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Endothelium-dependent relaxation of the pig aorta: relationship to stimulation of ^{86}Rb efflux from isolated endothelial cells

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1 Bradykinin, adenosine triphosphate (ATP) and acetylcholine each relaxed histamine-contracted strips of pig aorta in a dose-dependent manner. These relaxations were abolished when the endothelium was removed.

2 Relaxation induced by ATP was mimicked by adenosine diphosphate (ADP) but adenosine monophosphate (AMP) and adenosine were about 120 times less potent.

3 Relaxation induced by acetylcholine was antagonized by atropine in a competitive manner, and carbachol induced the same degree of relaxation as acetylcholine, but was about 10 times less potent.

4 The calcium ionophore, A23187, also induced a dose-dependent relaxation of pig aortic strips provided the endothelium was present, suggesting that a rise in the level of ionized calcium within the endothelial cells is one means by which vascular smooth muscle relaxation can be triggered.

5 Bradykinin, ATP, ADP, AMP, adenosine and A23187 each induced a dose-dependent increase in ^{86}Rb efflux from preloaded pig aortic endothelial cells. The dose-response curves for stimulation of ^{86}Rb efflux and for endothelium-dependent relaxation were similar for each individual compound. ADP was equipotent with ATP, but AMP and adenosine were about 120 times less potent.

6 Neither acetylcholine nor carbachol, in concentrations that induce endothelium-dependent relaxation, had any effect on ^{86}Rb efflux from isolated aortic endothelial cells.

7 Lanthanum, which blocks calcium influx, abolished the increases in ^{86}Rb efflux induced by bradykinin and ATP, and the calcium ionophore A23187 was the most effective stimulant of ^{86}Rb efflux, suggesting that the potassium transport induced by these agents is calcium-activated.

8 It is concluded that endothelial responses to bradykinin and ATP can be assessed by monitoring ^{86}Rb efflux, which probably reflects a calcium-activated efflux of potassium associated with the endothelium-dependent vascular relaxation induced by these agents. This pathway is apparently not involved in endothelial responses to acetylcholine.

Introduction

Bradykinin, adenosine triphosphate (ATP) and acetylcholine, despite being powerful systemic vasodilators (Dale, 1914; Rocha e Silva, Beraldo & Rosenfeld, 1949; Green & Stoner, 1950), often contract or have no effect on isolated vascular preparations (Furchgott & Bhadrakom, 1953; Furchgott, 1955; Kovalcík, 1962; Johnson, 1979). One possible explanation for this discrepancy was provided by Furchgott & Zawadzki (1980a) who demonstrated that relaxation of rabbit isolated aortic rings induced by acetylcholine was dependent upon the presence of the endothelium. Subsequent reports confirmed the requirement for endothelial cells in relaxation induced by acetylcholine, bradykinin and ATP on a range of isolated vascular preparations from several species (Altura & Chand, 1981; De Mey & Vanhoutte, 1981; Cherry, Furchgott, Zawadzki & Jothianandan, 1982). It has been suggested by these authors that the endothelium responds to these substances by transmitting a signal to the smooth muscle cells of the blood vessel wall which leads to relaxation. This implies that endothelial cells have receptors for these agents but this has not been extensively studied, although bradykinin is known to stimulate prostaglandin synthesis in cultured endothelial cells (Hong, 1980).

We have now shown that the isolated aorta of the pig also displays endothelium-dependent relaxation,

and we have directly examined the responsiveness of freshly-isolated pig aortic endothelial cells to vasodilator agents by monitoring agonist-induced potassium efflux (using ^{86}Rb). A preliminary account of some of these findings has already been published (Gordon & Martin, 1982b).

Methods

Aortic strips

Babraham piglets, 1–7 days old, were killed by a blow on the neck and exsanguination. A section of aorta was removed from between the renal and iliac arteries and cut into spiral strips 2 cm long by 2 mm wide as described by Furchgott & Bhadrakom (1953). Extreme care was taken during this procedure to minimize damage to the intimal surface of the vessel. Strips were made endothelium-free by scraping the luminal surface several times with a No. 11 scalpel blade. Strips with or without endothelium present were mounted under 1 g resting tension on glass hooks in 4 ml organ baths and bathed at 37°C in a Krebs solution of the following composition (mM): NaCl 119, KCl 3.1, $MgSO_4$ 0.6, $NaHCO_3$ 25, KH_2PO_4 1, $CaCl_2$ 1.3 and glucose 11.1. This solution was gassed with 5% CO_2 in O_2.

Tension was induced by histamine in aortic strips with and without endothelium, and cumulative dose-response curves were constructed to vasodilator drugs. Tension was measured with Gould Statham UC3 isometric transducers coupled to a Lectromed MX 212 pen recorder.

Isolation of aortic endothelial cells

Endothelial cells were isolated from piglets, 1–7 days old, as described by Pearson, Carleton, Hutchings & Gordon (1978). Briefly, small sheets of endothelial cells were obtained after treatment of the aortic lumen for 15 min at 37°C with a solution of 0.2% collagenase (type II, Sigma) in Dulbecco's modification of Eagles Medium (DMEM, Flow Laboratories, U.K.). About 200 μl of cell suspensions, in DMEM containing 20% foetal calf-serum (FCS, Sera-Lab), normally containing $2-5 \times 10^4$ cells, were placed on glass coverslips (15 × 5 mm); 10 μCi of ^{86}Rb Cl (Amersham International), was added to each coverslip resulting in concentrations of rubidium of 0.1–0.5 mM, and the cells were incubated overnight at 37°C under a humidified atmosphere of 5% CO_2 in air. Figure 1 shows a phase-contrast micrograph of endothelial cells isolated by the above procedure. In some experiments, when stated, endothelial cells were isolated without the use of digestive enzymes by immersing the artery in DMEM (containing 20% FCS to prevent the action of any endogenous proteases), scraping the intimal surface of the aorta with a scalpel blade and harvesting the cells adhering to the blade (see Ryan, Mortara & Whitaker, 1980).

