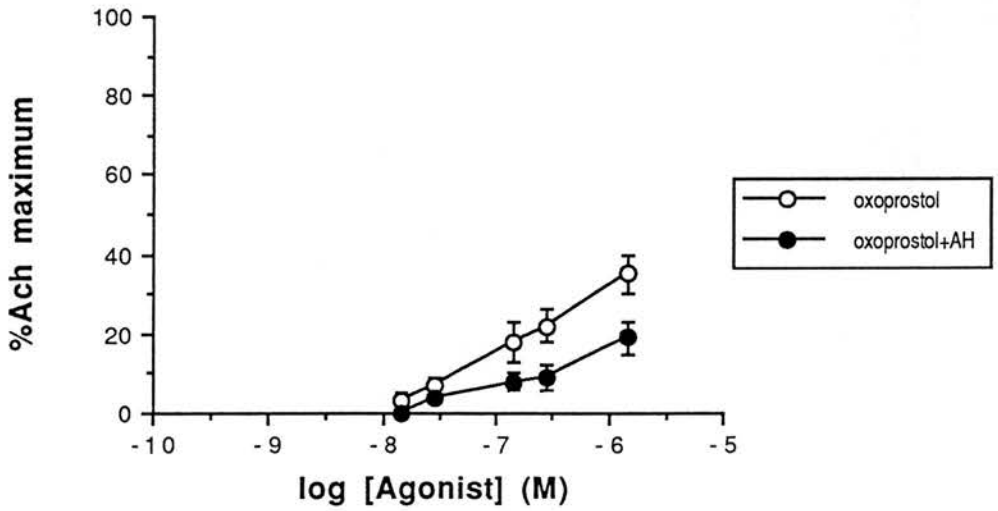
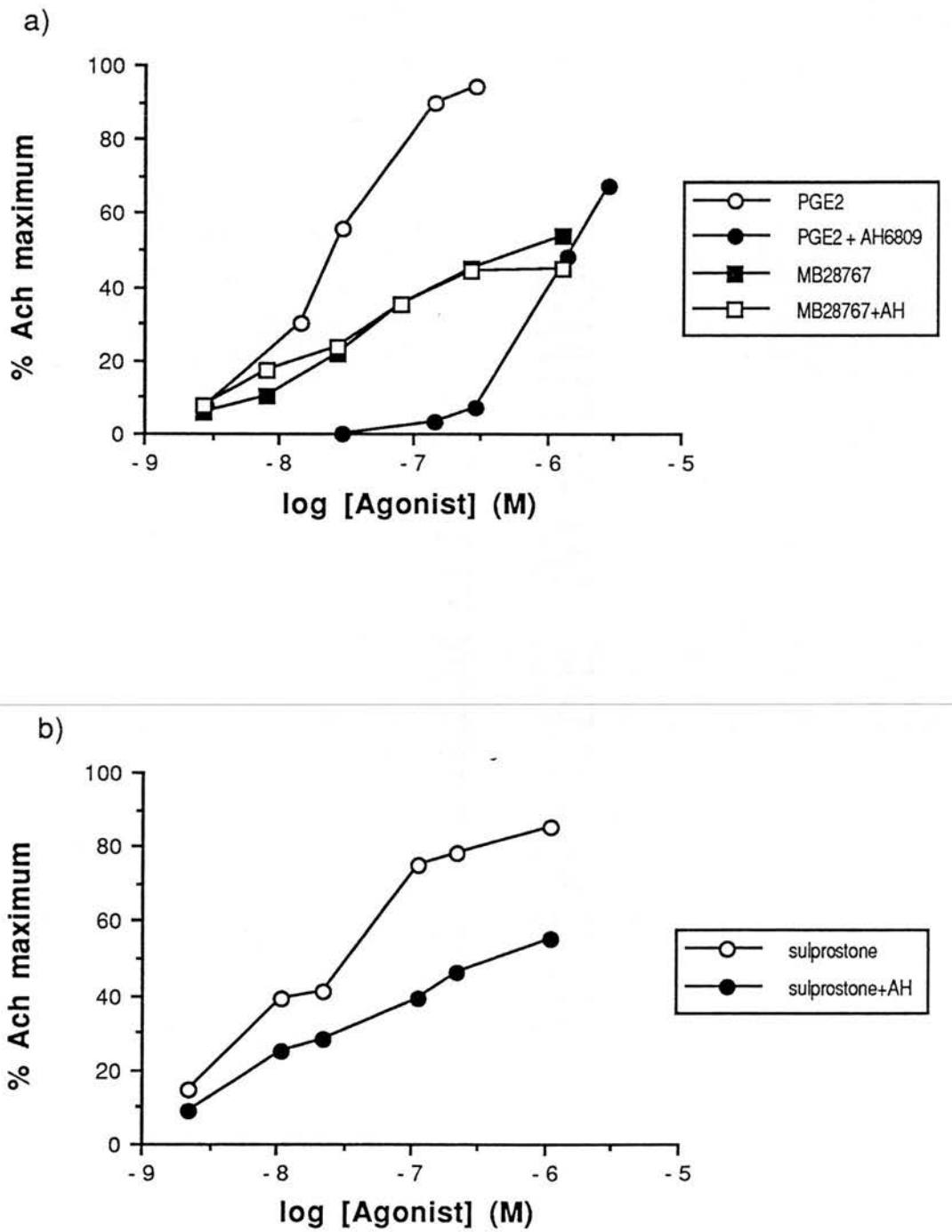

100
80
60
40
20
0
%Ach maximum
-10
-9
-8
-7
-6
-5
log [Agonist] (M)
oxoprostol
oxoprostol+AH

Figure 4.16: Effect of AH 6809 (10μM) on the concentration-response curves of (a) PGE_2 and MB 28767 and (b) sulprostone in the guinea-pig ileum (mean of 2 expts.).



Increasing the concentration of AH 6809 to 10 μM (Fig. 4.16) caused a greater shift in the curves of PGE_2 (dose ratio, 52), however, the curve of MB 28767 was not shifted.

Inhibition of Histamine-induced contractions

There was no evidence of inhibition of histamine responses with PGE_2 however, in some preparations potentiation of the histamine contractions could be observed at doses which produced no observable increase in the resting tone of the preparation (see Figure 4.17D). Butaprost had no effect on the contractions at any of the concentrations tested in 2/4 preparations (Figure 4.17D) but slightly potentiated the actions of histamine in the other two preparations.

FIgure 4.17A: Trace showing the effect of (a) TTX (100nM) and (b) AH 6809 (1μM) on the contractile action of PGE_2 (14nM) in the guinea-pig ileum.

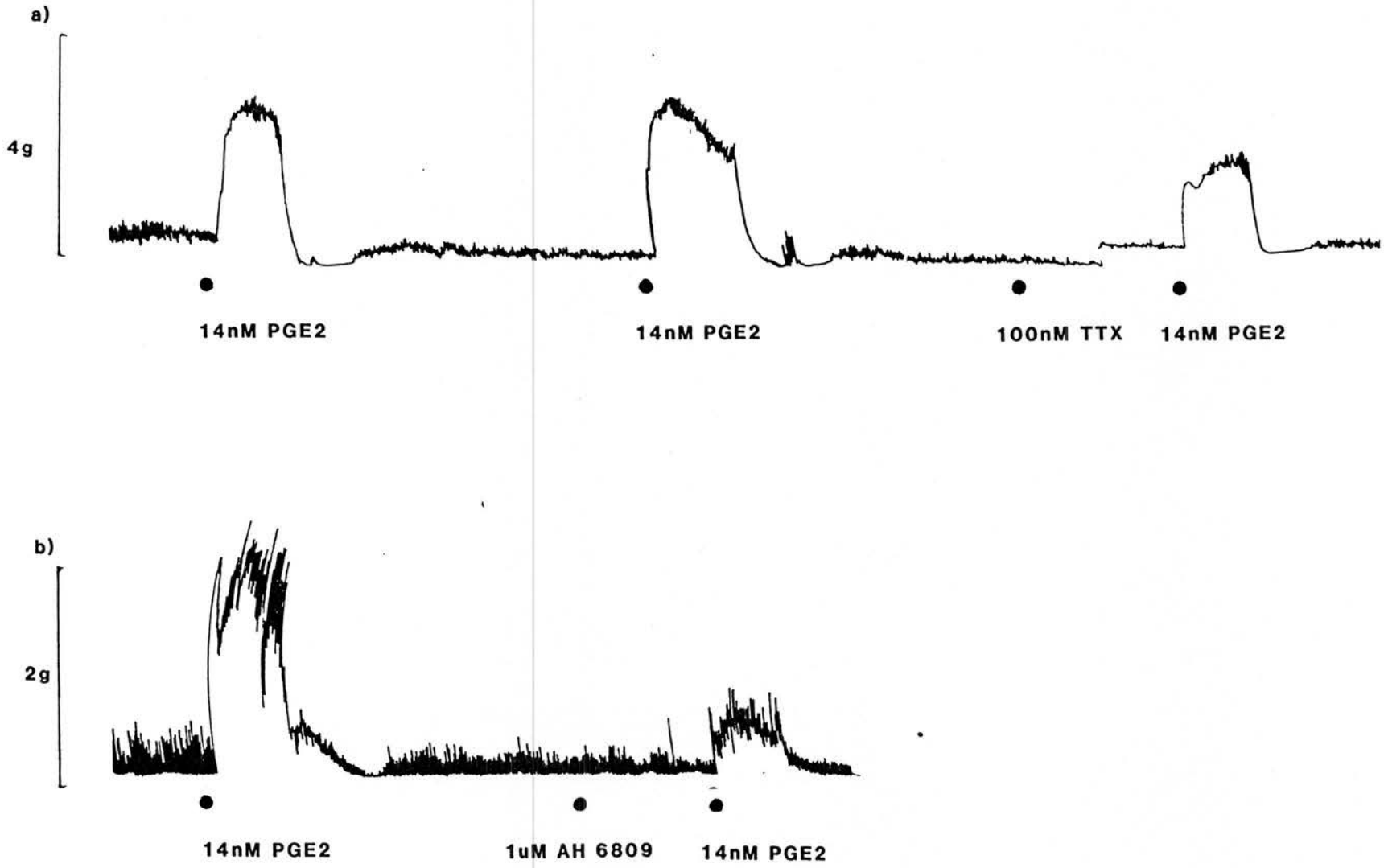

a)
4g
14nM PGE2
14nM PGE2
100nM TTX
14nM PGE2
b)
2g
14nM PGE2
1uM AH 6809
14nM PGE2

Figure 4.17B: Trace showing the effect of (a) SP 150 (1μM) and (b) SP 150 (1μM) in the presence of morphine (1μM) on the contractile action of PGE_2 in the guinea-pig ileum.

