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Vasodilator actions of acetylcholine, A23187 and bradykinin in the guinea-pig isolated perfused heart are independent of prostacyclin

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1 The involvement of prostacyclin (PGI_2) in the vasodilator responses to acetylcholine (ACh), A23187 and bradykinin (Bk) has been investigated in guinea-pig, isolated, Krebs-perfused hearts.

2 ACh (0.01–10 nmol), A23187 (0.1–1.0 nmol) and Bk (0.3–10 pmol) each elicited dose-related and shortlasting (~2 min) reductions in perfusion pressure. Larger maximal responses were obtained in preparations with coronary vascular tone elevated by platelet-activating factor (100 pmol) than in preparations at basal perfusion pressure.

3 Bk and A23187 elicited dose-related increases in the generation of PGI_2 as measured by its chemically-stable breakdown product, 6-oxo-$PGF_{1\alpha}$. Indomethacin (2.8 μM) prevented both basal and the stimulated generation of 6-oxo-$PGF_{1\alpha}$, whereas the magnitudes of the vasodilator responses were unaffected.

4 Attempts to identify the release of vasodilator materials by on-line superfusion bioassay of cardiac effluent were unsuccessful, indicating a possible role for a labile vasodilator such as endothelium-dependent relaxing factor (EDRF). In addition, the inhibitors of EDRF action/production, mepacrine (3 μM) or diethylcarbamazine (300 μM), attenuated vasodilator responses to ACh without altering those to the endothelium-independent vasodilator, verapamil (1 nmol).

5 Haemoglobin (10 μM) reduced vasodilator responses to ACh, Bk and verapamil and abolished those induced by A23187. Inhibition of the endothelium-independent vasodilator, verapamil, was significantly less than that for the other compounds.

6 The present data indicate the existence of an indomethacin-resistant vasodilator mechanism in the coronary microcirculation in response to ACh, A23187 and Bk. EDRF is a candidate for mediating these responses; however, a direct vasodilator action of these substances cannot be excluded.

Introduction

The observation that acetylcholine was able to relax rabbit aorta in the presence but not in the absence of an intact endothelium (Furchgott & Zawadzki, 1980) stimulated great interest in the possible modulatory role of endothelium on vascular reactivity. It has been subsequently shown that the endothelium releases a humoral factor called endothelium-derived relaxing factor (EDRF) (Griffith *et al.*, 1984; Cocks *et al.*, 1985; Gryglewski *et al.*, 1986) which has recently been shown to resemble closely nitric oxide (Palmer *et al.*, 1987). The release of EDRF from many large arteries including the coronary arteries of the pig and the dog has been confirmed (Cocks & Angus, 1983). However, progress towards identification of a role of EDRF in the microcirculation has been less rapid due to technical difficulties in the removal of the endothelium. Nevertheless, it has recently been shown that EDRF may contribute to the regulation of perfusion pressure in the rat perfused mesenteric bed which contains arteriolar resistance vessels (Byfield *et al.*, 1986). This latter study and another claiming to demonstrate EDRF-mediated vasodilator responses in the perfused rat heart (Hearse & Saldanha, 1986) have failed to exclude a possible contribution from prostacyclin

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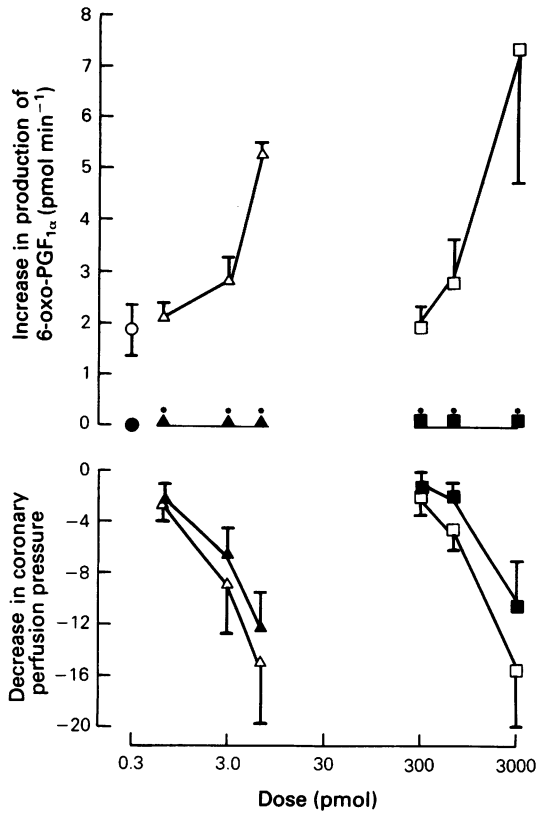