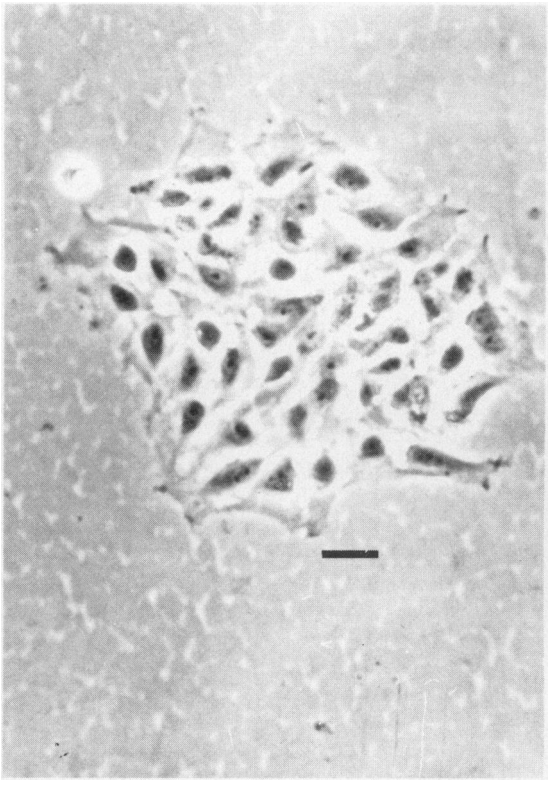

Figure 1 Phase-contrast micrograph of a sheet of endothelial cells isolated from the aorta of a pig by collagenase treatment; the bar represents 20 μm.

Measurement of ^{86}Rb efflux from endothelial cells

^{86}Rb efflux from coverslips of aortic endothelial cells was measured as previously described for isolated smooth muscle cells (Martin & Gordon, 1982b). Briefly, two coverslips of preloaded endothelial cells were placed back-to-back in a small chamber and superfused with Krebs solution at 37°C at a flow rate of 2.5 ml min^{-1}. The superfusate was collected in 2 min fractions and radioactivity was determined by gamma counting. Using a computer programme, a desaturation plot was constructed by adding, in reverse order, the radioactivity in each fraction to that remaining in the cells at the end of the experiment (Mauger, Moura & Worcel, 1978), and from these data the first-order rate constant of efflux (R) for each fraction was calculated and expressed in units of min^{-1}.

The effects of vasodilator drugs on ^{86}Rb efflux were measured by subtracting from the maximal rate of efflux during exposure, the mean rate in five fractions immediately prior to drug addition. This change in the efflux rate was termed ΔR.

In experiments in which cells were to be exposed to lanthanum chloride, the superfusion medium used was a HEPES-buffered Krebs solution, pH 7.4, of the following composition (mM): NaCl 144, KCl 4.1, $MgCl_2$ 0.6, $CaCl_2$ 1.3, glucose 11.1 and HEPES 5. $LaCl_3$ (1.3 mM) was added where indicated in the Results. This solution was bubbled with air.

Drugs

Acetylcholine chloride, bradykinin triacetate, carbachol (carbamylcholine chloride), adenosine diphosphate (ADP), adenosine monophosphate (AMP), adenosine triphosphate (ATP) and histamine dihydrochloride were purchased from Sigma, Dorset; ionophore A23187 was purchased from Calbiochem-Behring, Herts; adenosine was purchased from Koch-Light, Bucks; atropine sulphate was purchased from BDH, Dorset. The ionophore A23187 stock solution (10^{-2} M) was made up in dimethylsulphoxide and subsequent dilutions were made using distilled water. Similar dilutions of dimethylsulphoxide alone had no effect on Rb efflux from endothelial cells or on the tone of pig aortic strips. All drug concentrations are expressed in molar terms.

Results

Responses of isolated pig aortic strips to vasodilator agents

Bradykinin-induced relaxation Spiral strips of aorta, with and without endothelium present, were contracted with histamine (2×10^{-5} M). Bradykinin (10^{-10}–10^{-7} M), added cumulatively, relaxed aortic strips with endothelium present in a dose-dependent manner (Figure 2a). Relaxation was dependent on the presence of the endothelium since responses were abolished when the endothelial cells were removed (Figure 2a). Figure 2b shows the dose-response curves for the action of bradykinin on six prepara-