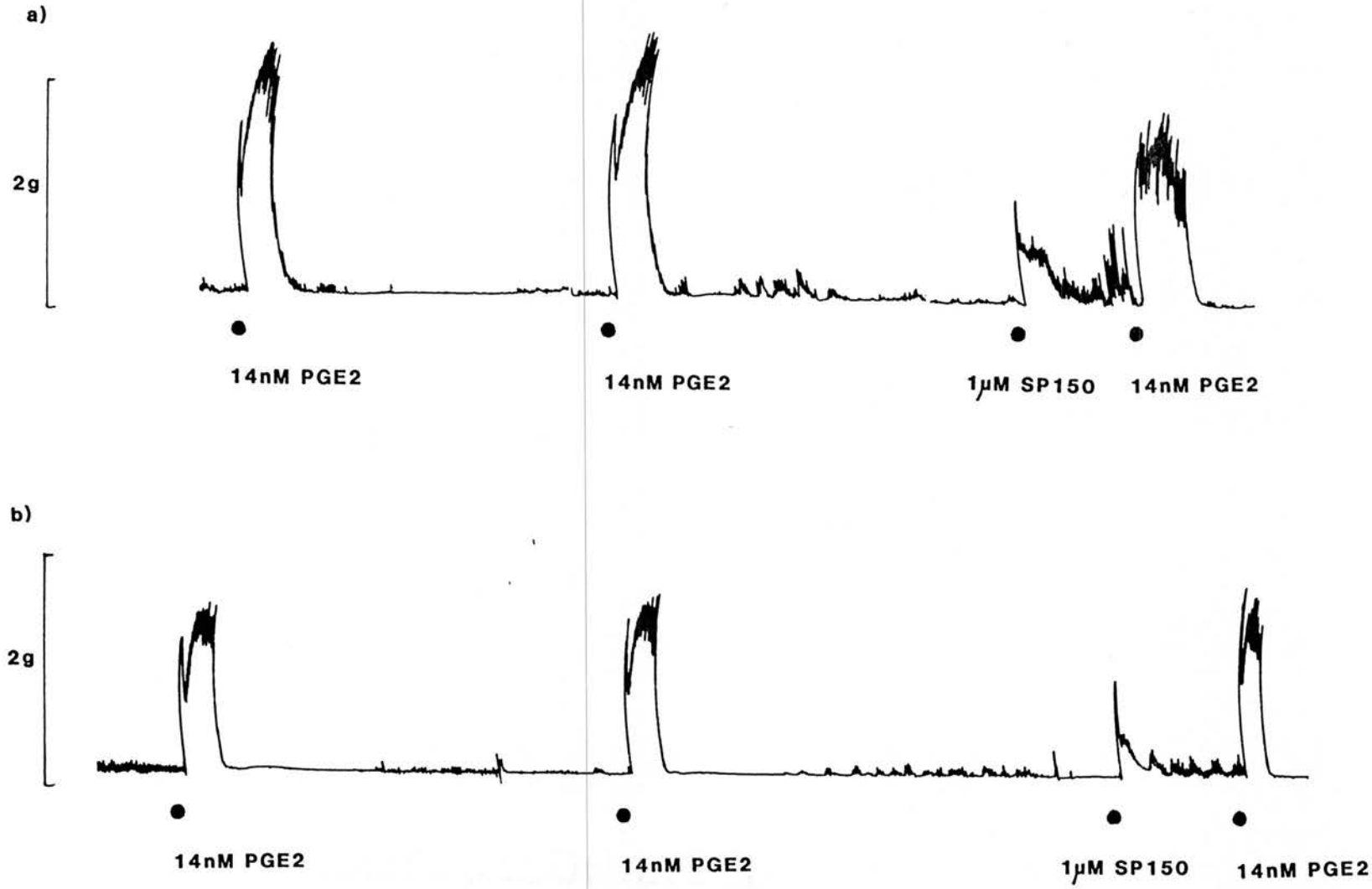

a)
2g
14nM PGE2
14nM PGE2
1μM SP150
14nM PGE2
b)
2g
14nM PGE2
14nM PGE2
1μM SP150
14nM PGE2

Figure 4.17C: Trace showing the effect of atropine (200nM) and SP 150 (1μM) on the contractile action of PGE_2 in the guinea-pig ileum.

142

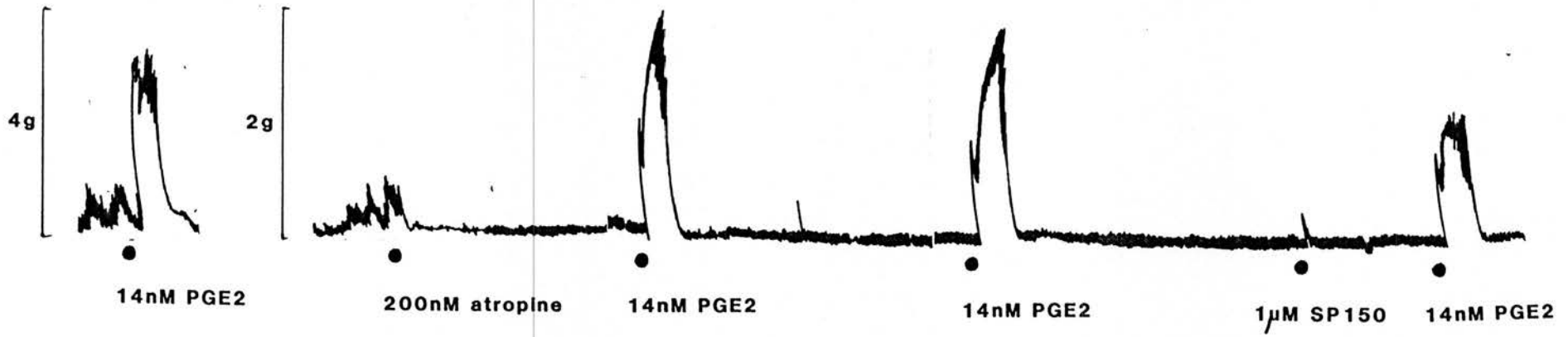
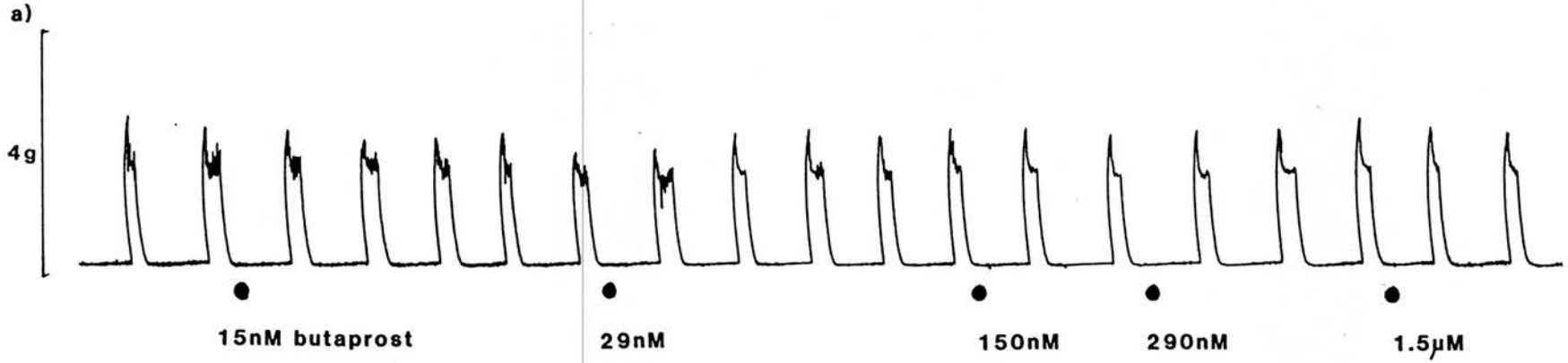
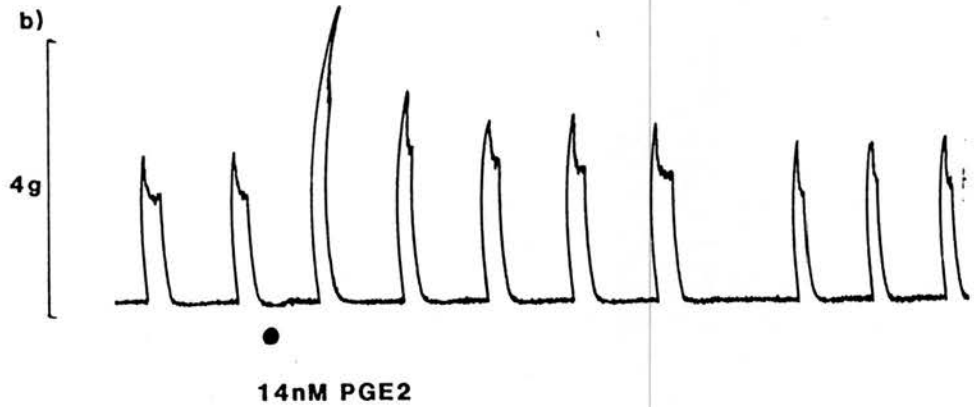

4g
2g
14nM PGE2
200nM atropine
14nM PGE2
14nM PGE2
1μM SP150
14nM PGE2

Figure 4.17D: Trace showing the effect of (a) butaprost and (b) PGE2 on contractions of the guinea-pig ileum induced by histamine. The tissues were bathed in Tyrodes solution containing AH 6809 (2μM) and morphine (1μM).


a)
4g
15nM butaprost
29nM
150nM
290nM
1.5μM


b)
4g
14nM PGE2

Chick ileum

The results obtained in the chick ileum (Table 4.3) appear to confirm that the tissue contains an EP_3 receptor: (1) contractions are only subject to a small shift (Dose ratio = 2.7) in the presence of AH 6809 and (2) those compounds which are highly active as inhibitors of the twitch response in the guinea-pig vas deferens (Jones, unpublished: Table 4.4A) are also active as contractile agents in this tissue (16,16-dimethyl PGE_2, ICI 80205, sulprostone, MB 28767 and misoprostol). However, there are a number of anomalies in the results obtained. Firstly, in the chick ileum a number of these PGE analogues have a considerably lower maximum than PGE_2: they are sulprostone, MB 28767 and oxoprostol. It is possible that these compounds are partial agonists at the EP_3 receptor, or that they have contractile and relaxant actions which effectively cancel one another out. However, the PGE_2 concentration-response curve in the presence of a supramaximal concentration (220 nM) of sulprostone fits that predicted for an additive interaction of the two compounds. Secondly, the PGE_1 analogue butaprost, which is inactive as an inhibitor of the twitch response in the guinea-pig vas deferens (Table 4.4A), is highly active as a contractile agent in this preparation. However, the maximum response of 47% at ~100 nM is again lower than that obtained for PGE_2. These results are at variance with those of Gardiner (1986) who found butaprost to be inactive in the chick ileum over a wide concentration range (2 nM - 200 μM). The experiments were carried out in Krebs solution containing a mixture of receptor antagonists (hyoscine 0.1 μg/ml, mepyramine 0.1 μg/ml, phenoxybenzamine 0.1 μg/ml, propranolol 3 μg/ml and methysergide 0.2 μg/ml) and indomethacin (3 μM). However, as shown in Figure 4.18 the presence of this cocktail had no effect on the activity of butaprost.

Butaprost has been shown (Gardiner, 1986) to be active in preparations containing EP_2 receptors (relaxation of the cat/guinea-pig trachea), but to have low EP_1 activity (contraction of the guinea-pig trachea, guinea-pig ileum). We have confirmed these results in the guinea-pig trachea (Jones, unpublished: Table 4.4B) and ileum. Thus, it may be postulated that the chick ileum contains an EP_2 receptor mediating contraction in

TABLE 4.3: pD_{25} values and equi-effective molar ratios (EMR) of prostanoids in the chick ileum (mean of 4-6 expts.). (± s.e.m)

PROSTANOID	pD_{25}	EMR
PGE analogues		
PGE_2	8.39 ± 0.06	1
16,16 dimethyl PGE_2	8.58 ± 0.06	0.32 ± 0.03
ICI80205	8.62 ± 0.45	0.44 ± 0.15
17-phenyl PGE_2	7.40 ± 0.13	13 ± 2
sulprostone	8.18 ± 0.41	1.9 ± 0.45
MB28767	8.52 ± 0.12	1.3 ± 0.1
oxoprostol	7.26 ± 0.16	12 ± 5
misoprostol	7.69 ± 0.21	4.9 ± 2.1
11-deoxy PGE_2 1-alcohol	6.68 ± 0.06	65 ± 20
butaprost	7.97 ± 0.19	2.5 ± 0.7
PGI analogues		
cicaprost	<5.89	_

TABLE 4.4A: pD_{50} values and equi-effective molar ratios (EMR) of PGE analogues for the inhibition of twitch of the guinea-pig vas deferens and contraction of the guinea-pig trachea (mean of 4 expts. except oxoprostol, 11-deoxy PGE_2 1-alcohol and butaprost, 3 expts). Jones, unpublished.

