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Amiloride analogues cause endothelium-dependent relaxation in the canine coronary artery *in vitro*: possible role of Na^+/Ca^{2+} exchange

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1 A number of amiloride analogues were used to test the proposal that Na^+/Ca^{2+} exchange may play a role in the secretion of endothelium-derived relaxing factor (EDRF). The analogues used were those substituted on either the 5-amino group or the terminal guanidino nitrogen atom. The former block both Na^+/Ca^{2+} and Na^+/H^+ exchange whilst the latter block the Na^+ channel and the Na^+/Ca^{2+} exchange.

2 Both series of compounds caused relaxation in isolated rings of dog coronary artery (EC_{50} values, 1–10 μM) presumably due to release of EDRF since removal of endothelium greatly attenuated the response.

3 Amiloride (1–100 μM) had little effect on either endothelium-intact or denuded arteries.

4 The guanidino substituted analogues also appeared to block selectively the relaxation response to acetylcholine in the coronary artery, independently of their EDRF-releasing activity.

5 It is proposed that endothelial cells have an active Na^+/Ca^{2+} exchange operating in the forward mode to extrude Ca^{2+}. This mechanism may be important in the control of EDRF release.

Introduction

The release of the powerful vasodilator, endothelium-derived relaxing factor (EDRF) from endothelial cells (for review see Furchgott, 1984) is generally thought to be a Ca^{2+}-dependent process. Extracellular Ca^{2+} appears to be important for the expression of endothelium-dependent relaxation to agents such as acetylcholine (Long & Stone, 1985a,b). However, the mechanism whereby intracellular free Ca^{2+} is either elevated in response to various EDRF-releasing stimuli or buffered upon cessation of the stimulus is poorly understood.

Winquist *et al.* (1985) reported that dichlorobenzamil, an amiloride analogue which inhibits Na^+/Ca^{2+} exchange in brain and heart plasma membranes (Kaczorowski *et al.*, 1985), selectively blocked the relaxation responses to acetylcholine and surprisingly, A23187 in the rat aorta. Similarly, Schoeffter & Miller (1986) found that either high concentrations of amiloride or replacement of Na^+ with choline or Li^+ blocked acetylcholine-induced relaxations in the rat aorta. Both studies concluded that Na^+/Ca^{2+} exchange, which has been proposed as an important mechanism for Ca^{2+} entry in cardiac muscle (Barry & Smith, 1982; Langer, 1982) may also be important in the agonist stimulated, Ca^{2+}-dependent mechanism of EDRF release from endothelial cells.

We examined a variety of amiloride analogues which are know to block Na^+/Ca^{2+} exchange in other cell systems (Siegl *et al.*, 1984; Kaczorowski *et al.*, 1985) and discovered that a number of them, including dichlorobenzamil, relaxed isolated rings of dog coronary artery only if the endothelium was left intact. If these drugs act as inhibitors of Na^+/Ca^{2+} exchange in endothelial cells as reported for other tissues, then a conclusion from our results is that the block of Na^+/Ca^{2+} exchange in endothelial cells leads to an increase in intracellular Ca^{2+} and thus the release of EDRF. Such a proposal is contrary to that of Winquist *et al.* (1985) and of Schoeffter & Miller (1986).

Methods

Tissue preparation

Greyhound circumflex coronary artery Male or female greyhounds (20–25 kg) were anaesthetized

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with sodium pentobarbitone (40 mg kg^{-1}, i.v.) and the heart removed and quickly placed in ice-cold Krebs solution (see below). The circumflex coronary artery was dissected from the heart and 3–4 mm ring segments cut from areas not having visible side branches. When required, the endothelium was removed from some rings of artery by gently rubbing the luminal surface with a Krebs-moistened filter paper taper (Cocks & Angus, 1983). Each artery ring was then mounted on two stainless steel hooks passed through the lumen. The bottom hook was attached to a support leg (methacrylate-vertex) which could be adjusted in a vertical plane by means of a micrometer. The upper hook was attached to a Grass force-displacement transducer (FT.03) to record isometric circumferential force in the artery ring. The tissue holder leg was immersed in a water jacketed organ bath (25 ml) containing Krebs solution at 37°C, pH 7.4. The composition of the Krebs solution was as follows (in mM): Na^+ 144, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 128.7, HCO_3^- 25, SO_4^{2-} 1.2, $H_2PO_4^-$ 1.2 and glucose 11, gassed with a mixture of 95% O_2, 5% CO_2. Each artery ring was then equilibrated for 1 h under an initial passive force of 4 g before active force was generated by adding a thromboxane A_2-mimetic, U46619 (30 nM). When a steady level of force was reached, concentration-response curves were constructed to either EDRF-releasing agonists such as acetylcholine, bradykinin and substance P, or Na^+/Ca^{2+} exchange inhibitors. The effectiveness of the technique for endothelium removal was assessed functionally by testing EDRF-releasing agonists. Rings that failed to relax to maximal concentrations of these drugs were regarded as being devoid of endothelium.