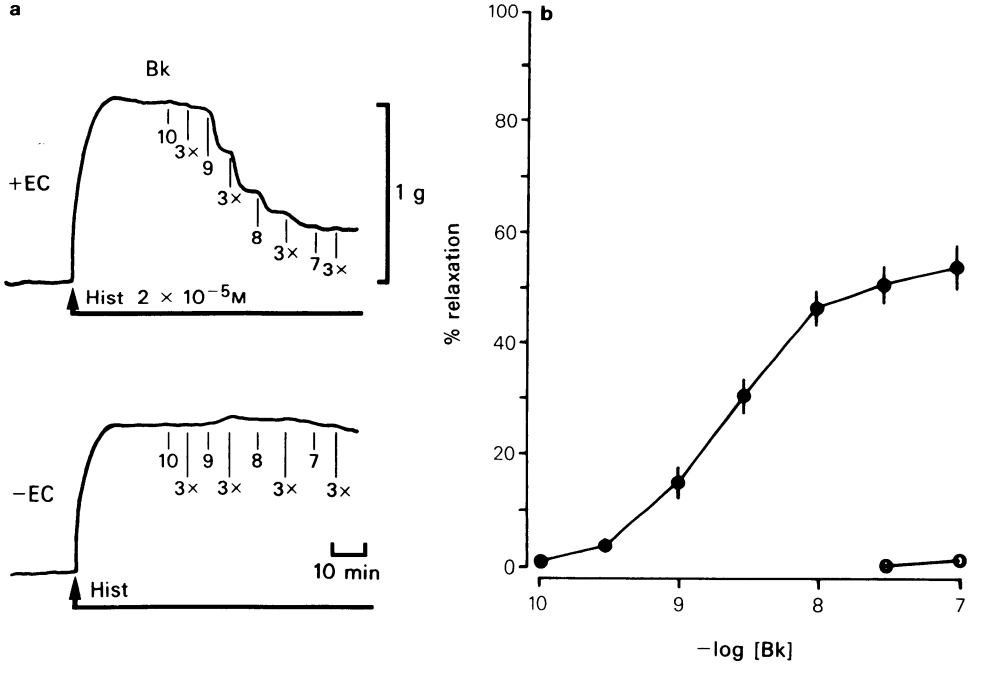

Figure 2 (a) The relaxant effect of bradykinin (Bk) on histamine (Hist)-contracted pig aortic strips, one with (+EC) and one without (−EC) the endothelium present. When tone had stabilised, cumulative dose-response curves were obtained beginning at 10^{-10} M and increasing in 3 fold increments (to 3×10^{-10} M, 10^{-9} M etc.). (b) Dose-response relationship showing relaxation produced by bradykinin on aortic strips with (●) and without (○) endothelium present. Results are plotted as percentage relaxation (mean of $n = 6$; s.e.mean shown by vertical lines) of histamine-induced tone. In this and subsequent figures, error bars are shown only when they exceed the size of the symbols used.

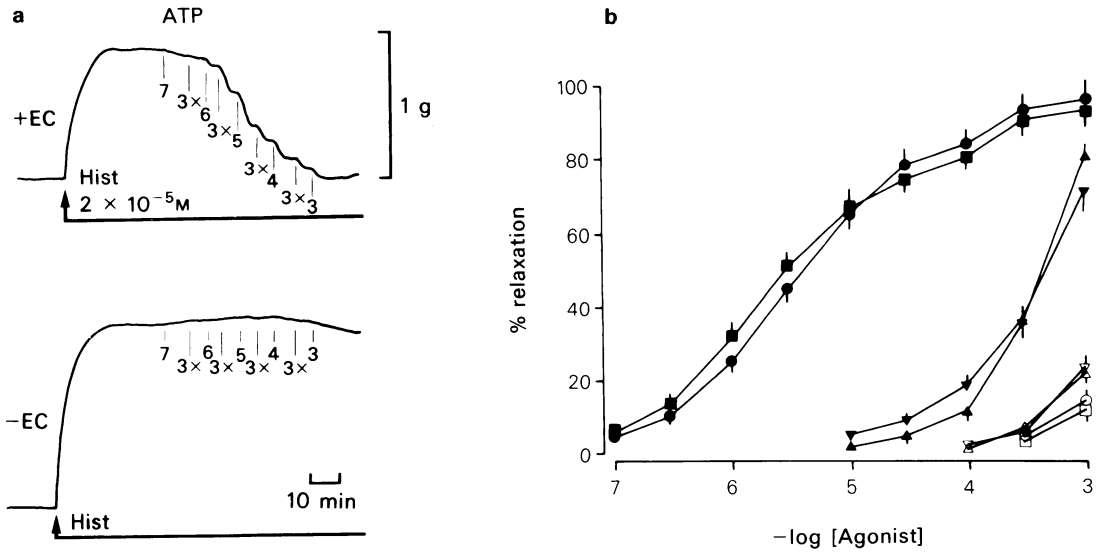

Figure 3 (a) The relaxant effect of ATP on histamine-contracted pig aortic strips, one with (+ EC) and one without (− EC) the endothelium present. (b) Dose-response relationships showing the relaxation induced by ATP (●), ADP (■), AMP (▲), and adenosine (▼) on aortic strips with endothelium present. Relaxations induced by each of these purine compounds on aortic strips without endothelium present are given by their respective open symbols. Each point is the mean of $n = 5-8$; s.e.mean shown by vertical lines.

tions with and without endothelium present; the maximum relaxation of histamine-induced tone was $54 \pm 4\%$.

ATP-induced relaxation ATP ($10^{-7}-10^{-3}$ M) produced a dose-dependent relaxation of histamine-contracted aortic strips with endothelium present but had no effect below 3×10^{-4} M on preparations lacking endothelial cells (Figure 3a); concentrations of 3×10^{-4} M and 10^{-3} M had a slight relaxant effect. This endothelium-independent relaxation possibly contributed to the small increment in the dose-response curve to ATP observed above 10^{-4} M on preparations containing endothelial cells (Figure 3b). The maximum relaxation induced by ATP was $95 \pm 5\%$ ($n = 7$); ADP was equipotent with ATP, but AMP and adenosine were both 120-times less potent when ED_{50} values were compared (Figure 3b).