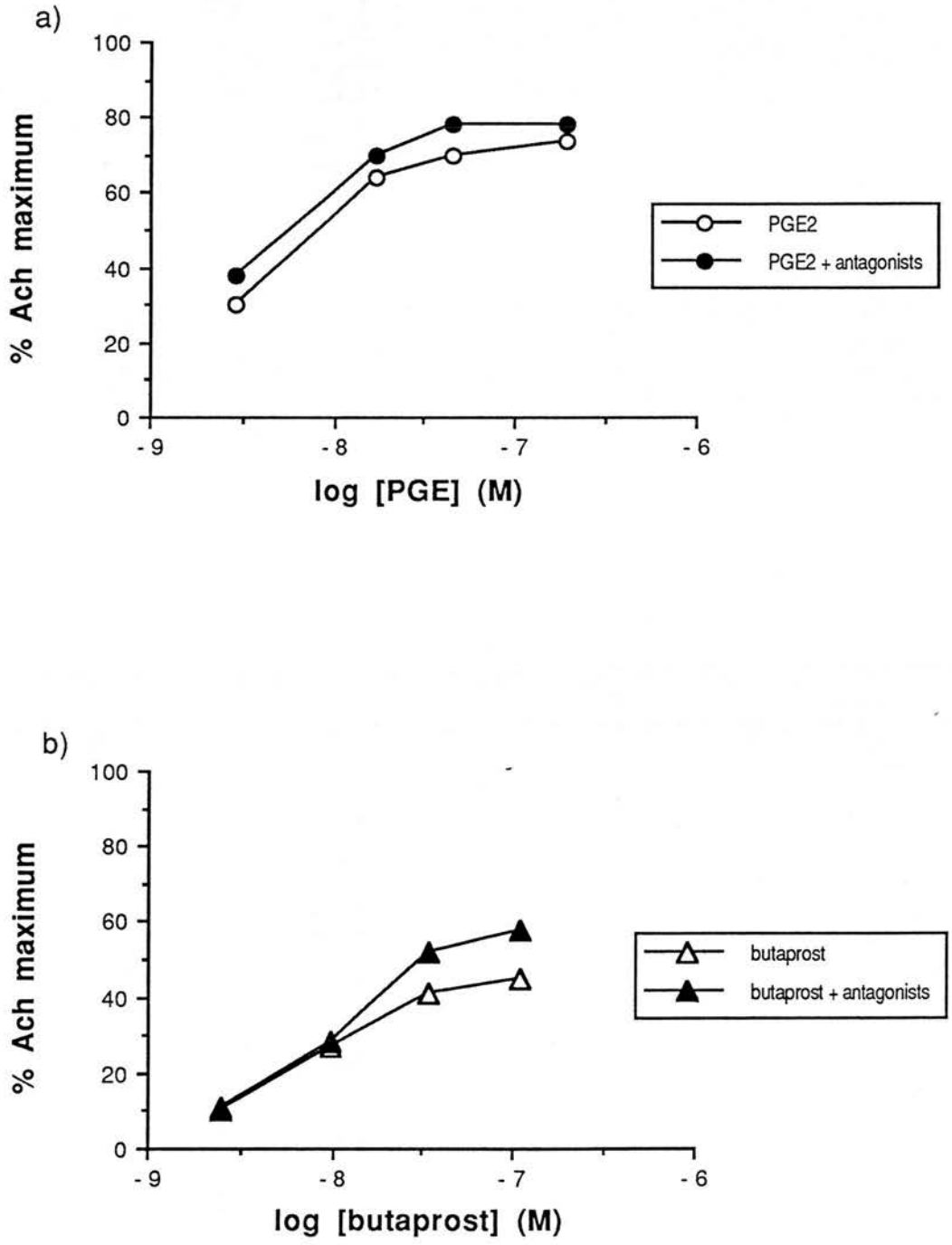
PROSTANOID	Guinea-pig vas deferens		Guinea-pig trachea
	pD_{50}	EMR	pD_{50}
PGE_2	8.76 ± 0.03	(1)	_
16,16 dimethyl PGE_2	9.62 ± 0.08	(0.13)	9.72 ± 0.06
ICI80205	9.02 ± 0.10	(0.76)	_
17-phenyl PGE_2	7.96 ± 0.06	(6.3)	8.68 ± 0.10
sulprostone	9.69 ± 0.03	(0.15)	8.14 ± 0.07
MB28767	8.97 ± 0.10	(0.72)	_
oxoprostol	8.38	(3.1)	_
misoprostol	9.12 ± 0.07	(0.51)	_
11-deoxy PGE_2 1-alcohol	7.81	(10.5)	_
butaprost	< 6.0	(>900)	_

Table 4.4B: Equi-effective molar ratios of PGE analogues for the relaxation of the guinea-pig trachea and cat trachea (3-4 expts).

Prostanoid	Equi-effective Molar Ratio	
	Guinea-pig trachea	Cat trachea
PGE_2	1.0	1.0
11-deoxy PGE_2 1-alcohol	9.5	-
11-deoxy PGE_1	11 *	13 *
Misoprostol	1.0	3.7 **
Butaprost	3.8	27 ***

* Dong et al, 1986; ** Coleman et al, 1988; *** Gardiner, 1986.

Figure 4.18: Effect of a cocktail of antagonists (see Gardiner, 1986) on the log concentration-response curves of (a) PGE_2 and (b) butaprost in the chick ileum (mean of 2 expts.).



addition to the EP_3 receptor. The existence of a second receptor in the tissue would also explain the low maximum of the EP_3-selective compounds: sulprostone, MB 28767 and oxoprostol. However, as will be discussed in the following section, the results obtained show butaprost to be considerably less active than PGE_2 (EMR ~700) at the EP_2-receptors in the rabbit jugular vein. In addition, the profile of action of 11-deoxy PGE_2 1-alcohol is the reverse of that of butaprost: it is only 4 fold less active than PGE_2 as a vasorelaxant in the rabbit jugular vein, but 65 times less active in contracting the chick ileum. On the basis of these results it is could be postulated that the EP_2 receptors present in the two tissues are different.

A number of problems must be addressed in relation to this hypothesis (1) why do the EP_1/EP_3 selective agonists, ICI 80205 and 17-phenyl PGE_2 reach a maximum response ? (2) why is there a significant shift in the log concentration-response curve of PGE_2 in the presence of the EP_1 receptor antagonist, AH 6809 ? One could postulate that the EP_2 receptor in the chick ileum is considerably more sensitive to ICI 80205 and 17-phenyl PGE_2 than that found in the RJV, however this seems unlikely as there is no evidence of relaxation of the guinea-pig trachea (Table 4.4A) or the cat trachea (Dong et al, 1986) with these agonists. It could however, be postulated that the chick ileum contains, in addition to the EP_3 receptor, a new EP receptor subtype. These hypotheses will remain purely conjecture until selective antagonists for these subtypes become available.

Rabbit jugular vein

The data obtained (Table 4.5) would suggest that relaxation of the rabbit jugular vein (RJV) by PGE analogues is mediated by an EP_2 receptor. Firstly, the responses are unaffected by the EP_1-receptor blocker, AH6809 at a concentration which produces a large shift in the concentration-response curve to PGE_2 in preparations containing EP_1 receptors (see *guinea-pig ileum*). In addition, the EP_1/EP_3 selective agonist, sulprostone is virtually inactive as a vasorelaxant in this preparation; earlier work has shown the compound to have little EP_2 activity (Coleman et al, 1987a). Finally, a number of compounds which displayed solely relaxant actions in the guinea-pig trachea (11-deoxy

TABLE 4.5: pD_{50} and equi-effective molar ratio (EMR) of prostanoids in the rabbit jugular vein (mean of 4-6 expts.).

PROSTANOID	pD_{50}	EMR
PGE analogues		
PGE_2	9.34 ± 0.11	1
16,16 dimethyl PGE_2	9.21 ± 0.19	2.1 ± 0.5
ICI80205	7.25 ± 0.17	83 ± 15
17-phenyl PGE_2	7.21 ± 0.11	200 ± 53
sulprostone	_	>3000
MB28767	_	414 ± 100 *
oxoprostol	_	>2000
misoprostol	8.21 ± 0.21	8.3 ± 1.7
11-deoxy PGE_2 1-alcohol	8.27 ± 0.16	4.6 ± 0.59
(nat) 11-deoxy PGE_1	9.35 ± 0.12	1.4 ± 0.59
(±) 11-deoxy PGE_1	9.22 ± 0.14	2.05 ± 0.64
butaprost	6.70 ± 0.10	685 ± 194
PGI analogues		
cicaprost	8.02 ± 0.19	_
iloprost	7.90 ± 0.08	_
carbacyclin	7.26 ± 0.19	_

* calculated using the IC_{25} values.

PGE_2 1-alcohol, 11-deoxy PGE_1 and misoprostol) are highly active in the RJV.

The preparation is highly sensitive to PGE_2 (IC_{50}, 0.61 nM), considerably more so than the cat trachea (IC_{30}, 40 nM: Dong et al, 1986; IC_{50}, 20 nM: Coleman et al, 1988a) or the guinea-pig trachea (IC_{30}, 10 nM: Dong et al, 1986). Thus, this preparation may prove very useful in study of the EP_2 activity of PGE analogues. The usefulness of the RJV is demonstrated in assessment of the potency of 17-phenyl PGE_2-ω-trinor (EMR, 200). An accurate estimate of the EP_2 activity of this compound would not have been possible from the cat trachea (due to the insensitivity of the preparation) or the guinea-pig trachea (due to the high potency of the compound on the EP_1 receptors mediating contraction).

The results obtained confirm those of Dong et al (1986) showing that 16,16-dimethyl PGE_2 has considerable activity at EP_2 receptors mediating relaxation of the cat trachea. ICI 80205 has less EP_2 activity in the rabbit jugular vein, again consistent with the results obtained by Dong et al (1986) on the relaxation of the guinea-pig/cat trachea.

The data is at variance with that of Eglen and Whiting (1988) who found that at 300 nM misoprostol elicited contractions of the guinea-pig trachea, with no evidence of relaxation. However, Coleman et al (1988a) found misoprostol to be highly active at EP_2 receptors mediating relaxation of the cat trachea. In addition, it has recently been shown (Jones et al, 1989) that at 200 nM the compound rapidly abolishes submaximal contractions of the guinea-pig trachea induced by persistent TP receptor agonists such as EP 171. A possible explanation for this discrepancy may be that Eglen and Whiting used zig-zag preparations while we have used rings, although this is unlikely to account for such a large difference.

11-Deoxy PGE_1 and 11-deoxy PGE_2 1-alcohol appear to be active EP_2 agonists. They also appear to be more selective for this subtype than misoprostol having less EP_1 (guinea-pig trachea/ileum) and EP_3 activity (guinea-pig vas deferens), and may prove useful in the investigation of EP_2 receptors.

As mentioned earlier, work by Gardiner (1986) suggested that butaprost is a highly selective EP_2 agonist. Studies by Jones (unpublished) have also shown that the compound is active as a relaxant in the guinea-pig trachea with no evidence of contractile activity. However, in the RJV butaprost appears to be considerably less active at EP_2 receptors than predicted from the above results. As will be discussed later, this preparation is known to contain TP-receptors mediating contraction, however the experiments were carried out in the presence of 10 μM GR 32191, a potent TP-receptor blocker. In addition, Gardiner (1986) found the compound to be inactive in the guinea-pig lung and the guinea-pig trachea - both known to contain TP receptors.

It may be that the potency of PGE_2 as a relaxant in the guinea-pig trachea is being vastly underestimated, thus making EP_2-selective agonists appear considerably more active relative to PGE_2 in this preparation. However, this would not explain the finding that in the guinea-pig trachea 11-deoxy PGE_2 1-alcohol is less active relative to PGE_2 than butaprost, while in the RJV it is considerably more active. Another possible explanation may be the presence of esterase activity in *one* of these tissues which produces the free acid form of butaprost from its methyl ester, the former having greater activity at EP_2 receptors. However, preliminary experiments (Jones, unpublished) show that the anticholinesterase, diisopropyl-fluorophosphonate (DFP) had no effect on the concentration-response curve of butaprost in the guinea-pig trachea. In addition, one would expect such enzyme activity to similarly affect misoprostol (see figure 4.1), however, this compound has the same order of activity in both preparations.

It may be that the RJV contains a subtype of the EP_2 receptor distinct from that found in the guinea-pig trachea, cat trachea and chick ileum. However, this hypothesis cannot be confirmed without highly selective EP_2 agonists and antagonists - which are not, as yet, available.

We have confirmed data obtained by Giles et al (1989) showing that the tissue contains a TP receptor, which is highly sensitive to U 46619 (EC_{50}, 4.6 nM). The contractile action of MB 28767 would appear to be mediated by an action on the thromboxane receptor as its actions are inhibited by

the TP receptor blocker GR 32191. The relative potency of the compound *vs* U 46619 (6.5 ± 0.7) is slightly higher than that obtained at thromboxane receptors of a range of tissues (Banerjee et al, 1986; Halushka et al, 1987).