Figure 1 Effects of bradykinin (△, 0.3–3.0 pmol) and A23187 (□, 0.1–1.0 nmol) on 6-oxo-$PGF_{1\alpha}$ generation and coronary perfusion pressure ($n = 4$) in the absence (open symbols) or presence (filled symbols) of indomethacin (2.8 μM). Basal 6-oxo-$PGF_{1\alpha}$ is indicated by the open circles. * $P < 0.05$ paired t test, compared to generation in the absence of indomethacin.

(PGI_2). In rat and guinea-pig perfused hearts PGI_2 is a potent vasodilator (Schror *et al.*, 1978; Belo & Talesnik, 1982). Furthermore, PGI_2 formation in endothelial cells is stimulated by many substances that are also capable of releasing EDRF such as bradykinin and the ionophore, A23187 (Weksler *et al.*, 1978; McIntyre *et al.*, 1985; Gryglewski *et al.*, 1986). Thus, several studies have been directed towards the identification of the relative roles of PGI_2 and EDRF in mediating endothelium-dependent relaxant responses (Forstermann & Neufang, 1984a).

The present study was undertaken to discover whether the coronary microvasculature undergoes vasodilatation in response to three well characterized stimulants of PGI_2 and EDRF generation, acetylcholine, A23187 and bradykinin. In addition, the importance of PGI_2 for vasodilator responses has been directly investigated by radioimmunoassay of the stable breakdown product of PGI_2, 6-oxo-$PGF_{1\alpha}$, indomethacin being used to prevent PGI_2 formation. These studies have identified an indomethacin-resistant vasodilator mechanism in response to acetylcholine, A23187 and bradykinin which shows some of the characteristics of EDRF (Piper & Stewart, 1987a).

Methods

Hearts, obtained from male Dunkin-Hartley guinea-pigs (350–500 g), were perfused at a constant flow rate (8 ml min^{-1}, 37°C, gassed with 95% O_2, 5% CO_2) with Krebs solution of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $NaHCO_3$ 25, $MgCl_2$ 0.5, NaH_2PO_4 1.0 and D-glucose 11.1. The aorta was cannulated retrogradely and perfusion commenced within 2 min. Coronary perfusion pressure was measured by attaching a side-arm of the aortic cannula to a pressure transducer (Elcomatic EM50) and was displayed on a Graphtec linearcorder Mark VII VR3101. An equilibration period of 30 min was allowed, during which time perfusion pressure stabilized.

Drugs were administered as bolus injections (10–100 μl) into the perfusate 2 cm proximal to the aortic cannula. In most experiments, a single bolus dose of platelet-activating factor (Paf, 100 pmol) was administered to increase the coronary perfusion pressure and thereby facilitate the measurement of vasodilator responses. The Paf-induced increase in perfusion pressure was stable for at least 3 h as previously observed (Piper & Stewart, 1986; 1987b). Indomethacin (2.8 μM) was added to the Krebs reservoir (1 ml l^{-1} of 1 mg ml^{-1} dissolved in 0.1 M Na_2CO_3 immediately before use) 20 min, before obtaining responses. Haemoglobin was prepared according to the method of Martin *et al.* (1985).

The generation of 6-oxo-$PGF_{1\alpha}$, reflected by its level in the cardiac effluent, was used as an index of cardiac PGI_2 formation. Samples (1 ml) of 2 min collections of cardiac effluent, obtained before and after administration of A23187 (0.1–1.0 nmol) or bradykinin (Bk, 0.3–3.0 pmol), were evaporated to dryness under reduced pressure and stored at −20°C under N_2 before resuspension in 100 μl of the radioimmunoassay buffer (50 mM Tris-HCl, pH 7.4, 0.1% w/v gelatin). The assay of 6-oxo-$PGF_{1\alpha}$ was carried out as previously described (Piper & Stewart, 1987a). None of the drugs used in this study interfered in the radioimmunoassay of 6-oxo-$PGF_{1\alpha}$.