Dog coronary artery rings: interactions between amiloride analogues and EDRF agonists For this series of experiments, endothelium intact rings of coronary artery were treated with amiloride analogues after the tissues had reached and maintained a steady level of contraction to U46619. The most efficacious of each of the guanidino- and 5-substituted analogues were examined; 2',3'-benzobenzamil and 5-(N-1-adamantyl)amiloride respectively. If no response to the compounds was obtained after 10 min incubation, cumulative concentration-response curves were constructed to the EDRF releasing agonists bradykinin, acetylcholine, substance P and the Ca^{2+} ionophore, A23187. If relaxation to the amiloride analogues occurred, the EDRF-agonist curves were then constructed upon plateau of these responses.

Rat aorta Male Wistar rats (200–250 g) were stunned by a blow to the head and exsanguinated. The thoracic aorta was quickly dissected into Krebs solution (25°C) and cleaned of connective tissue. Two 4 mm long rings from each rat were cut and suspended on fine wire hooks to record isometric circumferential force as described above for the dog coronary artery. One ring of each pair had its endothelium removed by carefully abrading the luminal surface as described above. Rings were stretched to a resting passive force of 2 g and left for 60 min before starting the experiment.

Guinea-pig terminal ileum and right atrium Male or female guinea-pigs (150–200 g) were stunned by a blow to the head and exsanguinated. The last 20–25 cm of the terminal ileum was removed and placed in Krebs solution at room temperature. It was then cut into ~3 cm lengths and each cleared of its luminal contents. Each segment was stretched over a glass rod and the longitudinal muscle coat was removed as a sheet. This was achieved by means of a fine longitudinal cut (being careful not to cut the circular layer) followed by gently rubbing off the outer longitudinal muscle coat with a Krebs-moistened cotton bud. Each sheet was then suspended in a 25 ml organ bath to record muscle activity auxotonically by means of a Grass FT.03 force-displacement transducer coupled to a 0.3 g cm^{-1} spring. Activity was amplified and displayed on a flatbed chart recorder. Tissues were allowed to equilibrate for 30 min at 37°C before being stretched to an initial tension of 0.5 g. They were then allowed a further 60 min equilibration period before the start of the experiment.

The right atrium was removed from the heart and suspended on wire hooks for recording of the spontaneous surface electrogram and atrial period as described previously (Angus & Harvey, 1981).

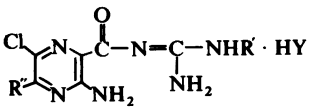
Data analysis

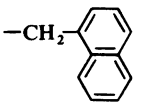
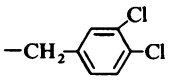
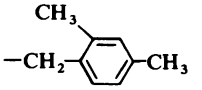
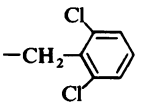
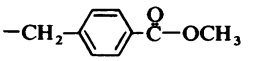
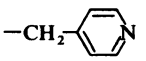
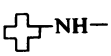
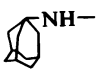
Cumulative concentration-relaxation curves to EDRF-releasing agonists were constructed in separate rings of coronary artery. Similar cumulative concentration-contraction curves were obtained in separate strips of ileal longitudinal smooth muscle. EC_{50} values were then obtained from each curve by computerized sigmoid curve fitting analysis (Nakashima *et al.*, 1982). Average values $\pm$ 1 s.e.mean were calculated; *n* values refer to the number of rings of artery from separate animals.

Drugs

The drugs used and their sources (in parentheses) were: acetylcholine bromide, indomethacin, histamine hydrochloride, substance P triacetate (Sigma; St. Louis, MO, U.S.A.), A23187 (Calbiochem,

Table 1 Structure-activity relationship of guanidino- and 5-amino-substituted amiloride analogues and endothelium-dependent relaxation in the canine coronary artery