Acetylcholine-induced relaxation Acetylcholine ($3 \times 10^{-9}-10^{-6}$ M) induced a dose-dependent relaxation of histamine-contracted aortic strips containing endothelial cells but had little effect on preparations without endothelium present (Figure 4a). The maximum relaxation of histamine-induced tone was $67 \pm 3\%$ ($n = 5$) as shown in Figure 4b. In preparations with endothelium present, atropine (10^{-8} M) caused a parallel displacement of the dose-response curve for acetylcholine to the right by a factor of 14 fold, with no change in the maximum response ($n = 8$); the muscarinic agonist, carbachol, induced a maximum relaxation of $57 \pm 3\%$ ($n = 5$) although its ED_{50} value was about 10 fold higher than for acetylcholine (Figure 4b).

A23187-induced relaxation The calcium ionophore A23187 ($10^{-8}-10^{-5}$ M) induced a dose-dependent relaxation of histamine-contracted aortic strips with endothelium present but had no effect on strips lacking endothelial cells (Figure 5a). The maximum relaxation of histamine-induced tone was $97 \pm 1\%$ ($n = 6$) (Figure 5b).

^{86}Rb efflux from isolated aortic endothelial cells of the pig

The spontaneous efflux of ^{86}Rb from preloaded pig aortic endothelial cells could be described by a single exponential function, indicating loss of isotope from a single compartment, namely, escape from the cytoplasm across the plasma membrane. The first-order rate constant for basal ^{86}Rb efflux from endothelial cells in 15 experiments was $1.9 \pm 0.05 \times 10^{-2}$ min^{-1} (mean ± s.e.mean).

Effect of bradykinin on ^{86}Rb efflux Bradykinin ($10^{-10}-10^{-7}$ M) induced a dose-dependent increase in the rate of ^{86}Rb efflux from preloaded aortic endothelial cells (Figure 6a). Each concentration of bradykinin was applied for 6 min to ensure a maximal

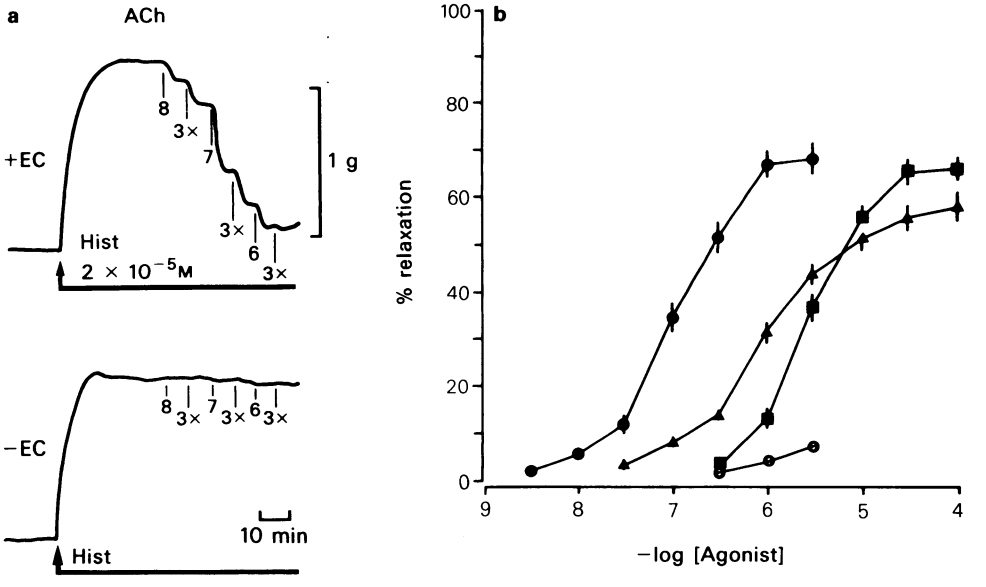

Figure 4 (a) The relaxant effect of acetylcholine (ACh) on histamine-contracted pig aortic strips, one with (+ EC) and one without (− EC) the endothelium present. (b) Dose-response relationships showing the relaxation induced by acetylcholine (●), carbachol (▲) and acetylcholine in the presence of 10^{-8}M atropine (■) on aortic strips with endothelium present, and for acetylcholine on strips without endothelium present (○). Each point is the mean of $n = 6-8$; s.e.mean shown by vertical lines.

effect, but responses usually began to decay before the compound was withdrawn. A slight degree of tachyphylaxis (< 10%) was seen in some but not all preparations when repeated additions of a single concentration were applied at 20 min intervals. The relationship between the concentration of bradykinin applied and the increase in the rate constant for ^{86}Rb efflux (ΔR, mean ± s.e.mean) is shown in Figure 6b.