All reagents used were of analytical grade. Drugs were obtained from the following sources: acetylcholine, bradykinin, haemoglobin, (Sigma); A23187 (Calbiochem); hexadecyl platelet-activating factor (Bachem, U.K.).

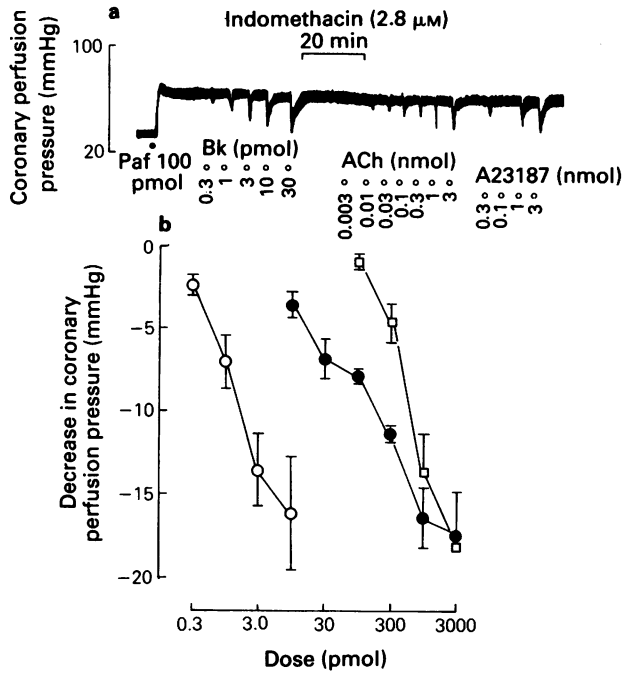

Figure 2 (a) Decreases in coronary perfusion pressure induced by acetylcholine (ACh), bradykinin (Bk) and A23187 in guinea-pig isolated heart. (b) Dose-response curves for vasodilator actions of ACh (●), Bk (○) and A23187 (□) ($n = 6$–8).

Results

Bradykinin (0.3–3.0 pmol) and the ionophore, A23187 (0.1–1.0 nmol) induced dose-related reductions in perfusion pressure in hearts that had previously received Paf (100 pmol) (Figure 1). These vasodilator responses were accompanied by a similarly dose-related increase in the cardiac generation of 6-oxo-$PGF_{1\alpha}$. Indomethacin reduced the resting generation (1.81 ± 0.25 pmol min^{-1}) to below the assay detection limit (0.05 pmol per sample). Although indomethacin also prevented Bk- or A23187-induced increases in the generation of 6-oxo-$PGF_{1\alpha}$, the associated reductions in perfusion pressure were not significantly affected ($P > 0.05$, Student's t test) by indomethacin pretreatment (Figure 1). In addition, at a dose eliciting a similar decrease in perfusion pressure, verapamil (1 nmol) had no effect on 6-oxo-$PGF_{1\alpha}$ generation (in pmol min^{-1}: basal, 1.57 ± 0.27; post-verapamil, 1.55 ± 0.51, $n = 4$). Bradykinin (0.3–10.0 pmol), A23187 (0.1–3.0 nmol) and acetylcholine (ACh 0.01–3.0 nmol) each induced a dose-related reduction in perfusion pressure in the presence of indomethacin (2.8 μM) (Figure 2). Bk was more potent than either ACh or A23187 whereas all these substances had

Table 1 Maximum reductions in basal perfusion pressure and in preparations with Paf-elevated perfusion pressure in response to acetylcholine (ACh), A23187 and bradykinin (Bk)

Vasodilator	(nmol)	*Maximum decrease in perfusion pressure* (mmHg)			
		n	*Basal*	n	*Paf* (100 pmol)
ACh	3	5	7.3 ± 1.0[a]	6	17.3 ± 2.5
A23187	3	5	5.4 ± 1.5	8	18.2 ± 5.2
Bk	0.01	5	5.2 ± 1.6	8	16.1 ± 3.4

[a] The response to acetylcholine at basal perfusion pressure was biphasic with vasodilatation followed by vasoconstriction ($+8.6 \pm 1.3$ mmHg).