Compound	*R'*	*R''*	*HY*	*Code*	EC_{50} (μM)
Amiloride	H	NH_2			
2',3'-Benzobenzamil		NH_2	$(CH_3)_2NHCH$	G_1	7.1 ± 1.7
3',4'-Dichlorobenzamil		NH_2		G_2	8.7 ± 1.0
2',4'-Dimethylbenzamil		NH_2		G_3	10 ± 1.0
2',6'-Dichlorobenzamil		NH_2	$\frac{1}{2}(CH_3)_2NCH$	G_4	57 ± 30
4'-(Methoxycarbonyl)benzamil		NH_2	$\frac{1}{2}H_2O$	G_5	170 ± 30
4'-Azabenzamil		NH_2		G_6	
5-(N-cyclododecyl)amiloride	H			A_1	1.0 ± 0.4
5-(N-1-adamantyl)amiloride	H			A_2	1.1 ± 0.3
5-(N,N-dodecamethylene)amiloride	H			A_3	2.0 ± 1.3
5-(N-ethyl-N-isopropyl)amiloride	H	$C_2H_5[(CH_3)_2CH]N-$		EIPA	>10

The EC_{50} values are means ± s.e.means from at least 5 experiments.

La Jolla, CA, U.S.A.), bradykinin triacetate (Fluka, Switzerland), U46619 (Upjohn, Kalamazoo, U.S.A.) 3',4'-dichlorobenzamil, 2',6'-dichlorobenzamil, 2',3'-benzobenzamil, 2',4'-dimethylbenzamil, 4'-(methoxycarbonyl)-benzamil, 4'-azabenzamil, 5-(N-cyclododecyl)amiloride, 5-(N-1-adamantyl)amiloride, 5-(N,N-dodecamethylene)amiloride and 5-(N-ethyl-N-isopropyl)amiloride were synthesized by methods described previously (Cragoe *et al.*, 1967; 1981). A23187 was dissolved in absolute ethanol to a final concentration of 1 mM. All the amiloride derivatives were dissolved in dimethyl sulphoxide (DMSO) to a final concentration of 100 mM. These were then diluted to 10 and 1 mM with 50% DMSO. All other drugs were dissolved in distilled water. All drugs were made up fresh from stock solutions before each experiment.

Results

Rat aorta: effect of 3',4'-dichlorobenzamil

In three experiments methoxamine (3 μM) caused an active contraction of between 1 and 5 g in both endothelium intact and denuded rings of aorta. In each experiment, the effectiveness of removing the endothelium was confirmed by the complete aboli-

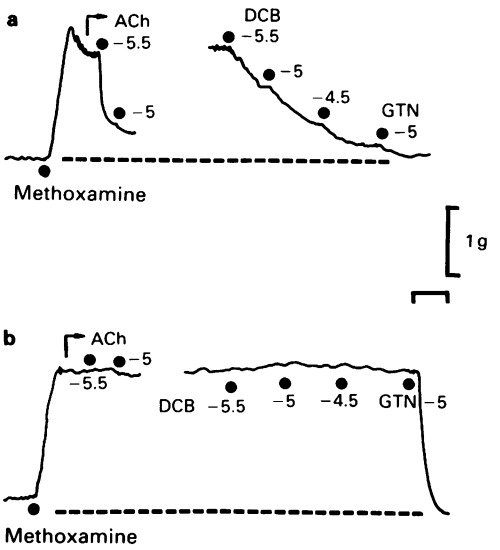


Figure 1 The effect of 3',4'-dichlorobenzamil (DCB) on endothelium-intact (a) and denuded (b) rings of rat thoracic aorta. Both rings were contracted to a steady level of active force with methoxamine (3 μM), then cumulative concentrations (log M) of acetylcholine (ACh) were added to test for functional removal of the endothelium. The break in each trace represents washout of ACh and recovery for 60 min during which time the tissues were contracted again with methoxamine. Cumulative concentrations (log M) of DCB were then added. Note that only the endothelium-intact ring relaxed. Glycerol trinitrate (GTN) was added to indicate maximum relaxation. The horizontal calibration bar represents 10 min before and 2 min after the arrow.

tion of the relaxation response to a maximal concentration of acetylcholine (Figure 1). After washout of acetylcholine, 3',4'-dichlorobenzamil (3–30 μM) caused graded relaxations only in the endothelium-intact artery (Figure 1).

Dog coronary artery: effects of amiloride analogues

Both amiloride (1–100 μM) and the vehicle (DMSO) had only small effects ($\leqslant$10% relaxation for 100 μM amiloride; $n = 4$) on U46619-contracted, endothelium-intact rings of coronary artery. In similar endothelium-denuded rings, amiloride (100 μM) caused either no response or a concentration-dependent slow relaxation ($\leqslant$10% relaxation; $n = 4$). In endothelium-intact rings ($n = 3$) amiloride (100 μM) did not affect the endothelium-dependent response to substance P.