It is notable that the pA_2 (7.2) of GR 32191 against U 46619 is significantly lower than that reported for other tissues (Lumley et al, 1987) - this finding is discussed in detail in Chapter 5. However, the shift of ~200 in the concentration-response curve produced by 10 μM GR 32191 is sufficient to give an indication of the EP_2 activity of MB 28767 and to eliminate the TP agonist action of ICI 80205 at the doses producing relaxation. In the presence of the blocker the concentration-response curve for relaxation of histamine-induced contractions of MB 28767 is bell-shaped, indicating that the EP_2 relaxant activity is being overcome by the TP contractile action at high doses.

In conclusion, the rabbit jugular vein appears to contain a highly sensitive EP_2 receptor system mediating relaxation. In the presence of thromboxane receptor antagonists the preparation is most useful in the study of the EP_2 selectivity of a range of PGE analogues. However, the nature of the EP_2 receptor in relation to those present in other preparations (guinea-pig trachea and cat trachea) requires further study.

Guinea-pig ileum

As previously reported (Sanner,1971; Kădlec et al, 1971) we have found that the contractile response of the guinea-pig ileum to PGE_2 has a component due to the action of activation of enteric neurones (Table 4.6), which is inhibited to a similar extent by both TTX and morphine. Studies with atropine and the substance P antagonist, SP 150 indicate that both acetylcholine and substance P are released. As was found for the IP system (see Chapter 3) the effects of the blockers is additive, and neither has any further activity in the presence of 1 μM morphine.

The concentration-response curves for misoprostol, 11-deoxy PGE_2 1-alcohol, 11-deoxy PGE_1 and butaprost are subject to a large shift in the presence of 1 μM morphine (Table 4.7). As discussed above these

Table 4.6: The pD_{25} values for PGE analogues in the absence and presence of Morphine (1μM) and AH6809 (2μM).

PROSTANOID	MAX. (% ACH)	pD_{25}	pD_{25} MORPHINE (1μM)	pD_{25} AH6809 (2μM)
PGE_2	88 ± 3	8.96 ± 0.17	8.42 ± 0.17	6.88 ± 0.13
16,16 dimethyl PGE_2	90 ± 2	9.38 ± 0.11	9.19 ± 0.11	7.66 ± 0.10
ICI80205	96 ± 1	9.54 ± 0.27	9.28 ± 0.24	7.95 ± 0.11
17-phenyl PGE_2	96 ± 1	8.10 ± 0.10	8.11 ± 0.10	7.31 ± 0.12
sulprostone	75 ± 4	7.92 ± 0.07	7.86 ± 0.06	7.39 ± 0.23
MB28767	67 ± 4	8.50 ± 0.11	7.85 ± 0.19	7.58 ± 0.20
oxoprostol	45 ± 3	7.03 ± 0.16	6.34 ± 0.28	5.69 ± 0.14
misoprostol	70 ± 7	8.10 ± 0.09	6.55 ± 0.09	>5.89
11-deoxy PGE_2 1-alcohol	35 ± 5	7.11 ± 0.14	<5.52	_
11-deoxy PGE_1	45 ± 6	6.87 ± 0.22	<5.70	_
butaprost	36 ± 5	6.67 ± 0.28	<5.92	_

Table 4.7 : The effect of Morphine (1μM) and Atropine (200nM) on the dose-response curves of PGE_2 analogues in the guinea-pig ileum (mean of 4-6 expts.).

PROSTANOID	DOSE RATIO: Morphine (1μM)	Atropine (200nM)
PGE_2	3.58 ± 0.38 *	4.66 ± 2 *
16,16 dimethyl PGE_2	1.62 ± 0.24	2.40 ± 0.24 *
ICI80205	1.96 ± 0.19	_
17 phenyl PGE_2	0.94 ± 0.04	_
sulprostone	1.15 ± 0.03	1.40 ± 0.27
MB28767	5.2 ± 1.4 *	_
oxoprostol	4.0 ± 1.2	_
misoprostol	42 ***	_
11-deoxy PGE_2 1-alcohol	>50	_
11-deoxy PGE_1	>20	_
butaprost	>10	_

*** $P < 0.001$

** $P < 0.01$

* $P < 0.05$

compounds have been shown to be active at the EP_2 receptors in a range of tissues: rabbit jugular vein, guinea-pig trachea (Jones, unpublished Table 4.4), cat trachea (Dong et al, 1986; Coleman et al, 1988; Gardiner, 1986). In contrast, those compounds whose activity was not significantly affected by morphine (ICI 80205, sulprostone, 17-phenyl PGE_2-ω-trinor and oxoprostol) have low activity at the EP_2 receptors of the RJV, and are contractile agents in the guinea-pig trachea. On the basis of these results one could suggest the receptor subtype mediating the indirect action of PGE analogues in the guinea-pig ileum is EP_2.

Poll et al (1988a) showed that in the presence of 10 μM AH 6809 (see introduction), PGE_2 caused a concentration-related potentiation of electrically-induced contractions, and reversed inhibition produced by indomethacin (1-30 μM). The authors suggest that excitatory neuronal responses to PGE_2 in the guinea-pig ileum are mediated by a receptor other than EP_1. However, although their data suggests the existence of a receptor additional to EP_1, a neuronal site of action cannot be definitely concluded from these experiments (see Hedqvist, 1980). Recently, however, the group (Poll et al, 1988b) have demonstrated that this potentiating action of PGE_2 is inhibited by cyclopentyladenosine, known to act presynaptically to inhibit transmitter release (Gintzler & Musachio 1975; Fredholm & Dunwiddie, 1988.).

Classification of the neuronal EP receptor, at this stage, must be purely tentative for a number of reasons. Firstly, studies lack a compound which is a potent and selective EP_2 receptor agonist / antagonist. In addition, the situation is made more complex by the conflicting results obtained in studies of the EP_2 receptors of the rabbit jugular vein, cat trachea and guinea-pig trachea which raise the possibility of the existence of subtypes of the receptor.

Some of the results presented on the action of butaprost in this preparation are at variance with those of Gardiner (1986). Firstly, Gardiner (1986) found that the concentration-response curve for this compound was bell-shaped - the maximum response being <20% of the tissue maximum at 1μM. In contrast, the results presented in this thesis show that the concentration-response curve of butaprost has no

evidence of belling and 36% of the tissue maximum is reached at the highest concentration tested, 3μM. Secondly, Gardiner (1986) reported an inhibitory action of the compound on histamine-induced contractions at concentrations of 3 and 10μM. However, as is shown in Figure 4.17 no such inhibition was evident in the results obtained.

The experiments of Gardiner (1986) involve concentrations of butaprost of up to 200μM, which would require aqueous stock solutions of ~2mg/ml. However, in our hands when a 2mg/ml solution of the compound, in 100% ethanol, was diluted to 100μg/ml in saline opalescence appeared. The solution did not clear on warming. It should be noted that butaprost is a methyl ester thus the addition of sodium bicarbonate does not increase its aqueous solubility as with free acid prostanoids. Thus the aqueous top stock used in the experiments presented here was 50μg/ml - a dilution into saline of a 1mg/ml ethanol solution. It may be that Gardiner (1986) has used a high proportion of ethanol/DMSO to ensure solubility of high concentrations of butaprost and these agents are responsible for the inhibitory effects observed.

Our studies have shown that the EP_1 blocker, AH 6809, causes a parallel rightward shift in the concentration-response curve of PGE_2, consistent with a pA_2 of 7.08 ± 0.14 (6.3-7.4). AH 6809 also produced a large shift in the concentration-response curves of 16,16-dimethyl PGE_2, ICI 80205 and 17-phenyl PGE_2-ω-trinor (Table 4.8), this is not unexpected as these compounds are all highly active at EP_1 receptor sites in the guinea-pig trachea (Table 4.4). However, certain results indicate the presence of another contractile receptor in addition to EP_1: both sulprostone (at concentrations giving 10-30% responses) and MB 28767 are resistant to block by 2μM AH 6809. Similar results were obtained using 10 μM AH 6809.

It is unlikely that this effect is due to an action of the compounds on TP receptors as the TP-receptor agonist, U 46619 is inactive in this preparation. In addition, the contractile action of $PGF_{2\alpha}$ is blocked by AH 6809 indicating that it is acting at EP_1 receptors. Both sulprostone and MB 28767 are highly active in the chick ileum and the guinea-pig vas deferens, indicating that the AH 6809-resistant receptor of the

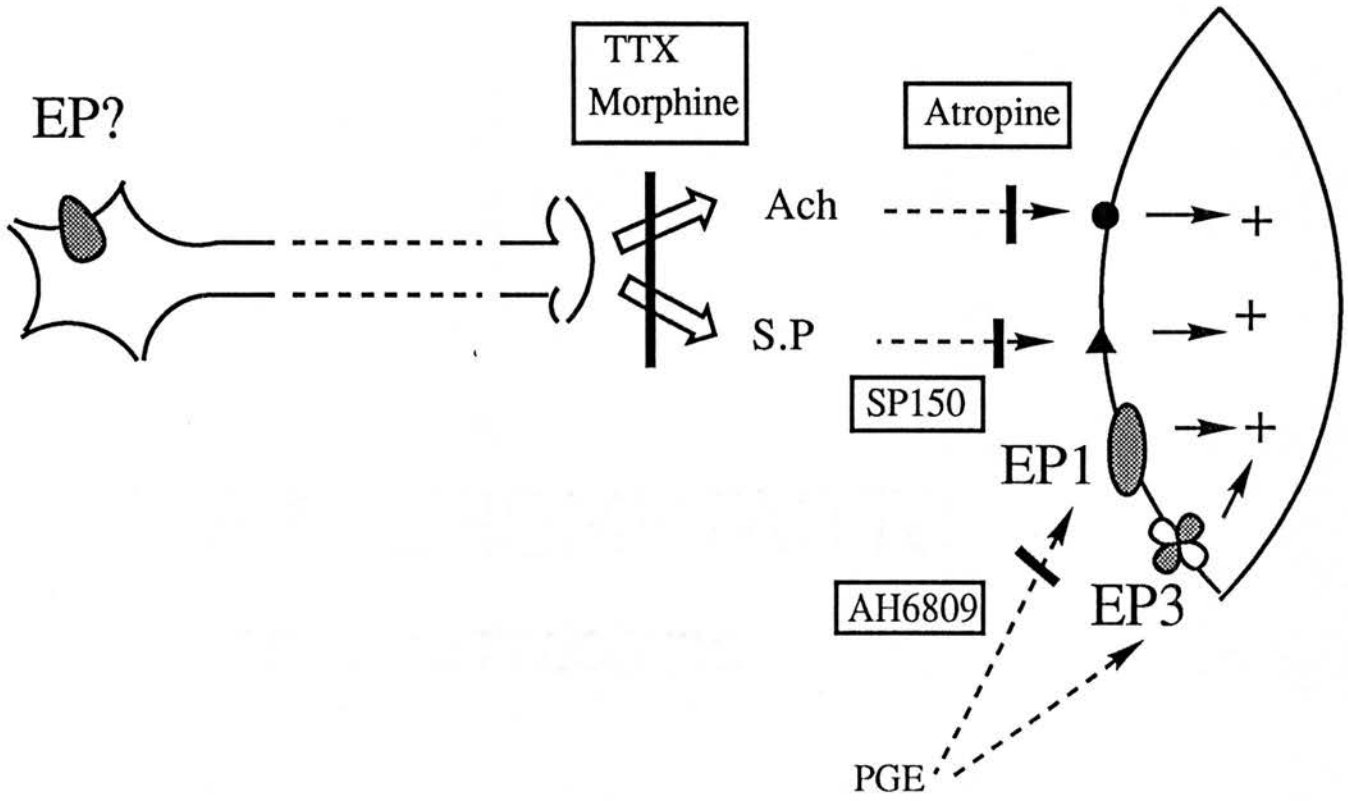
Table 4.8: Effect of AH 6809, 2 μM (mean of 4-6 expts) and 10μM (2 expts) on the concentration-response curves on PGE and PGI analogues in the guinea-pig ileum.