Table 2 Effects of mepacrine (3 μM), diethylcarbamazine (300 μM) and haemoglobin (10 μM) on vasodilator responses

			Maximum decreases in perfusion pressure (mmHg)		
Vasodilator	(nmol)	n	*Control*	*Mepacrine*	*Difference*
ACh	1–3	5	17.0 ± 3.0	7.6 ± 1.7	−9.4 ± 3.3[a]
A23187	1	5	16.8 ± 2.8	10.6 ± 2.0	−6.2 ± 2.8
Bk	0.01	5	13.3 ± 2.3	10.6 ± 4.7	−2.7 ± 2.8
Verapamil	1–3	5	13.9 ± 2.6	16.7 ± 3.8	+2.8 ± 3.5
			Control	*Diethylcarbamazine*	*Difference*
ACh	1–3	4	15.5 ± 2.2	7.5 ± 1.2	−8.0 ± 1.3[a]
A23187	1	4	17.0 ± 3.8	14.7 ± 2.1	−2.4 ± 2.3
Bk	0.01	4	18.0 ± 2.9	12.9 ± 1.5	−5.1 ± 1.5[a]
Verapamil	1–3	4	8.2 ± 1.7	9.0 ± 2.0	+0.9 ± 1.2
			Control	*Haemoglobin*	*Difference*
ACh	1–3	4	16.5 ± 1.8	6.4 ± 2.5	−10.1 ± 1.9[a]
Bk	1	4	15.0 ± 1.9	4.7 ± 1.7	−10.3 ± 1.4[a]
A23187	0.01	4	14.1 ± 2.6	0	−14.1 ± 2.6[a]
Verapamil	1–3	4	12.8 ± 1.1	6.9 ± 1.8	−6.1 ± 1.4

[a] $P < 0.05$, unpaired Student's t test compared to the difference obtained with the endothelium-independent vasodilator, verapamil, after antagonist treatment.

similar maximum responses. Over the same dose-ranges these substances also induced dose-related reductions in basal (i.e. not elevated by Paf) perfusion pressure even though the maximum responses were considerably less (Table 1). Furthermore, under these conditions, the response to ACh was biphasic with the vasodilatation being followed by a small vasoconstrictor response.

The inhibitors of EDRF production/action, mepacrine (3 μM), haemoglobin (10 μM) and diethylcarbamazine (300 μM) were used in experiments in which vasodilator responses of similar magnitudes were obtained before treatment with either mepacrine or diethylcarbamazine. Indomethacin (2.8 μM) was present throughout these experiments. A 20 min treatment time was allowed before obtaining vasodilator responses to the same doses of agonist as previously used. Mepacrine significantly reduced the response to ACh only, whereas diethylcarbamazine inhibited both ACh and Bk (Table 2). Neither treatment significantly altered the response to A23187, nor was there any apparent effect on the response to the endothelium-independent vasodilator, verapamil. On the other hand, haemoglobin caused more marked inhibition of vasodilatation induced by ACh and Bk, abolished the response to A23187 and attenuated that to verapamil (Table 1).

Further attempts to characterize these vasodilator responses included the use of on-line superfusion bioassay in which the cardiac effluents were directed over a series of rabbit aortic strips denuded of endothelium and precontracted with 5-hydroxytryptamine (5-HT, 10 μM).

Administration of ACh, A23187 or Bk at doses of up to 2 orders of magnitude greater than those eliciting a maximum decrease in perfusion pressure in indomethacin-treated hearts failed to release any relaxant material detectable on the vascular strips ($n = 3$). In a further series of experiments, the cardiac effluent was regassed and rewarmed before perfusing another guinea-pig isolated heart in which perfusion pressure was monitored. The recipient heart was continuously exposed to atropine (1 μM) to prevent a direct action of the ACh and tone was increased in both hearts with Paf (100 pmol). ACh (10 nmol), which elicited a maximal decrease in the donor heart perfusion pressure, failed to affect the perfusion pressure in the recipient heart. The transit time between the 2 preparations was approximately 20 s.