Three of the amiloride analogues, 2',3'-benzobenzamil, 3',4'-dichlorobenzamil and 2',4'-dimethylbenzamil, caused rapid, dose-dependent relaxations of coronary artery rings only if the endothelium was present (Figures 2 and 3; Table 1). In rings of artery without endothelium ($n = 6$) these three analogues caused slow, progressive relaxations of the arterial smooth muscle at concentrations $\geqslant 3\,\mu$M. These were markedly slower in both onset and time to plateau than those for endothelium-intact rings of artery. At 100 μM, maximal relaxations were obtained in 20–30 min compared with 2–5 min for the endothelium-intact rings.

Two guanidino-substituted amiloride analogues, 2',6'-dichlorobenzamil and 4'-(methoxycarbonyl)benzamil also caused rapid, concentration-dependent relaxations only in rings of artery with intact endothelium but at ten fold higher concentrations than the first three (Figure 3, Table 1). Their direct relaxing effect on the artery smooth muscle (endothelium-denuded rings) also occurred at higher concentrations (30–300 μM). However, the time course for these relaxations was similar to the other more active analogues.

4'-Azabenzamil was, in concentrations up to 100 μM, ineffective at causing relaxations in both endothelium-intact and denuded rings of artery (Table 1). Above 100 μM it caused similar slow relaxations in both types of artery.

Another group of amiloride analogues bearing large hydrocarbon ring substituents on the 5-amino nitrogen atom were examined. Each caused endothelium-dependent relaxations that were both rapid in onset and in time to reach maximum. All three analogues were approximately 8–10 times more potent than the most active guanidino analogues (Figure 3, Table 1). 5-(N-ethyl-N-isopropyl)-amiloride (EIPA), which possesses a relatively small 5-substituent on the 5-amino nitrogen atom and is the most potent Na^+/H^+ exchange inhibitor reported by Frelin *et al.* (1984) also caused endothelium-dependent relaxations in this tissue. However, EIPA was approximately 10–30 times less active than the other three 5-substituted analogues, and was the only analogue which caused concentration-dependent tonic contractions in rings of artery without endothelium (Figure 4).

Pretreatment of the coronary artery with indomethacin (10 μM) for 30 min did not affect the endothelium-dependent relaxation responses to either the guanidino- or 5-substituted analogues (data not shown).

Dog coronary artery: interactions of amiloride analogues and EDRF agonists

2',3'-Benzobenzamil at 1.0, 3.0 and 10.0 μM caused 0, 9.0 $\pm$ 2.2 ($n = 19$) and 52 $\pm$ 4.5 ($n = 17$) % relaxation