PROSTANOID	DOSE RATIO IN THE PRESENCE OF AH6809	
	2μM	10μM
PGE analogues		
PGE_2	31.2 ± 7 ***	52.5
16,16 dimethyl PGE_2	24.2 ± 5 ***	_
ICI80205	12.6 ± 1 ***	_
17-phenyl PGE_2	11.5 ± 2 ***	_
sulprostone	3.5 ± 2	4.6
MB28767	1.8 ± 0.5	1.6
oxoprostol	3.9 ± 1 *	_
misoprostol	>10 *	
PGI analogues		
iloprost	245 ± 57 ***	_
carbacyclin	24.9 ± 8 ***	_

*P< 0.05

***P<0.001

Figure 4.19: Schematic representation of the EP receptor sites in the guinea-pig ileum



guinea-pig ileum may be EP_3. The shift in the sulprostone curve at higher concentrations reflects its activity at EP_1 receptors, also seen in the guinea-pig trachea (Table 4.4a).

In conclusion, the data presented demonstrates that the guinea-pig longitudinal muscle contains EP receptors additional to the EP_1 subtype (see Fig 4.19). Thus, data claiming EP_1 selectivity of a compound on the basis of high activity in this preparation should be interpreted with caution. In order to state unequivocally the receptor subtype involved one should investigate the effect of both morphine and AH 6809 on the contractile action. Making use of these blockers, however, the guinea-pig ileum remains a useful tissue in assessing the EP selectivity of PGE analogues. It is notable that other tissues used to measure EP_1 activity (see introduction) have added complications: the guinea-pig trachea has highly sensitive EP_2 receptors mediating relaxation and recent work by Jones (unpublished) suggests that the guinea-pig fundus may have an additional population of EP receptors which are resistant to block by AH 6809 (possibly EP_3).

Structure-Activity Relationships

Previous work has shown that certain structural features are essential for EP receptor agonists activity. These are as follows:

(a) An α chain of 7 carbon atoms (Grudzinskas et al, 1980).

(b) A cyclopentane ring (Grudzinskas et al ,1980).

(c) A substituent at C9, usually carbonyl (Lin et al, 1976; Kimball et al, 1979; Carpio et al, 1987) .

(d) A hydroxyl group at C15 or C16 (Grundzinskas et al, 1980; Floyd et al, 1980).

All the analogues fulfil these criteria, however there is considerable variation in the activity of these compounds at the various EP receptor subtypes studied.

The results confirm earlier findings (Grundzinskas et al, 1980; Hess et al, 1980; Schaaf et al, 1981) that the substitution of a methanesulphonamido group at C1 (sulprostone) greatly reduces EP_2 activity, leaving EP_1 and EP_3 unaffected. Loss of the 11-hydroxy group (11-deoxy PGE_2 1- alcohol, 11-deoxy PGE_1, MB 28767 and oxoprostol)

reduces the EP_1 activity of these analogues but is not essential for EP_2/EP_3 activity (see Grudzinskas et al 1980; Carpio et al, 1987; Floyd et al, 1987). The 16-methyl group (16,16-dimethyl PGE_2) appears to enhance both EP_1 and EP_3 activity, with only small loss of EP_2 activity. The results are consistent with those of Banerjee et al (1981a) showing that the presence of the 16-phenoxy group (ICI 80205, sulprostone, MB 28767 and oxoprostol) leads to potent EP_3 activity but reduces EP_2 actions. The addition of a halogen (ICI 80205) appears to enhance the potency at EP_1 and EP_3 receptors. Finally, substitution of the terminal n-propyl unit by phenyl leads to EP_1 selectivity, reduced activity at EP_3 receptors and little EP_2 receptor activity.

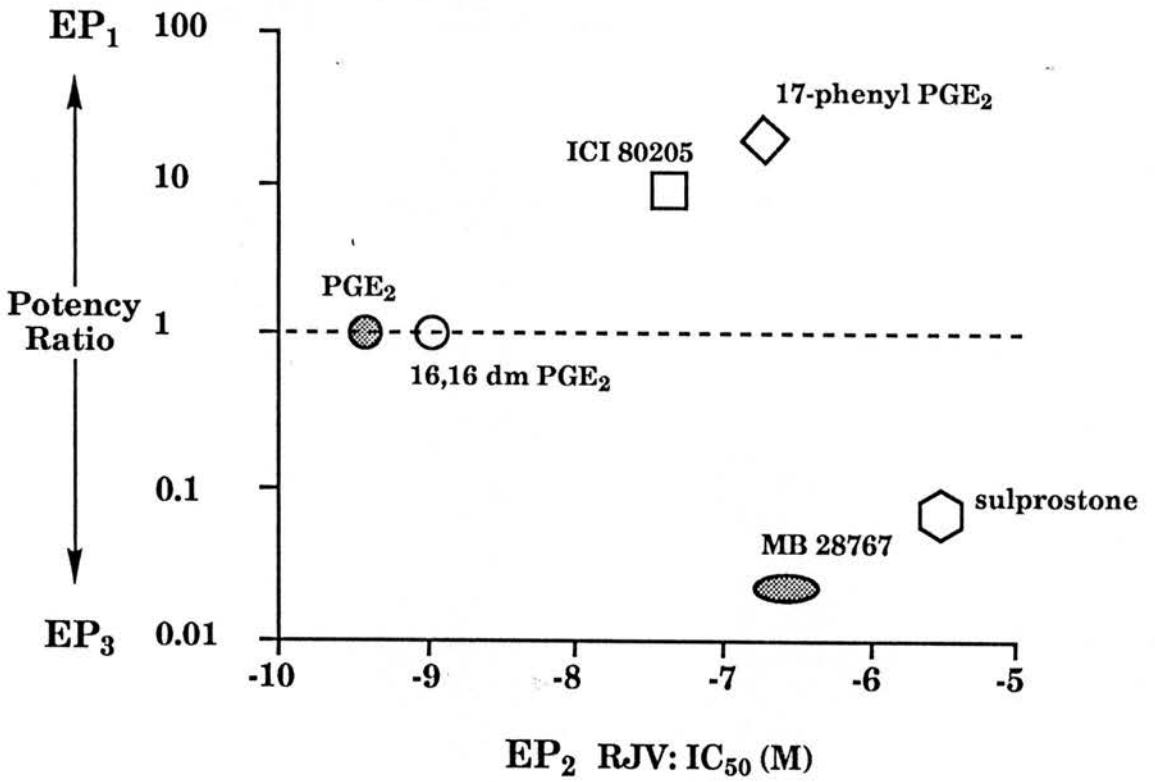
In summary, the results indicate that a number of the compounds studied may prove useful in the classification of EP receptors (see Fig 4.20):

EP_1: 17-phenyl PGE_2-ω-trinor appears to be the most selective EP_1 agonist tested: it is highly active in the guinea-pig trachea and ileum and is 200 times less active than PGE_2 in the rabbit jugular vein. However, the compound retains a certain degree of EP_3 activity.

EP_2: As previously demonstrated (Gardiner, 1986) the results show the butaprost has no activity at EP_1 or EP_3 receptors, thus it is useful in the study of EP_2 receptors. However, in certain EP_2 systems (rabbit jugular vein) it is 700 times less active than PGE_2. 11-deoxy PGE_2 1-alcohol and 11-deoxy PGE_1 are potent EP_2 agonists, however, they lack the selectivity of butaprost.

EP_3: The data confirms that sulprostone is devoid of activity in the most sensitive EP_2 system as yet characterised (rabbit jugular vein), however, it retains potent agonist action at EP_1 receptors. MB 28767 may prove more useful an EP_3 selective agonist than sulprostone: it has low EP_2 activity and shows no evidence of EP_1 agonist action in the guinea-pig trachea or guinea-pig ileum. However, experiments show that the analogue has considerable agonist action at TP receptor.

Figure 4.20: Agonist selectivity of EP receptor systems. EP_1 activity measured in the guinea-pig trachea (contraction), EP_3 activity measured in the field stimulated guinea-pig vas deferens (inhibition of the twitch response) and EP_2 activity measured in the rabbit jugular vein.



Chapter 5

TP-Receptor Studies

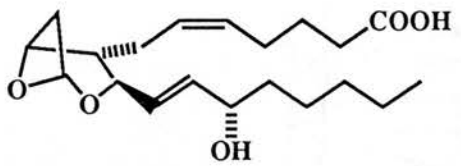
INTRODUCTION

The first description of thromboxane A_2 was based on superfusion bioassay experiments carried out by Piper and Vane (1969). They detected the release from the passively sensitized guinea-pig lung, when challenged with antigen, of a labile material which contracted the rabbit aorta and affected the other tissues much less than stable prostaglandins. The substance was called rabbit aorta contracting substance (RCS). Later, Hamberg et al (1974) identified and named the major component of RCS as thromboxane A_2 (TXA_2), a highly unstable intermediate in the conversion of prostaglandin G_2 to thromboxane B_2.

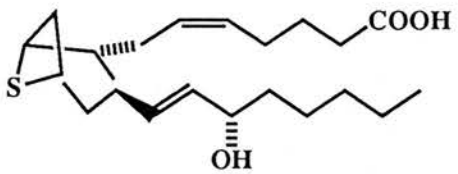
TXA_2 is highly potent as a stimulator of platelet aggregation (Svensson et al, 1976; Kinlough-Rathborne et al, 1977; Moncada and Vane, 1978) and a constrictor of vascular and other types of smooth muscle (Hamberg et al, 1975; Needleman et al, 1976; Moncada et al, 1976; Tuveno et al ,1976; Svensson and Fredholm, 1977; Ellis et al, 1977; Dusting et al 1978). In addition, TXA_2 has been implicated in the pathophysiology of a range of conditions including: thrombosis and occlusive vascular disease (Fitzgerald et al, 1987; Green and Vesterqvist, 1986; Catella et al, 1987).

As mentioned above, TXA_2 is highly unstable with a half-life of about 30 seconds at 37°C and pH 7.4. By comparison with the time courses of action of the other natural prostanoids, it would seem unlikely that TXA_2 establishes equilibrium occupancy at TP receptors within its half-life. This poses a major difficulty in studying the pharmacology of TP receptors and in particular the properties of TP receptor antagonists. It has therefore been necessary to develop stable and specific TXA_2 mimetics (TP receptor agonists) (Fig. 5.1A). One of the first to be synthesized was 11,9- epoxymethano PGH_2 (U 46619). Although strictly an analogue of PGH_2, it has been shown that this compound has a profile of activity very similar to that of TXA_2 (Fitzpatrick et al, 1978; MacIntyre et al, 1978) and as yet, it has the most favourable combination of potency, specificity and rapidity of action (Jones at al, 1989). Jones and Marr (1977) showed that replacing the terminal 4 carbon unit of $PGF_{2\alpha}$ with a *p*-flurophenoxy group produced a compound with a 50 fold

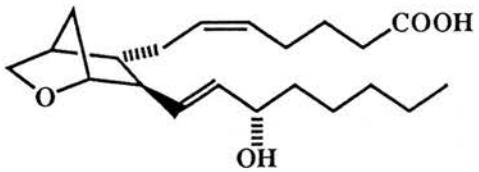
Figure 5.1A: Structure of TP-receptor agonists