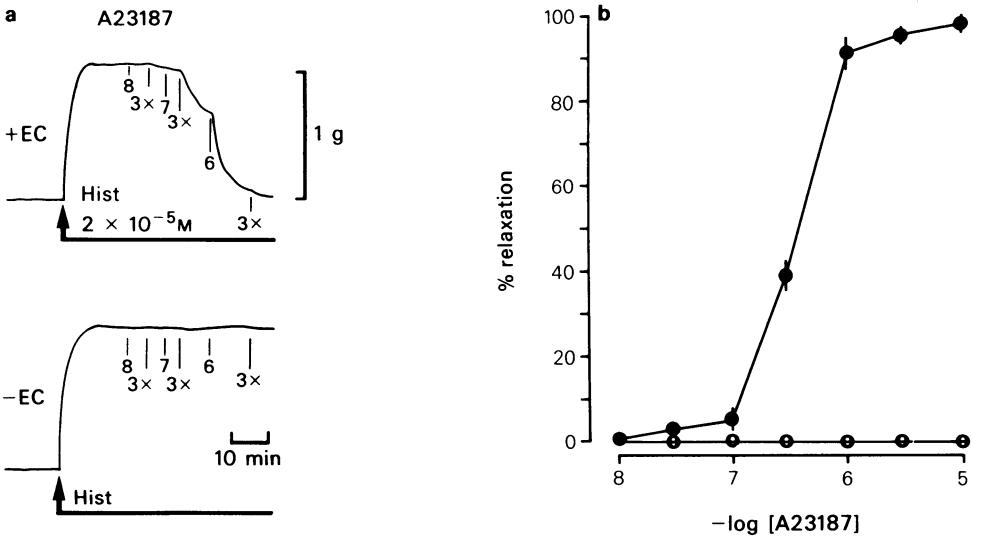

Figure 5 (a) The relaxant effect of ionophore A23187 on histamine-contracted pig aortic strips, one with (+ EC) and one without (− EC) the endothelium present. (b) Dose-response relationship showing the relaxation induced by A23187 on aortic strips with (●) and without (○) endothelium present. Each point is the mean of $n = 6$; s.e.mean shown by vertical lines.

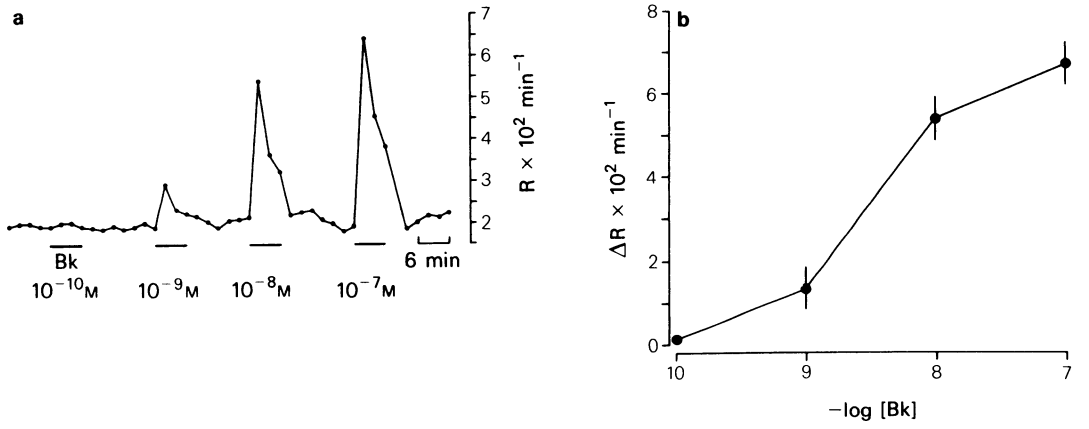

Figure 6 (a) The effect of bradykinin (Bk) on the rate of ^{86}Rb efflux (R) from preloaded pig aortic endothelial cells. Each point represents the first-order rate constant for efflux, units of min^{-1}, in 2 min fractions of superfusate. (b) Dose-response relationship showing the increase in the rate constant for ^{86}Rb efflux (Δ R) induced by bradykinin. Each point is the mean of $n = 5$; s.e.mean shown by vertical lines.

The maximum increase in ^{86}Rb efflux induced was 4.5 times the basal rate.

We wished to determine the mechanisms by which bradykinin induces ^{86}Rb efflux and considered the possibility that it might involve a calcium-activated potassium efflux mechanism similar to that described for other cell types (Gardos, 1956; Haylett, 1976; Putney, 1976; Burgess, Claret & Jenkinson, 1981). The most direct way to test this was to remove calcium from the superfusion medium, but this led to