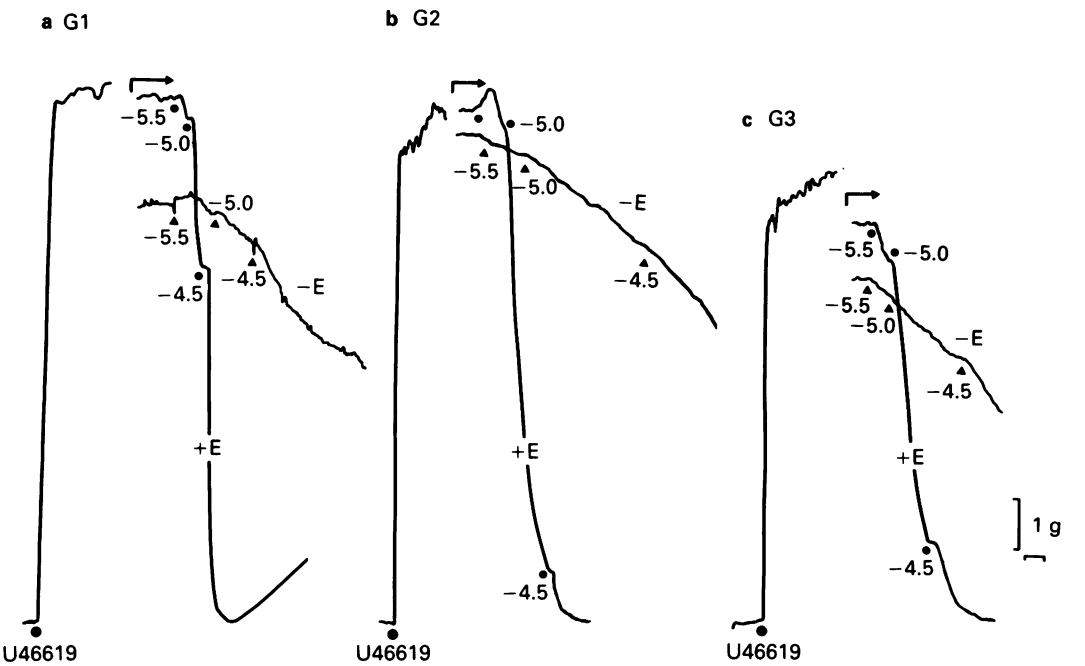


Figure 2 The effect of three guanidino-substituted amiloride analogues. (a) 2',3'-benzobenzamil (G1), (b) 3',4'-dichlorobenzamil (G2) and (c) 2',4'-dimethylbenzamil (G3) on endothelium-intact (+E) and denuded (−E) rings of greyhound coronary artery contracted to a steady level of force with U46619 (30 nM). In each −E ring the initial U46619 contraction has been omitted for clarity and the plateau levels superimposed on the +E traces. Cumulative concentrations are given as (log M). The horizontal bar represents 10 min before and 2 min after the arrow.

respectively (means ± s.e.mean) of endothelium-intact coronary arteries. At 10 μM, 2',3'-benzobenzamil blocked the relaxation responses to acetylcholine without affecting those to A23187 (Figure 5). The effects of increasing concentrations of 2',3'-benzobenzamil on the relaxation response curves to (a) the receptor-linked EDRF agonists acetylcholine, substance P and bradykinin and (b) A23187 and an endothelium-independent relaxant, glyceryl trinitrate are shown in Figures 6 and 7 respectively. From these curves it can be readily seen that 2',3'-benzobenzamil selectively blocks, in a concentration-dependent manner, the relaxation responses to acetylcholine. The family of curves for each agonist was then normalized (% relaxation) and computer fitted to determine the EC_{50} values.

At 0, 1, 3 and 10 μM 2',3'-benzobenzamil, the EC_{50} values (means ± s.e.mean) for acetylcholine were 42 ± 10 nM, 0.3 ± 0.09 μM, 1.5 ± 0.5 μM and 3.4 ± 1.3 μM respectively ($n = 5$). These represent corresponding 7, 36 and 81 fold shifts in the location (EC_{50}) of the relaxation curve for acetylcholine. Paired EC_{50} values (means ± s.e.mean) for substance P, bradykinin, A23187 and glyceryl trinitrate at 0 and 10 μM 2',3'-benzobenzamil were as follows: substance P (37 ± 22 pM; 27 ± 8.9 pM; $n = 3$), bradykinin (2.8 ± 1.4 nM; 2.4 ± 1.6 nM; $n = 6$), A23187 (19.6 ± 2.5 nM; 13.7 ± 3.4 nM; $n = 3$), glyceryl trinitrate (61.1 ± 3.7 nM; 37.8 ± 16.2 nM; $n = 3$). Atropine (0.1 μM) did not have any effect on the relaxation response curves to 2',3'-benzobenzamil and 5-(N-1-adamantyl)amiloride whilst in separate experiments atropine caused an approximate 100 fold rightwards shift in the relaxation curve to acetylcholine. Similarly, in a further three experiments, complete and specific desensitization of both bradykinin and substance P receptors failed to affect the response to either 2',3'-benzobenzamil or 5-(N-1-adamantyl)amiloride (data not shown).

In contrast to 2',3'-benzobenzamil, 5-(N-1-adamantyl)amiloride (0.1–1.0 μM) did not affect the relaxation responses to any of the EDRF-releasing agonists, including acetylcholine and A23187. The highest concentration of 5-(N-1-adamantyl)amiloride used, 1 μM, itself caused 51 ± 9.5% relaxation ($n = 4$).