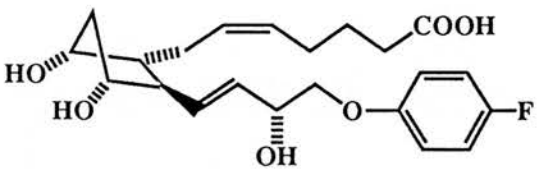
TXA_2



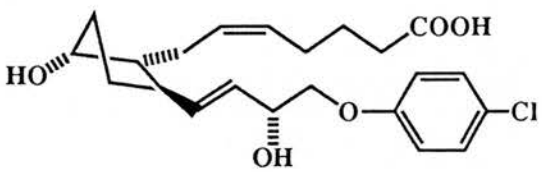
STA_2



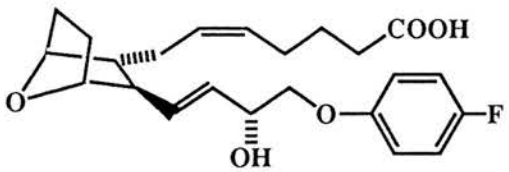
U 46619



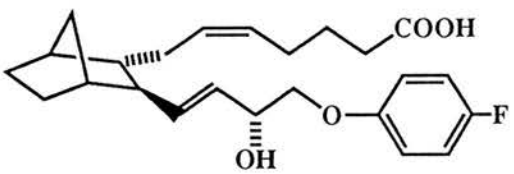
ICI 79939



11-deoxy-16-p-chlorophenoxy
ω-tetranor $PGF_{2\alpha}$

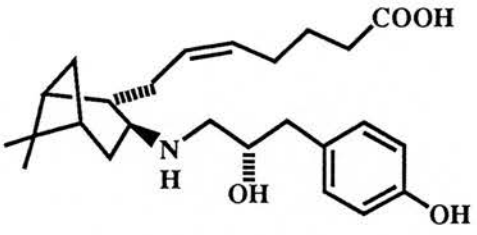


EP 171

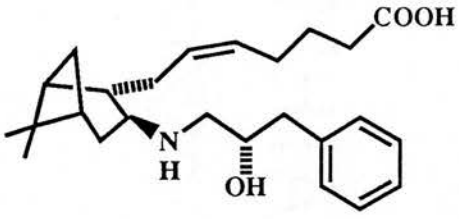


EP 031

Figure 5.1B: Structure of TP-receptor antagonists



PTA-OH



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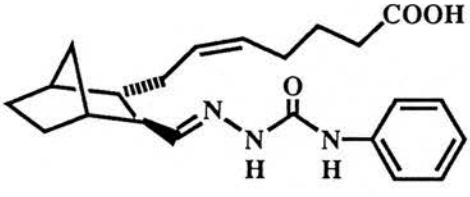

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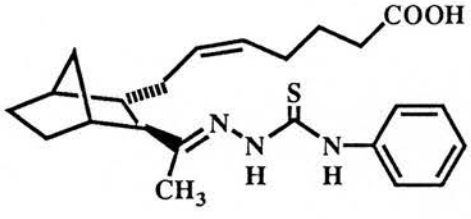

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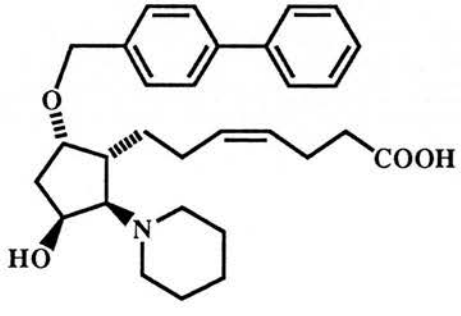
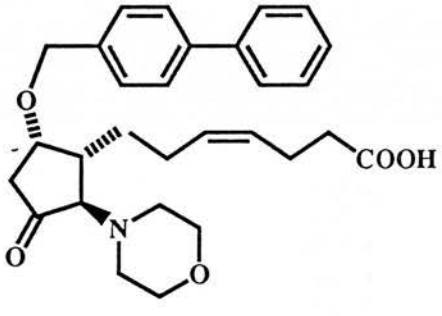
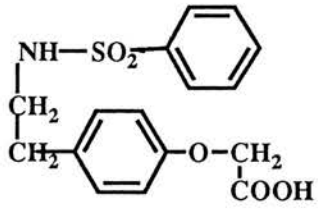


GR 32191



AH 23848



BM 13177

increase in potency at TP receptors in various isolated preparations (ICI 79939 - Dukes et al, 1974). A similar substitution on ring analogues of PGH_2 has led to synthesis of EP 031 and EP 171, both highly potent TXA_2 mimetics (Jones et al,1989). Other potent TXA_2 mimetics include a thia derivative of TXA_2, 11a-carba-9,11-thia TXA_2 (STA_2) (Katsura et al, 1983).

A range of structurally diverse compounds have been synthesized which specifically antagonise the actions of thromboxane mimetics in both platelet and vasculature of a number of species (Fig 5.1B). The TP receptor antagonists can be divided into three distinct categories. Firstly, those which are modified thromboxane / endoperoxide structures. The original TXA_2 antagonists were of this type: LeBreton et al (1979) demonstrated that 13 azapostanoic acid (APA) inhibited aggregation to PGH_2 and a similar profile was demonstrated for pinane TXA_2 (PTA_2) (Nicolaou et al, 1979). However, subsequent studies (Jones et al, 1984) have shown that PTA_2 is, in fact, a partial agonist. Jones et al (1984) and Wilson and Jones (1985) produced the TP receptor antagonists EP 045 and the more potent EP 092, which retain the natural α chain and bicyclo [2.2.1] heptane ring but have a semicarbazone modification to the ω chain. Finally, ONO 11120 (Katsura et al, 1983) is a TXA_2 antagonist developed from the combination of 2 classes of antagonist, the aza prostanoids and the pinane series. The second category of antagonists include compounds with prostanoid structures bearing little resemblance to thromboxane/endoperoxides. AH 23848 (Brittain et al, 1985) and GR 32191 (Lumley et al,1987) nominally have the PGD ring structure with a modified ω chain and addition to the C9 hydroxyl of a 4-phenylbenzyl group.

The final group of antagonists bear no relation to the prostanoid structure; this group includes BM 13,177 (Patscheke et al, 1984) which is a sulphonamide derivative.

Lefer et al (1981) were first to claim a dissociation between the platelet and vascular actions of thromboxane: they found that carbacyclic TXA_2 (CTA_2) was a vasoconstrictor in the cat coronary artery but antagonised

arachidonic acid-induced aggregation of human platelets (platelet rich plasma, PRP). A number of other early studies (Gorman et al, 1981b; LeDuc et al, 1981) have also suggested differences between the TP receptor on the platelet and vasculature. However the results have to be interpreted with caution for the following reasons. Firstly, many of the platelet studies employed PRP. Analogues which are lipophylic will bind to the plasma proteins present in PRP and this will result in a considerable decrease in the effective free concentration. Secondly, these groups did not investigate the ability of compounds to induce platelet shape change, which would indicate partial agonist properties. In the platelet, partial agonists may cause shape change but not a full aggregation wave. The shape change and even small aggregation waves are not always obvious if the test compound is added to the PRP at room temperature before placing in the heated cell block of the aggregometer. It is notable that CTA_2 has been shown to induce a rapidly reversing aggregation wave and also inhibits the aggregating wave of U 46619, indicating partial agonist activity (Armstrong et al, 1986). Finally, the platelet contains IP and DP receptors which mediate inhibition of aggregation - agonist activity of thromboxane analogues at these receptors may reduce their effectiveness as aggregatory agents.

Studies by Mais et al (1985a, 1988) have demonstrated a difference in the rank order of potency of a range of 13-azapinane TP receptor antagonists in the platelet and vasculature (saphenous vein) of both the human and the dog. The problem of plasma protein binding has been eliminated from these later studies as washed platelets have been used. In addition, this group (Mais et al, 1985c) have resolved three of the analogues used in the above study (ONO 11120, PTA-OH and I-PTA-OH), and found that in the saphenous vein one of the enantiomers, of all three compounds, was ~3 times more potent than the other, while in the platelet the epimers were of equal potency. The authors concluded that there was a difference in the TP receptor present in the platelet and vasculature.

More recent investigation of a wide range of structurally distinct agonist and antagonists in both platelet and vasculature (Swayne et al, 1988; Jones et al, 1989) show no differences in the rank order of potency at the

two TP receptors involved. Thus, the hypothesis for the existence of distinct receptors on the platelet and vasculature is based on differences seen within a close series of 13-azapinane TP receptor antagonists.

A number of groups (Burke et al, 1983; Jones et al, 1987; Narumiya, et al, 1986; Swayne et al,1988) have found the rabbit platelet to be 20 - 300 times less sensitive to TP-receptor antagonists than those of other species. Recently, Jones et al (1989) have demonstrated that the pA_2 values of a wide range of structurally distinct TP receptor antagonists are significantly lower in the rabbit platelet and aorta as compared to platelet and vessels from other species, however no such difference is observed with TP receptor agonists.

As yet, studies on the nature of the vascular TP receptor in the rabbit have been restricted to the aorta. We have extended the study to include investigation of the action of TP receptor agonists and antagonists in the jugular vein. One reason for this is the requirement to effectively block TP receptors in this preparation when studying the EP_2 receptor mediating relaxation (see Chapter 4).

Several attempts have been made to characterise platelet TP receptors biochemically using radiolabelled analogues. Hung et al (1983) used [^{3}H]-13-azaprostanoic acid (APA) and found two classes of binding activity on the human platelet membrane: one with a K_d of ~100 nM and the other with a K_d of ~3.5 μM. Halushka et al (1985) used [^{125}I] cis-APA and described a single class of binding sites, however, the compound had a high K_d of 1.48 μM. Armstrong et al (1983) investigated the binding of [^{3}H]-15(S) 9,11- epoxymethano PGH_2. They found that the K_d was approximately 70 nM and binding could be displaced by a wide range of agonists and antagonists. However, the compound had a large component of non-displaceable binding. Similar results were obtained by Kattleman et al (1986) who demonstrated binding of [^{3}H]-11,9-epoxymethano PGH_2 (U 46619) to a single class of binding site on the human platelet (K_d, 108 nM), which was displaced by a series of TP receptor antagonists. However, the above assays have been made difficult by the low affinity and specific activity (<20 Ci/mmol) of the ligands used.

Mais et al (1985b) prepared radioiodinated 13-aza-13,14-dihydro-16 (*p* - hydroxy-*m*-iodophenyl)-ω-tetranor PTA_2 ([^{125}I]-PTA-OH) and showed that the process did not affect the biological activity of the compound (antagonism of platelet aggregation). They found that [^{125}I]-PTA-OH bound to a single class of receptor with a K_d of 21 nM and specific binding of 40-50%. The epimers of the iodinated ligand had equal activity on human platelets. These results have since been confirmed by Narumiya et al (1986) and Halushka et al (1987) who also investigated the displacement of the ligand by a range of analogues and compared the IC_{50} values with those obtained for platelet aggregation *in vitro*. The results showed that the rank order of potency for agonists correlates well. Thus, [^{125}I]-PTA-OH appears to be a most useful high affinity, high specific activity (2000 Ci/mmol) ligand for the study of TP receptors. More recently, however, the TP receptor antagonist SQ 29, 548 (Ogletree et al, 1985) has been radiolabelled with tritium and the binding to human platelets characterised (Hedberg et al, 1988). The compound was shown to bind to a single class of receptors with a K_d of 5nM and specific binding was 90-97% of the total binding.

We have used [^{125}I]-PTA-OH to investigate the binding of various TP receptor agonists and antagonists to the human platelet. The aim of the study is two fold. Firstly, to further characterise agonist binding at TP receptors, particularly that of EP 171, the most potent TP receptor agonist so far reported (Jones et al, 1989). EP 171 is similar in structure to EP 031 (see Fig.5.1) which has been shown to have a particularly slow rate of onset and offset. The assumption was made that this was due to the removal of the highly lipophyllic analogue from the extracellular space into adjacent lipid domains. However, studies have shown that EP 171, although considerably less lipophyllic, also has a very slow rate of onset and offset such that concentration-response curves often cannot be obtained by cumulative addition of EP 171. Secondly, we were approached by Glaxo to see if we would be willing to measure the K_i of two of their potent TP receptor antagonists, AH 23848 and GR 32191 and the EP_1 receptor antagonist, AH 6809 on human platelets. The arrangement included Glaxo paying for the expensive radioligand.

RESULTS

Ligand Binding

Non-specific binding: The non specific binding, calculated by including 1 μM ONO 11120 in the assay mixture, was 50-60% of the total binding. This value and the K_d of 69 ± 14 for U 46619 correspond to those obtained in previous studies.