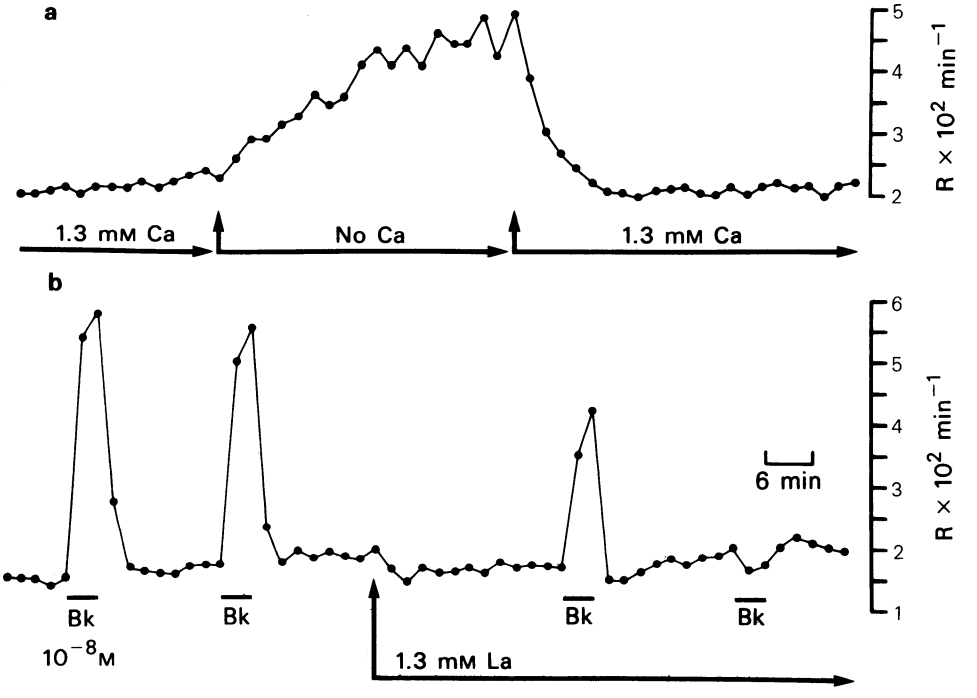

Figure 7 (a) The effect of removing calcium on the basal rate of ^{86}Rb efflux from aortic endothelial cells and the reversal of this effect when calcium was restored. (b) The blocking effect of 1.3 mM lanthanum chloride on bradykinin-induced ^{86}Rb efflux from aortic endothelial cells.

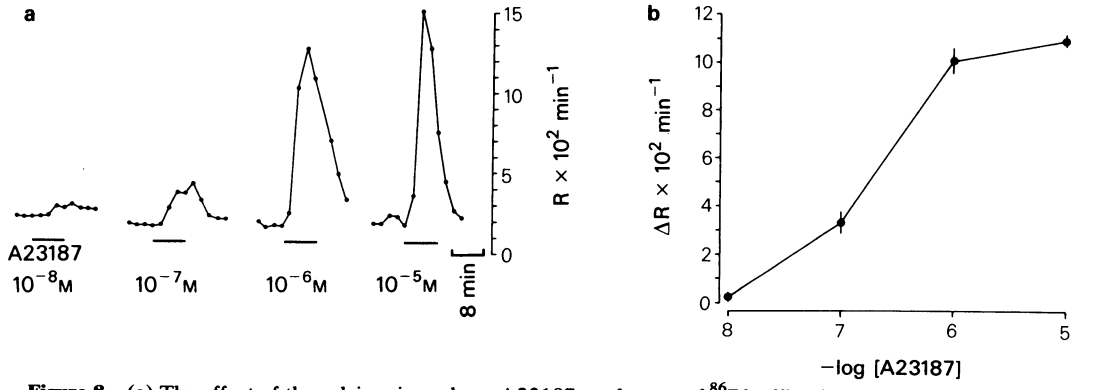

Figure 8 (a) The effect of the calcium ionophore A23187 on the rate of ^{86}Rb efflux from aortic endothelial cells. This agent produced a high degree of tachyphylaxis and so one response only was obtained from each preparation of cells. (b) Dose-response relationship showing the increase in the rate constant for ^{86}Rb efflux (Δ R) induced by A23187. Each point is the mean of $n = 5$; s.e.mean shown by vertical lines.

an increase in the basal rate of ^{86}Rb efflux and thus made it difficult to compare bradykinin-induced responses before and after calcium withdrawal. This rise in the basal rate of ^{86}Rb efflux was probably due to loss of the membrane-stabilizing action of calcium (Frankenhauser & Hodgkin, 1957; Shanes, 1958) and was completely reversed when calcium was restored (Figure 7a). We therefore attempted to block transmembrane calcium movements with lanthanum chloride (Weiss & Goodman, 1969; Goodman & Weiss, 1971), which at 1.3 mM had little effect on the basal rate of ^{86}Rb efflux but reduced the response to the first subsequent challenge with bradykinin and completely blocked responses to later challenges