Discussion

Three well characterized EDRF secretagogues evoked dose-related decreases in perfusion pressure in isolated hearts with resting or Paf-elevated vascular tone. These observations suggest a vasodilator action on the microvasculature of the guinea-pig perfused heart.

It is well established that PGI_2 decreases coronary flow in isolated hearts from rabbits, rats and guinea-pigs and that PGI_2 generation is increased by a range of stimuli including exogenous arachidonic acid (Schror *et al.*, 1978; Belo & Talesnik, 1982), hypoxia (Wennmalm, 1979), Paf (Piper & Stewart, 1986) and ATP (Fleetwood & Gordon, 1987). Since the ability of both bradykinin and the ionophore, A23187, to stimulate PGI_2 generation from cultured endothelial cells (McIntyre *et al.*, 1985; Gryglewski *et al.*, 1986; Gerritsen, 1987) and isolated perfused lungs (Bakhle *et al.*, 1985) is well recognized, it was important to determine whether these stimuli had a similar effect in the perfused heart and also to identify a possible contribution to the observed vasodilator responses. Even though both Bk and A23187 elicited an increase in the generation of PGI_2 over the same dose-range giving a dose-related decrease in perfusion pressure, indomethacin, which prevented PGI_2 generation, failed to alter the magnitude of the vasodilator response. This observation suggests that PGI_2 released by Bk and A23187 does not contribute to the peak of the vasodilator response. Similarly, EDRF responses are known to occur independently of the action of PGI_2 (Furchgott & Zawadzski, 1980; Forstermann & Neufang, 1984a).

Under conditions of constant flow, variations in perfusion pressure reflect changes in coronary microvascular resistance in the absence of changes in cardiac contractility or rate (Fleetwood & Gordon, 1987). The present observations do not appear to result from extravascular influences on perfusion pressure, since all three compounds reduced either basal or Paf-elevated perfusion pressure whereas ACh had negative chronotropic actions, A23187 had little effect on either rate or force and Bk elicited increases in force.

In vitro preparations of large coronary arteries retaining an intact layer of endothelium, relax in response to the agents used in this study with the same rank order of potency as that observed for reductions in perfusion pressure (Bk > ACh > A23187) (Cocks *et al.*, 1985). Thus, Bk-, ACh- and A23187-induced vasodilator responses in the guinea-pig perfused heart are consistent with a mechanism involving the release and action of EDRF. In addition, the selective attenuation of vasodilator responses to ACh by the putative inhibitors of EDRF production/action, mepacrine and diethylcarbamazine (Furchgott & Zawadzki, 1980; Forstermann & Neufang, 1984b) suggests a role for EDRF. This suggestion is further supported by the inhibition of vasodilatation by haemoglobin (Martin *et al.*, 1985) even though this inhibitor slightly reduced responses to verapamil. If the vasodilators used in this study caused their effect by release of EDRF then equal extents of inhibition with the compounds used to inhibit EDRF could be expected. The differential inhibitory effects observed may suggest that the vasodilator actions are only partly attributable to EDRF or that the EDRF inhibitors have agonist-selective, non-specific actions.

The inability to bioassay a relaxant material from maximally dilated hearts is consistent with the labile nature ($t_{1/2}$ 6–40 s) of EDRF in Krebs solution (Griffith *et al.*, 1984; Cocks *et al.*, 1985; Gryglewski *et al.*, 1986). Despite their lack of relaxant effects on endothelium-denuded large coronary arteries, the possibility that ACh, Bk and A23187 have a direct relaxant action on the coronary microvasculature, cannot be excluded.

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References

BAKHLE, Y.S., MONCADA, S., DE NUCCI, G. & SALMON, J.A. (1985). Differential release of eicosanoids by bradykinin, arachidonic acid and calcium ionophore A23187 in guinea-pig isolated perfused lung. *Br. J. Pharmacol.*, **86**, 55–62.