K_d(nM)		specific binding	Reference
[^{125}I]-PTA-OH	U 46619	(% of maximum)	
21 ± 5	84 ± 10	60%	Mais et al (1986)
22 ± 3	125 ± 17	40%	Narumiya et al (1986)
~20	140 ± 13	40 - 50%	Halushka et al (1987)

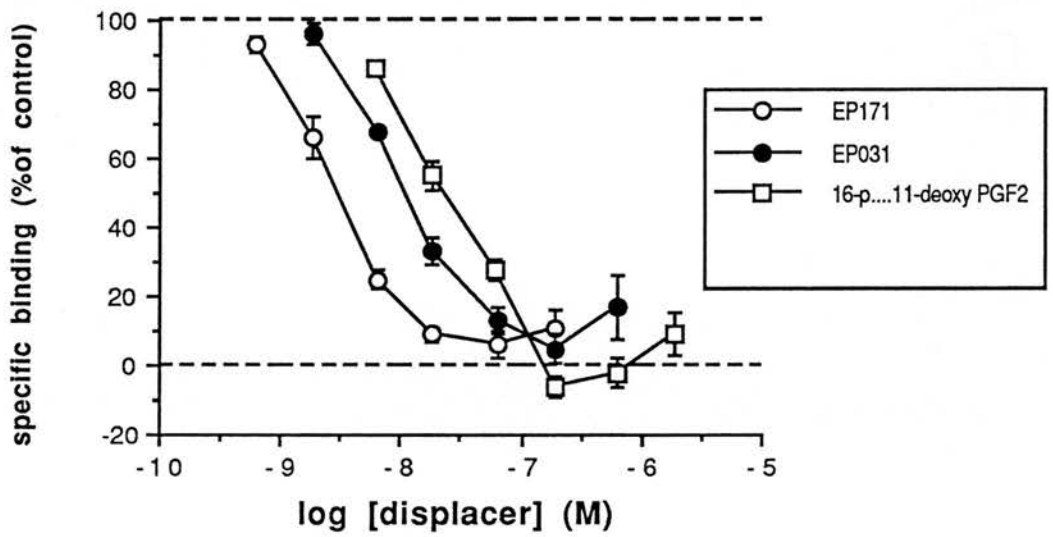
Agonist displacement curves: The displacement curves are shown in Figure 5.2. The rank order of affinity of the compounds tested was: EP 171 > EP 031 > 16-*p*-chlorophenoxy-*w*-tetranor-11-deoxy $PGF_{2\alpha}$ > STA_2 > U 46619 > ICI 79939. The log concentration-inhibition curves for a number of compounds (EP 171, EP 031, STA_2, 19-*p*- chlorophenoxy $PGF_{2\alpha}$) turned back slightly at highest concentration tested.

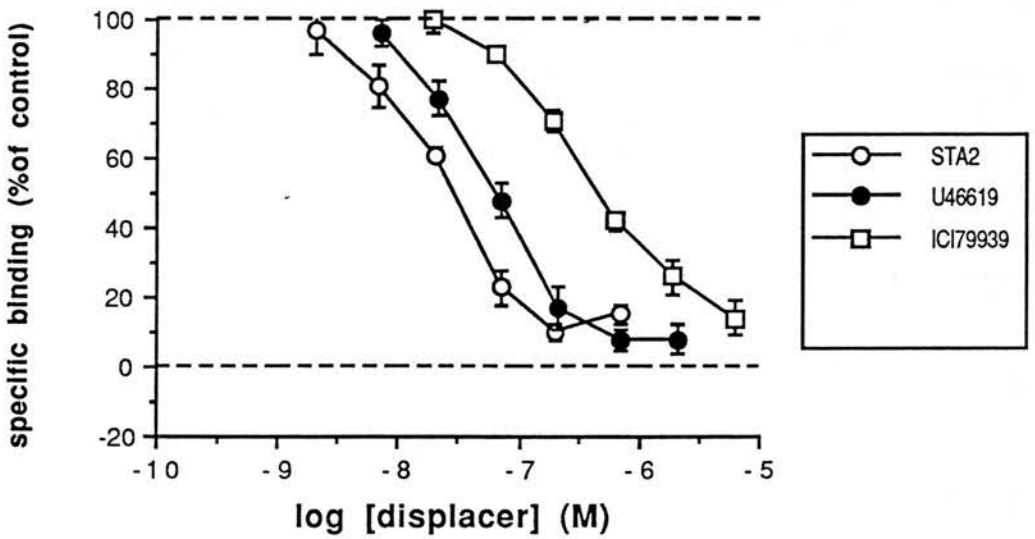
Antagonist displacement curves: The rank order of affinity of the compounds tested was: GR 32191 > AH 23848. Again the log concentration-inhibition curves of both compounds reversed at higher concentrations.

Displacement curves for the EP_1 receptor agonist, AH 6809: AH 6809 caused a maximum inhibition of specific binding of 20% at 25 μM, the curve then turned back dramatically, giving counts of 200% of the control maximum binding at 250 μM.

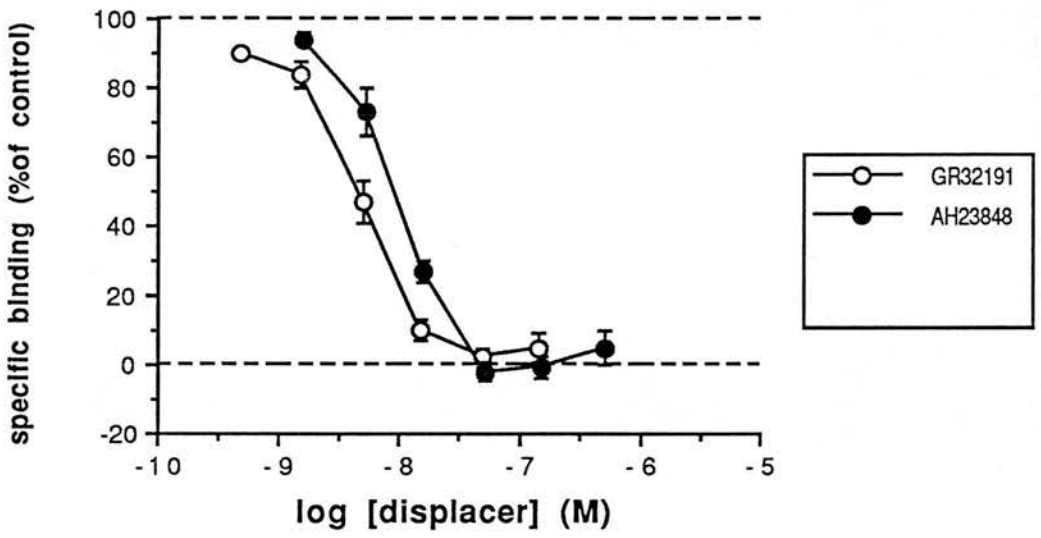
Figure 5.2: Inhibition of [^{125}I]-PTA-OH binding to human platelets by (a) TP-receptor agonists and (b) TP-receptor antagonists (means of 4 expts). (bars represent the s.e.m)

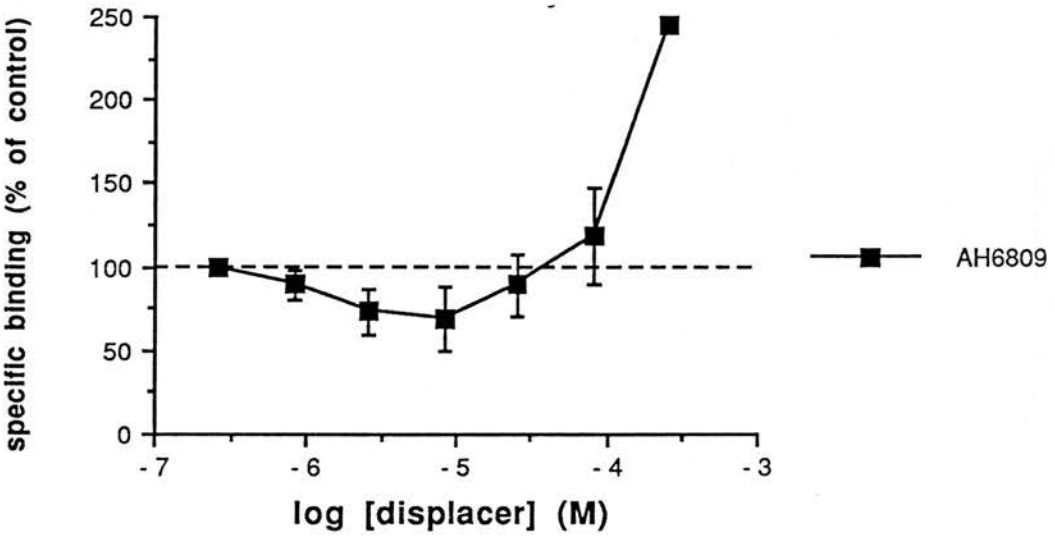
a)





b)


specific binding (%of control)
100
80
60
40
20
0
-20
-10
-9
-8
-7
-6
-5
log [displacer] (M)
GR32191
AH23848


specific binding (% of control)
250
200
150
100
50
0
-7
-6
-5
-4
-3
log [displacer] (M)
AH6809

Rabbit jugular vein

The vessel was highly sensitive to U 46619, which had an EC_{50} of ~6 nM. Both STA_2 and EP 171 contracted the preparation, the EMR values were 0.26 and 0.0217 respectively (Fig 5.3). The response to EP 171 was very slow, the lower doses taking 40-50 minutes to reach a stable level of tone. The log concentration-response curve of U 46619 was subject to a parallel rightward shift in the presence of 10 μM GR 32191 (Fig 5.4), consistent with a pA_2 of 7.22 ± 0.10.

Figure 5.3: Log concentration-response curves for contraction of the rabbit jugular vein by EP 171, STA_2 and U 46619 (mean of 4 expts).

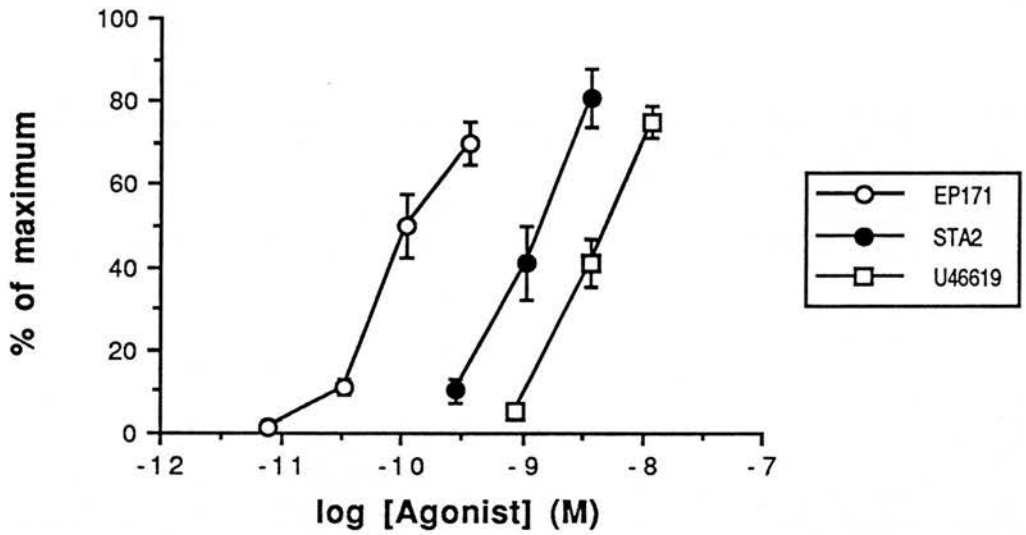
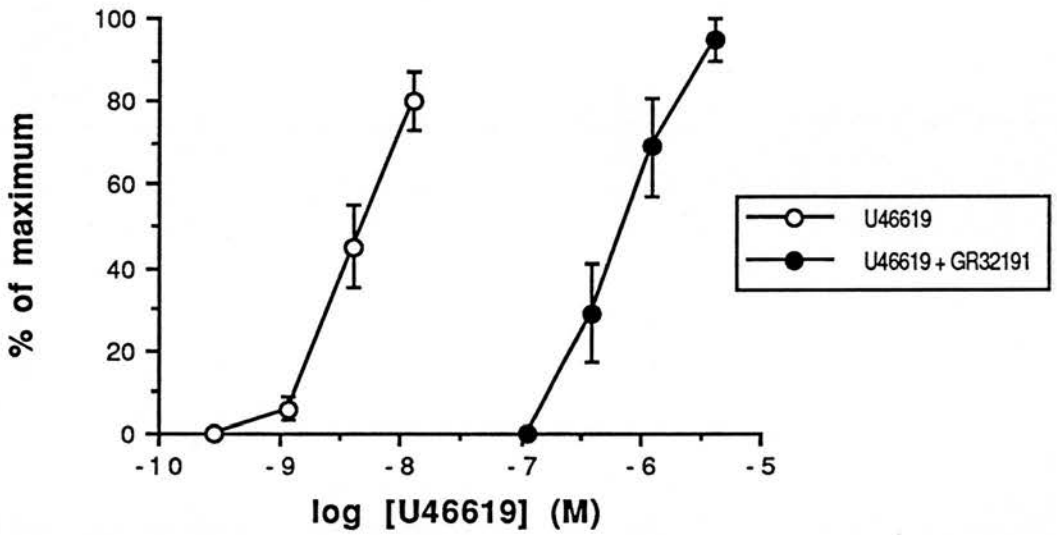


Figure 5.4: Effect of GR 32191 (10μM) on the log concentration-response curve of U 46619 in the rabbit jugular vein (mean of 4 expts).