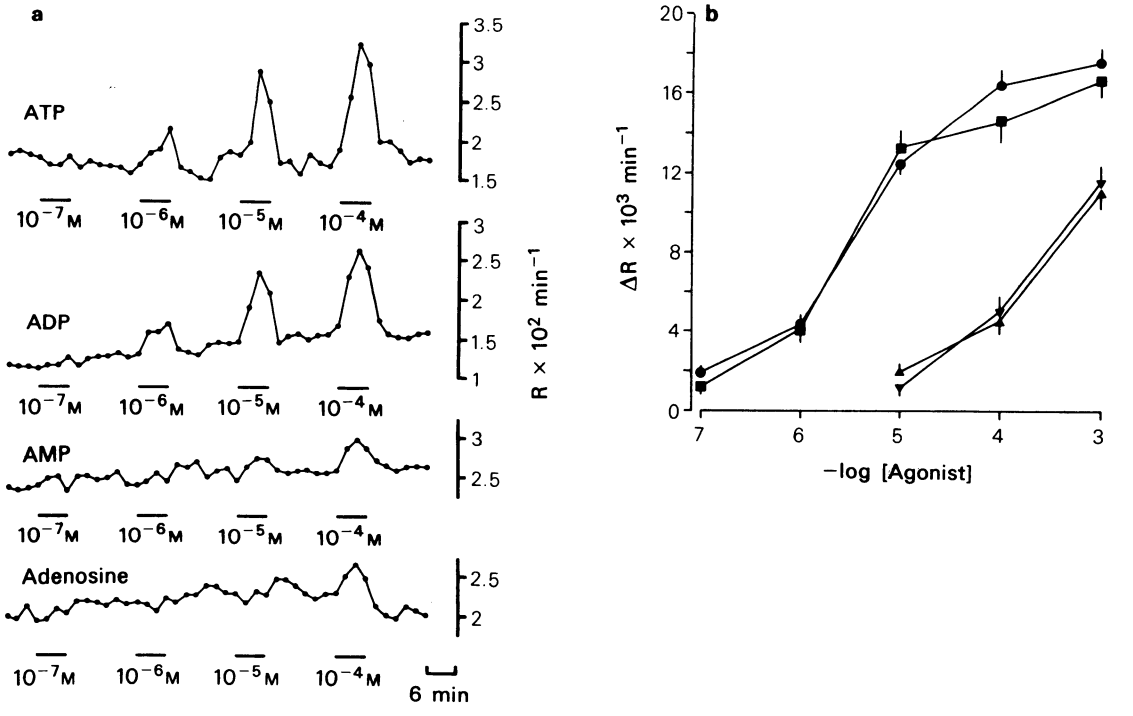

Figure 9 (a) The effects of ATP, ADP, AMP and adenosine on the rate of ^{86}Rb efflux from aortic endothelial cells. (b) Dose-response relationships showing the increase in the rate constant for ^{86}Rb efflux (Δ R) induced by ATP (●), ADP (■), AMP (▲) and adenosine (▼). Each point is the mean of $n = 5$–8; s.e.mean shown by vertical lines.

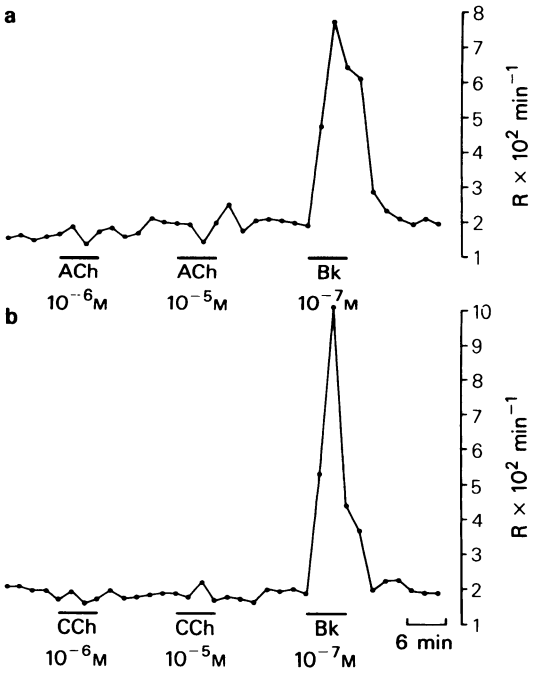

Figure 10 (a and b) The lack of effect of acetylcholine (ACh) and carbachol (CCh) on ^{86}Rb efflux from aortic endothelial cells. Both these preparations of cells responded to bradykinin (Bk).

(Figure 7b). Verapamil (10^{-5}M), which blocks voltage-sensitive calcium channels (Fleckenstein, 1971) had no effect on bradykinin-induced ^{86}Rb efflux (data not shown). In order to investigate further the possibility that aortic endothelial cells possessed a calcium-activated potassium efflux mechanism, we examined the effect of the calcium ionophore A23187.

Effect of A23187 on ^{86}Rb efflux A23187 (10^{-8}–10^{-5}M) induced a dose-dependent increase in ^{86}Rb efflux from preloaded aortic endothelial cells (Figure 8a). Responses to this compound exhibited a high degree of tachyphylaxis and so only one response from each preparation of cells could be obtained. A23187 was the most effective stimulus of ^{86}Rb efflux tested; the maximum increase in efflux induced was 6.8 times the basal rate (Figure 8b).

Effect of ATP on ^{86}Rb efflux ATP (10^{-7}–10^{-3}M) induced a dose-dependent increase in ^{86}Rb efflux from preloaded aortic endothelial cells (Figure 9a). The maximum increase in the rate of ^{86}Rb efflux was 1.9 times the basal rate (Figure 9b). ADP was equipotent with ATP but AMP and adenosine were about 120 times less potent when ED_{50} values were compared (Figures 9a and b). Lanthanum chloride (1.3 mM) blocked ATP-induced ^{86}Rb efflux.

Effect of acetylcholine on ^{86}Rb efflux Acetylcholine and carbachol, in concentrations of 10^{-8}–10^{-4}M, had only small and inconsistent effects on ^{86}Rb efflux from preloaded aortic endothelial cells in six separate experiments; the same preparations of cells did, however, respond to bradykinin (Figure 10a and b). Several batches of endothelial cells were harvested without the use of proteolytic enzymes by scraping the intimal surface of the aorta (Ryan *et al.*, 1980) to test whether the apparent lack of responsiveness to acetylcholine was due to digestion of the cholinoceptor during cell isolation. These cells also did not respond to either acetylcholine or carbachol but did respond to bradykinin.

Discussion

Our results show that in the aorta of the pig, as with several isolated arterial and venous preparations from other species, bradykinin, ATP, acetylcholine and the calcium ionophore A23187 induce relaxation by an endothelium-dependent mechanism (Furchgott & Zawadzki, 1980a; Zawadzki, Cherry & Furchgott, 1980; Altura & Chand, 1981; De Mey & Vanhoutte, 1981; 1982; Cherry *et al.*, 1982). The most likely explanation of these results is that endothelial cells respond to each of these agents by generating a signal which causes the smooth muscle cells of the vessel wall to relax, but measurements of endothelium-dependent relaxation *per se* cannot establish the nature of this signal, nor determine whether all the agents act via the same pathway.