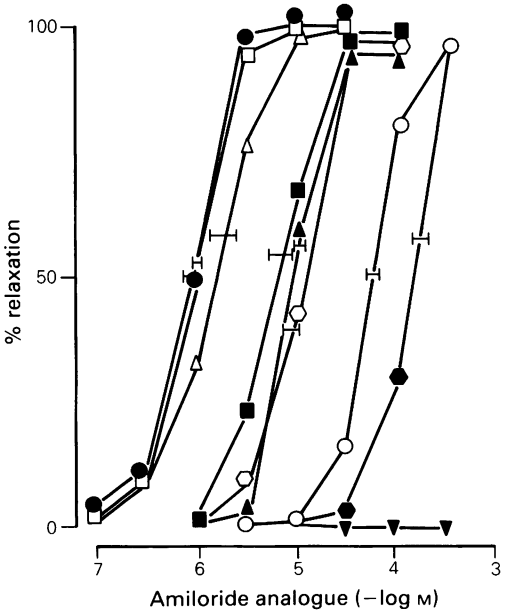


Figure 3 Cumulative concentration-relaxation curves for the six guanidino (G1–6) and three 5-amino (A1–3) substituted amiloride analogues (see Table 1) tested in greyhound coronary artery with endothelium: (●) A1, (□) A2, (△) A3, (■) G1, (▲) G2, (○) G3, ((○) G4, (●) G5, (▼) G6. Each curve was constructed in endothelium-intact rings of artery from experiments depicted in Figure 2. Horizontal bars are s.e.means of the EC_{50} values derived from individually computer fitted curves ($n > 5$). 100% relaxation represents complete relaxation of the U46619 contraction as shown in Figure 2.

Guinea-pig ileum and atrium

Both 2',3'-benzobenzamil (1–10 μM) and 5-(N-1-adamantyl)amiloride (1–10 μM) caused a small (<5% maximum), short lasting contraction of the ileal longitudinal smooth muscle preparation. At these concentrations, chosen from the EC_{50} values for endothelium-dependent relaxation of the dog coronary artery (see above), 2',3'-benzobenzamil (10 μM) caused a small but significant ($P < 0.01$) 3.2 fold rightward shift in the contraction curve to bethanechol (Figure 8) whereas 5-(N-1-adamantyl)-amiloride was without effect (data not shown).

In two preliminary experiments in the guinea-pig atrium, concentration-bradycardia curves to bethanechol (3–30 μM) were unaffected by 2',3'-benzobenzamil at both 10 and 30 μM. In each case the atria ceased beating at 30 μM bethanechol, the same concentration as in untreated tissues. The resting rate was then fully restored by the addition of atropine (0.2 μM) within 3 min.

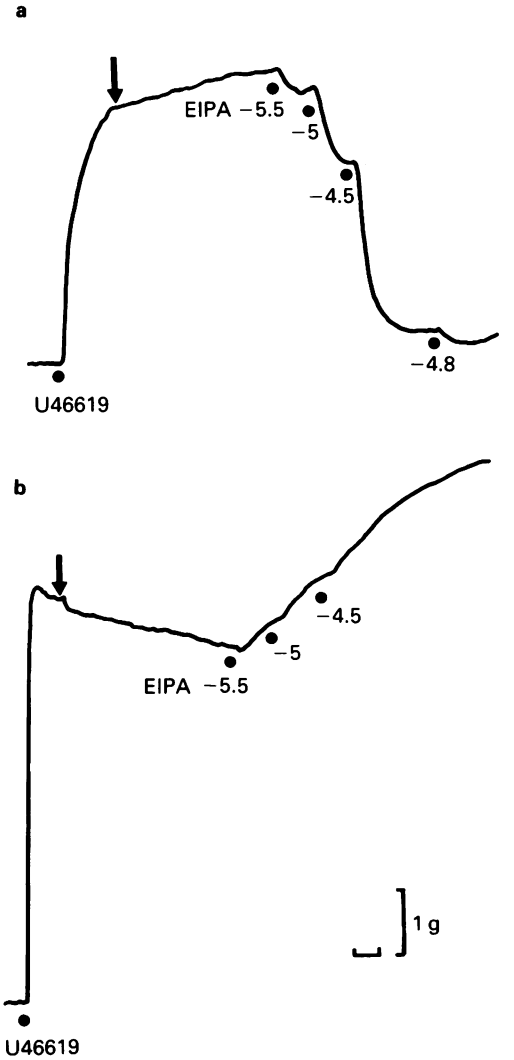


Figure 4 The effect of 5-(N-ethyl-N-isopropyl)amiloride (EIPA) on endothelium intact (a) and endothelium-denuded (b) rings of greyhound circumflex coronary artery. Each ring was contracted to a steady level of active force with U46619 (30 nM). The horizontal bar represents 10 min before and 2 min after the arrow.

Discussion

In this study we found that certain amiloride analogues caused endothelium-dependent relaxations of isolated coronary artery of the dog. The second, unexpected finding was the selective blockade of acetylcholine-induced release of EDRF by the guandino analogues.