DISSCUSSION

Ligand Binding

The IC_{50} value of 69 nM obtained for displacement of [^{125}I]-PTA-OH binding by U 46619 is comparable to that obtained by Mais et al (1985) (84 nM), Narumiya et al (1986) (125 nM) and Halushka et al (1987) (140nM). The data in Table 5.1 and 5.2 shows that the rank order of potency of a series of agonists and antagonists as displacing agents correlates well with their potency in the platelet. A similar correlation was demonstrated with a different series of antagonists by Halushka et al (1987).

The agonist ranking (Table 5.3) for EP 171, STA_2 and U 46619 is the same as that obtained on measuring the potency of these compounds as contractile agents in various smooth muscle preparations (Jones at al, 1989) and the rabbit jugular vein. Similarly, the K_i values obtained for the antagonists, GR 32191 and AH 23848 correlate with those obtained in the vasculature (Lumley et al, 1987; Brittain et al, 1985). Thus, the data present no evidence that the TP receptors on the platelet differ from those in the vasculature.

There are a number of possible reasons why the true K_i of some of the prostanoids may be lower than that measured in ligand binding experiments. Firstly, a number of the compounds used (EP 171, EP 031, 16-p-chlorophenoxy $PGF_{2\alpha}$, AH 23848) are racemic and it is possible that only one enantiomer competes effectively with the radioligand in the binding assay. It is notable that it has previously been shown (Sprague et al, 1985) that in the case of the parent compound the isomer formally related to PGH_2/TXA_2 is ~100 fold more active than the mirror image as an activator of platelets. Secondly, there may be a reduction in the initial free concentration of the displacing agent due to TP receptor binding. For example, assuming the platelet has 1700 TP receptors (Armstrong et al, 1983), the concentration of TP receptors will be about 0.7 nM. At the IC_{50} for the compound the concentration of receptors in the free and bound form will be equal (i.e 0.35 nM) hence, at the IC_{50} value of 1.45 nM (assuming only one enantiomer is active) the active species of EP 171 will be reduced to about 1 nM. This will only affect

Table 5.1: Comparison of the potency of TP receptor agonists in terms of shape change production and inhibition of [^{125}I]-PTA-OH binding in human washed platelets (mean of 4-6 expts.).(± s.e.m)

Agonist	EC_{50} for shape change: (nM)	Inhibition of [^{125}I]-PTA-OH binding: K_i (nM)
EP 171	0.065 ± 0.011 *	2.9 ± 0.4
EP 031	0.55 ± 0.08 *	11 ± 1.0
16-*p*-Chlorophenoxy-ω-tetranor-11-deoxy $PGF_{2\alpha}$	2.7 ± 0.6 *	23 ± 14
STA_2	1.8 ± 0.4 **	27 ± 4
U 46619	5.4 ± 0.9 *	69 ± 14
ICI 79939	27 ± 8 *	440 ± 32

* Jones et al (1989)

**Lawrence et al (1988)

Table 5.2: Comparison of the potency of TP receptor antagonists in terms of inhibition of aggregation and inhibition of [^{125}I]-PTA-OH binding in washed human platelets (mean of 4-6 expts.).

Antagonist	Inhibition of platelet aggregation: K_d † (nM)	Inhibition of [^{125}I]-PTA-OH binding: K_i (nM)
GR 32191	1.7 ± 0.2 *	4.6 ± 0.7
AH 23848	5.4 ± 1.1 *	8.8 ± 1.0

* Lawrence et al (1988)

† K_d = antilog pA_2 *vs* U 46619 platelet aggregation

high potency compounds, as the initial free concentration of less potent displacing agents would be reduced by less than 10%. The K_d of 1nM for EP 171 may account for its slow onset of action on platelets. For example, if the association rate constant, k_1 is $1x10^7$ $M^{-1}s^{-1}$ and the dissociation rate constant k_2 is $0.01s^{-1}$ (from K_d of 1nM) then at a concentration of 0.05 nM the calculated occupation half time is 45 seconds. The measured half time of onset of shape change for EP 171 (Jones et al, 1989) is 38 ± 3 seconds and for U 46619, 9.4 ± 0.4 seconds.

The IC_{50} values for the agonists obtained in the ligand binding assay are significantly higher (10-20 fold) than the EC_{50} values for inducing shape change, while the K_i values for antagonists are only slightly (~2 fold) higher than those obtained for inhibition of platelet aggregation. A similar difference in IC_{50} values obtained by radioligand binding and EC_{50} for platelet aggregation was observed by Halushka et al (1987). The existence of spare receptors would have predictable consequences for the comparison of binding and functional data for agonists. If spare receptors are present, the biological response could still be a linear function of the number of receptors occupied, but the response would be maximal when the number of receptors occupied is less than the total number of receptors by the number of spare receptors. In this case the biological response for agonists is platelet shape change and it has previously been shown (Armstrong et al, 1983) that only approximately 5% of receptors are occupied by U 46619 at the EC_{50}. Hence, the concentration if ligand that elicits a half maximal response in the radioligand binding experiment (i.e inhibits 50% of [^{125}I]-PTA-OH specific binding) will be considerably higher than the EC_{50} measured biologically.

It is notable that the K_i values obtained for the antagonists, GR 32191 and AH 23848 obtained from the ligand binding study compare well with those obtained functionally. In the case of antagonists the biological response is inhibition of a full aggregation wave of U 46619, thus, the agonist receptor occupancy may be near maximal and the proportion of spare receptors considerably lower than that present at the EC_{50} level for shape change. Hence, one would expect the Ki values for the antagonists obtained in the radioligand binding experiment to

correspond more closely to those obtained biologically. Similar results have been reported in studies of both opioid and adrenergic receptors (Tallarida, 1988): K_i values for agonists obtained in functional studies were consistently 1-2 log units lower than those obtained from binding studies, in contrast, for antagonists the two values were very similar. However, the author explains these findings in terms of a two site receptor model. In this model the agonist binding converts the receptor to a second state for which agonists have a considerably lower affinity and produces little response on binding. Antagonists, however, have equal affinity for both states.

The finding that the EP_1 receptor antagonist, AH 6809 displaces [^{125}I-PTA-OH agrees with data showing that this compound inhibits U 46619-induced human platelet aggregation with a pA_2 of 4.4 (Kerry and Lumley, 1988). However, the K_i cannot be estimated due to the dramatic turning back of the concentration-response curve at concentrations of 20-200 μM.

The data have not been analysed using the Hill equation due to the tendency of the binding curves of a number of displacing agents (both agonists and antagonist) to reverse slightly with increasing concentration. It is not clear whether this phenomenon is related to the more marked belling of the curve of AH 6809. There is no obvious explanation for this effect. It is possible that the presence of high concentrations of displacing agents unmasks additional binding sites for the radioligand.

Rabbit Jugular Vein

Investigation of the contractile action of EP 171 and STA_2 in the rabbit jugular vein (Table 5.3) shows that the activity in terms of EMR (*vs* U 46619) and absolute potency compare with those obtained in other smooth muscle systems (Jones et al,1989). However, the pA_2 value (7.2) for GR 32191 appears to be considerably lower than those previously reported for the compound (Table 5.4). It seems that the RJV is similar to other rabbit tissue (both platelet and vasculature) in having a low sensitivity to TP receptor antagonists but not agonists (Tymkewcz et al, 1989). It is notable that BM 13.177 has been shown to have similar

Table 5.3 : Comparison of the agonist potencies of EP 171, STA_2 and U 46619 at TP receptors in the rabbit jugular vein with those of other smooth muscle preparations (mean of 4-6 expts).

Preparation	EC_{50} for EP171 (pM)	Equieffective molar ratio (U46619 = 1) EP171	STA_2
Rabbit aorta	138	0.0136 ± 0.0025 *	0.23 ± 0.06*
Rat aorta	45	0.0094 ± 0.0006 *	0.15 ± 0.02*
Pig pulmonary artery	70	0.0093 ± 0.0008 *	0.21 ± 0.03*
Dog saphenous vein	120	0.0302 ± 0.0034 *	0.30 ± 0.05*
Guinea-pig trachea	57	0.0096 ± 0.0012 *	0.35 ± 0.04*
Rabbit jugular vein	125	0.0217 ± 0.0047	0.26 ± 0.06

* Jones at al (1989)

Table 5.4: Comparison of the pA_2 values obtained for GR 32191 and AH 23848 in the rabbit jugular vein with that obtained in other vascular preparations (mean of 4-6 expts).

Preparation	pA_2	
	GR 32191	AH 23848
Human pulmonary vessels	8.2 *	7.7 **
Guinea-pig aorta	8.7 *	_
Rat aorta	7.9 *	7.9 **
Rabbit Jugular vein	7.22 ± 0.1	

* Lumley et al (1987)

** Brittain et al (1985)

activity in rabbit tissues (aorta and platelets) to those of other species (Tymkewcz and Jones, unpublished data). Giles at al (1989) found that the pK_B (6.01 ± 0.10) of this blocker in the rabbit jugular vein was similar to that obtained in both the rabbit aorta and human platelet.

The experimental finding of equal pA_2 values of an antagonist measured against two different agonists is taken to indicate that the agonists are binding to the same receptor site, while different pA_2 values indicate that the agonists are acting at different receptors. The assumption is made that both agonist and antagonist bind to precisely the same site. However, if the antagonists bind to accessory sites adjacent to the agonist binding site then different pA_2 values may be obtained despite identical agonist binding sites (see Raffa et al, 1989). This would arise in the case of multiple antagonist accessory binding sites or with multiple subtypes of the antagonist receptor. Such a situation may exist with TP receptors: the antagonist binding site in the rabbit may differ from that found in other species. In addition, the findings of Mais et al (1985a,c ; 1988) showing a different rank order of potency for 13-azapinane TP receptor antagonists in the platelet and vasculature of humans and dogs suggest that the binding sites in the two systems may differ.

Similar observations have been made in other receptor systems. In the 5HT system the data obtained with agonists in various isolated preparations is not consistent with antagonist data (Leff and Martin, 1988). While the affinity values for 5HT are all found to be within a relatively narrow range, there is a wide variation (200 fold) in the antagonist affinity measurements. Also, antagonists of the histamine, H_1 and dopamine, D_1 receptor show a wide structural diversity such that they have been classified on the basis of chemical subgroups, and it has been proposed that they interact with different areas of the transmembrane section of one agonist receptor molecule (Ariens, 1987).

It could, of course, be argued that the range of TP receptor agonists tested to date is too small and by chance we have selected those which show the smallest differences. However, the difficulties associated with

agonists such as EP 171 may discourage the experimenter from a much larger screening process.

We have demonstrated, however, that both the TP receptor antagonists and agonists displace [^{125}I]-PTA-OH binding to human platelets. It is notable that Narumiya et al (1986) failed to demonstrate specific binding of [^{125}I]-PTA-OH to rabbit platelets. It would be interesting to study the binding of a radiolabelled agonist to rabbit platelets and determine if both agonists and antagonists displace the compound. EP 171 may be useful in a study of this nature although the radioligand would have to have a high specific activity since the free ligand concentration would be 1-10 nM.

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