From these indirect measurements of endothelial cell responsiveness, using pig aortic strips, we found that the relaxation induced by acetylcholine was antagonized competitively by atropine and was mimicked by carbachol, thus confirming that the cholinoceptor mediating endothelium-dependent relaxation is muscarinic, as had been previously found in the rabbit aorta (Furchgott & Zawadzki, 1980a). ATP and ADP were equipotent and were about 120 times more potent than AMP and adenosine at inducing endothelium-dependent relaxation. This is similar to the rank order of potency in relaxing the taenia coli of the guinea-pig (Axelsson & Holmberg, 1969; Burnstock, Campbell, Satchell & Smythe, 1970; Brown & Burnstock, 1981) and suggests that the major purinoceptor involved in endothelium-dependent relaxation is of the P_2-type (Burnstock, 1978). AMP and adenosine also induced more relaxation of pig aortic strips when the endothelium was present, in contrast to the results obtained on the rabbit aorta and dog femoral artery (Furchgott & Zawadzki, 1980b; De Mey & Vanhoutte, 1981).

We confirmed the finding of Zawadzki *et al.* (1980) that A23187 induces endothelium-dependent relaxation, which suggests that a rise in the level of ionised calcium within the endothelial cells can trigger relaxation of smooth muscle cells in the vessel wall.

Because of the difficulty of measuring intracellular calcium directly we sought an alternative means of assessing this parameter. In a previous study we found that vasoactive agents increased the rate of ^{86}Rb efflux from vascular smooth muscle cells by a calcium-dependent mechanism (Gordon & Martin, 1982a; Martin & Gordon, 1982a, b) and therefore applied the technique of assessing ^{86}Rb efflux to isolated endothelial cells.

Bradykinin, ATP and A23187 each induced an increase in the rate of ^{86}Rb efflux from preloaded pig aortic endothelial cells but acetylcholine or carbachol did not. The lack of effect of acetylcholine was apparently not due to digestion of the cholinoceptor during isolation of cells, since cells harvested without the use of enzymes were similarly unresponsive in terms of ^{86}Rb efflux. ATP and ADP were equipotent at inducing ^{86}Rb efflux whereas AMP and adenosine were about 120 times less so; this is similar to the rank order of potency at inducing endothelium-dependent relaxation and thus confirms directly that there is a purinoceptor of the P_2-type on pig endothelial cells.

For each of these vasodilators which did increase ^{86}Rb efflux, the dose-response relationship for efflux paralleled closely that for endothelium-dependent relaxation if log concentration was plotted against percent response. However, the rank order of potency of the agents as efflux stimulators was different from that of their potency as vascular relaxants: A23187 was the most potent in both respects, but ATP was a more effective relaxant than bradykinin although it was less potent at inducing ^{86}Rb efflux. Furthermore, acetylcholine, which did not stimulate ^{86}Rb efflux at all, was also a more effective relaxant than bradykinin. The effectiveness of A23187 indicates that endothelium-dependent relaxation can be induced by an increased level of ionised calcium within the endothelial cells and that this is associated with stimulation of potassium efflux. The results obtained with the other three agents are consistent with bradykinin inducing relaxation through this pathway, whereas acetylcholine uses a different, as yet undefined, mechanism. The observation that ATP stimulates ^{86}Rb efflux indicates that the calcium-dependent pathway probably plays a role in the relaxation induced by ATP, but as ATP is relatively ineffective as an inducer of efflux, another pathway may also be involved.

Efflux of ^{86}Rb induced by bradykinin and ATP was blocked by lanthanum, which prevents transmembrane calcium movements (Weiss & Goodman, 1969; Goodman & Weiss, 1971). These results support the concept that bradykinin and ATP induced ^{86}Rb efflux by a calcium-activated potassium transport mechanism similar to that described for a range of cells including erythrocytes, hepatocytes and parotid gland acinar cells (Gardos, 1956; Haylett, 1976; Putney, 1976; Burgess *et al.*,1981). After lanthanum treatment bradykinin and ATP induce one ^{86}Rb efflux response before a complete block is established, analogous to the effect of lanthanum on noradrenaline-induced contractions of the rabbit aorta (Deth & Van Breemen, 1974; Martin & Gordon, 1982a). This suggests that the endothelium has an intracellular calcium store which can be utilized by bradykinin and ATP. Verapamil, which blocks voltage-sensitive calcium channels in muscle cells (Fleckenstein, 1971), did not affect bradykinin-induced ^{86}Rb efflux from endothelial cells, although it inhibits agonist-induced ^{86}Rb efflux from aortic smooth muscle cells (Martin & Gordon, 1982). It is therefore unlikely that bradykinin induces a depolarization of endothelial cells, unless endothelium possesses calcium channels unlike those in muscle. A detailed study of membrane potential and voltage-sensitive channels in endothelial cells would be required to clarify this point.

In conclusion, monitoring ^{86}Rb efflux provides a direct assessment of endothelial cell responsiveness to some, but not all, agents that induce endothelium-dependent relaxation. For those agents that stimulate ^{86}Rb efflux, the dose-response relationships for relaxation and efflux are essentially identical, but results obtained with acetylcholine, which induces relaxation but not efflux, suggest that at least one other pathway can mediate endothelium-dependent relaxation. Further studies are needed to elucidate the mechanisms involved, but applying the continuous superfusion technique described here to measure other cellular functions such as prostaglandin production, already known to be stimulated by some vasoactive agents (Gimbrone & Alexander, 1975; Weksler, Ley & Jaffe, 1978; Hong, 1980), may facilitate the kinetic analysis of endothelial responses and thus increase our understanding of endothelial reactivity and vasoregulation.

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