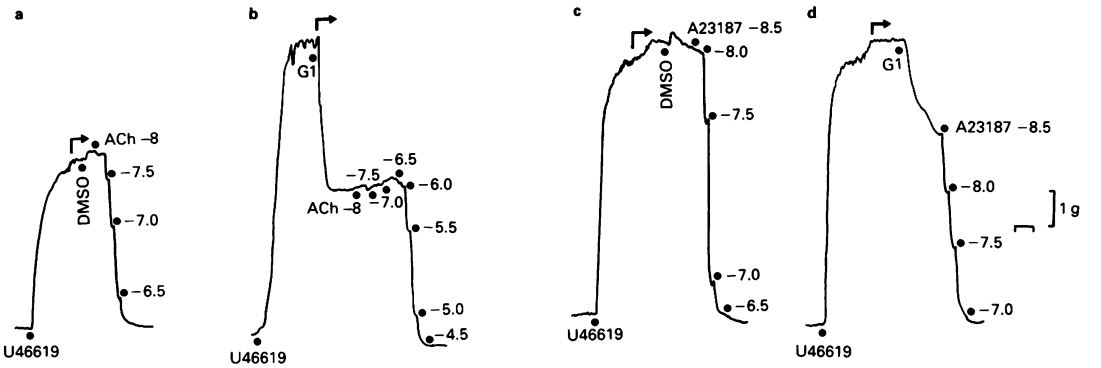


Figure 5 The effect of 2',3'-benzobenzamil (G1) on the cumulative relaxation responses to acetylcholine (ACh) and A23187 (log M) in the greyhound coronary artery contracted with U46619 (30 nM). Records shown are typical of at least 4 experiments. (a) and (c) Controls (DMSO), (b) and (d) 2',3'-benzobenzamil (10 μM). The horizontal calibration bar represents 10 and 2 min before and after the arrows respectively.

Na^+/Ca^{2+} exchange inhibition

Given the Na^+/Ca^{2+} exchange inhibitory profile of the amiloride analogues used here, albeit in other tissues (Siegl *et al.*, 1984; Kaczorowski *et al.*, 1985), our results are consistent with the hypothesis that endothelial cells possess an active Na^+/Ca^{2+} exchange operating in the 'forward' mode (see Pott, 1986) to extrude Ca^{2+} from the cell. This is in direct contrast to the proposal put forward by both Win-

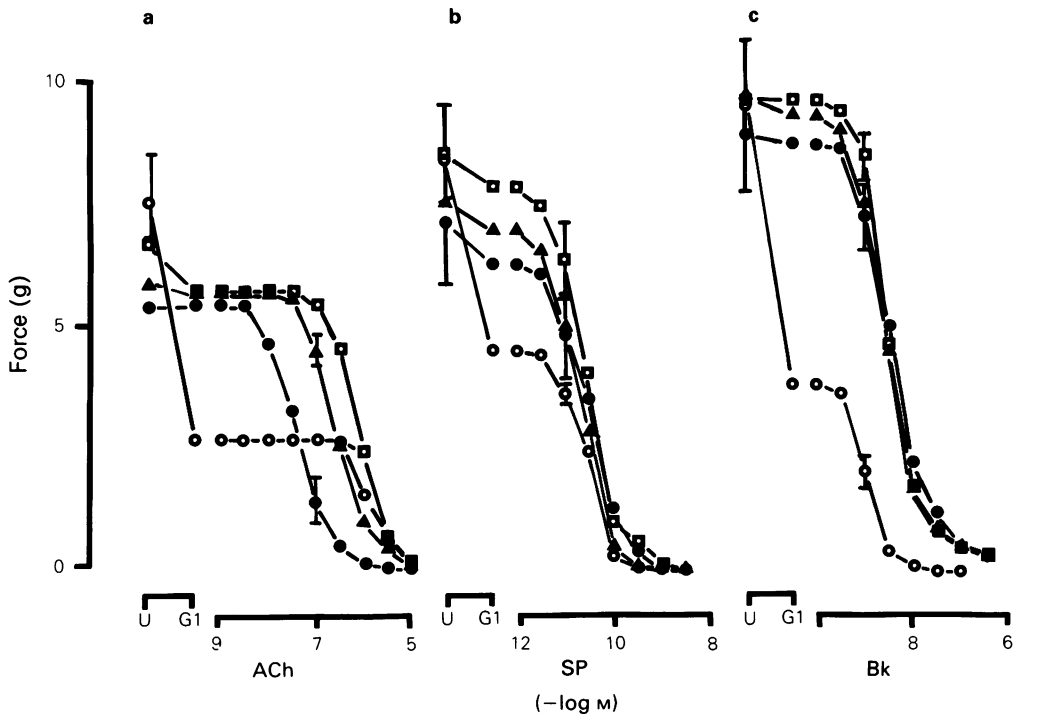


Figure 6 The effect of 2',3'-benzobenzamil (G1) on the relaxation curves to (a) acetylcholine (ACh), (b) substance P (SP) and (c) bradykinin (Bk) in the greyhound coronary artery. U (U46619: 30 nM); G1 (after treatment with G1); (●) 0, (▲) 1, (□) 3 and (○) 10 μM G1. Points on the curves are means (and in some cases vertical bars show s.e.mean) from at least 4 experiments. Error bars are excluded from some points for clarity of presentation.