BELO, S.E. & TALESNIK, J. (1982). Coronary vasoconstrictor and vasodilator actions of arachidonic acid in the isolated perfused heart of the rat. *Br. J. Pharmacol.*, **75**, 269–286.

BYFIELD, R.A., SWAYNE, G.T.G. & WARNER, T.S. (1986). A method for the study of endothelial derived factor (EDRF) in the isolated perfused rat mesentery. *Br. J. Pharmacol.*, **88**, 438P.

COCKS, T.M. & ANGUS, J.A. (1983). Endothelium-dependent relaxation of coronary arteries by noradrenaline and serotonin. *Nature*, **305**, 627–630.

COCKS, T.M., ANGUS, J.A., CAMPBELL, J.H. & CAMPBELL G.R. (1985). Release and properties of endothelium-derived relaxing factor (EDRF) from endothelial cells in culture. *J. Cell Physiol.*, **123**, 310–320.

FLEETWOOD, G. & GORDON, J.L. (1987). Purinoceptors in the rat heart. *Br. J. Pharmacol.*, **90**, 219–227.

FORSTERMANN, U. & NEUFANG, B. (1984a). The endothelium-dependent vasodilator effect of acetylcholine: characterization of the endothelial relaxing factor with inhibitors of arachidonic acid metabolism. *Eur. J. Pharmacol.*, **103**, 65–70.

FORSTERMANN, U. & NEUFANG, B. (1984b). Inhibition by diethylcarbamazine of acetylcholine-induced endothelium-dependent relaxation of rabbit aorta: are leukotrienes involved? *J. Pharm. Pharmacol.*, **36**, 696–697.

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

GERRITSEN, M.E. (1987). Eicosanoid production by the coronary microvascular endothelium. *Fed. Proc.*, **46**, 47–53.

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

GRYGLEWSKI, R.J., MONCADA, S. & PALMER, R.M.J. (1986). Bioassay of prostacyclin and endothelium-derived relaxing factor (EDRF) from porcine aortic endothelial cells. *Br. J. Pharmacol.*, **87**, 685–694.

HEARSE, D.J. & SALDANHA, C.B.R. (1986). Quinacrine inhibits vasodilatation caused by 5-HT but not papaverine in the rat isolated heart. *Br. J. Pharmacol.*, **87**, 12P.

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

McINTYRE, T.M., ZIMMERMAN, G.A., SATOH, K. & PRESCOTT, S.M. (1985). Cultured endothelial cells synthesize both platelet-activating factor and prostacyclin in response to histamine, bradykinin and adenosine triphosphate. *J. Clin. Invest.*, **76**, 271–280.

PALMER, R.M.J., FERRIGE, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327**, 524–526.

PIPER, P.J. & STEWART, A.G. (1986). Coronary vasoconstriction in the rat, isolated perfused heart induced by platelet-activating factor is mediated by leukotrine C_4. *Br. J. Pharmacol.*, **88**, 595–605.

PIPER, P.J. & STEWART, A.G. (1987a). Vasodilator actions of acetylcholine, A23187 and bradykinin in the guinea-pig isolated perfused heart: possible relationship to endothelium-derived relaxing factor. *J. Physiol. (Lond.)*, **384**, 54P.

PIPER, P.J. & STEWART, A.G. (1987b). Antagonism of vasoconstriction induced by platelet-activating factor in guinea-pig perfused hearts by selective platelet-activating factor antagonists. *Br. J. Pharmacol.*, **90**, 771–783.

SCHROR, K., MONCADA, S., UBATUBA, F. & VANE, J.R. (1978). Transformation of arachidonic acid and prostaglandin endoperoxides by the guinea-pig heart. Formation of RCS and prostacyclin. *Eur. J. Pharmacol.*, **47**, 103–114.

WEKSLER, B.B., LEY, C.W. & JAFFE, E.A. (1978). Stimulation of endothelial cell prostacyclin production by thrombin, trypsin and the ionophore, A23187. *J. Clin. Invest.*, **62**, 923–930.

WENNMALM, A. (1979). Prostacyclin-dependent coronary vasodilatation in rabbit and guinea-pig hearts. *Acta. Physiol. Scand.*, **106**, 47–52.

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