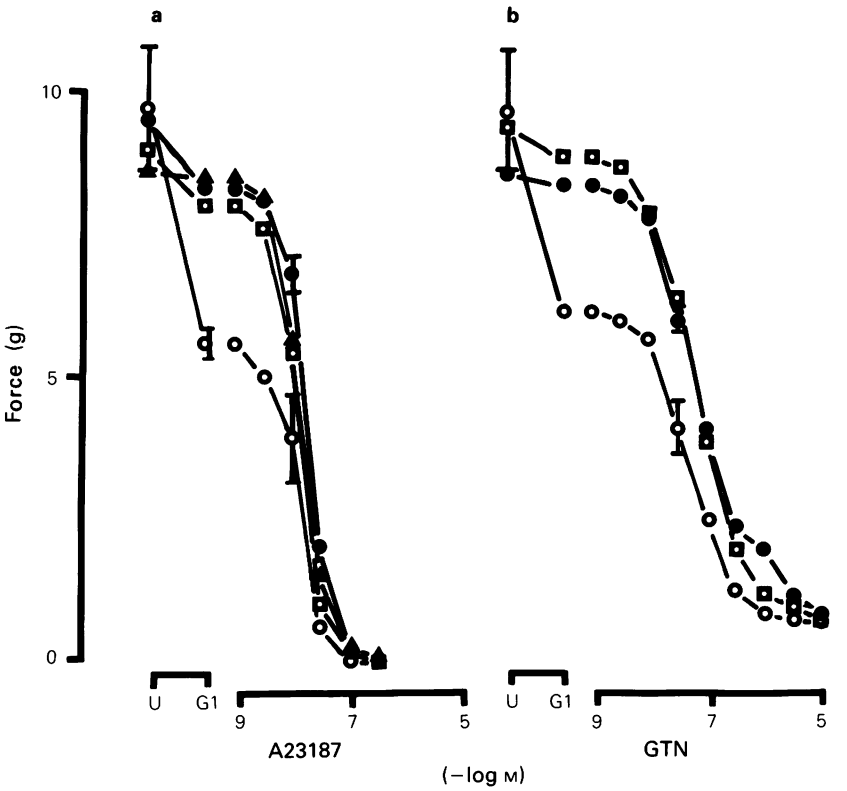


Figure 7 The effect of 2',3'-benzobenzamil (G1) on the relaxation curves to (a) A23187 and (b) glyceryl trinitrate (GTN). Notation is as for Figure 6.

quist *et al.* (1985) and Schoeffter & Miller (1986). They suggested that block of EDRF-mediated responses to acetylcholine and the Ca^{2+} ionophore, A23187 by either 3',4'-dichlorobenzamil (Winquist *et al.*, 1985) or replacement of Na^+ with choline (Schoeffter & Miller, 1986) could be taken as evidence that Na^+/Ca^{2+} exchange is involved in the release mechanism of EDRF in response to these stimuli, particularly acetylcholine. Thus they concluded that block of Na^+/Ca^{2+} exchange prevented the release of EDRF whereas our results indicated that such block, assuming a similar mechanism of action of 3',4'-dichlorobenzamil as they did, caused release of EDRF. Whilst we were able to demonstrate block of EDRF responses in the dog coronary artery to acetylcholine with 2',3'-benzobenzamil (but see below) we were, however, unable to show any affect of 2',3'-benzobenzamil against the EDRF-mediated responses to A23187, bradykinin or substance P at concentrations which caused approximately 50% relaxation. Nor could we show any block of all four endothelium-dependent agonists with any of the other more active 5-amino-substituted amiloride analogues. The block of A23187-induced relaxation responses in the endothelium-intact rat aorta by higher concentrations of 3',4'-dichlorobenzamil (30 μM, see Winquist *et al.*, 1985) most probably represented physiological antagonism. In both the rat aorta and the dog coronary artery the same concentration of 3',4'-dichlorobenzamil resulted in maximal relaxation which probably indicated maximal release of EDRF. Such EDRF-releasing activity would explain the finding in the rat aorta that following preincubation with 3',4'-dichlorobenzamil, much higher concentrations of the contracting agonist (i.e. methoxamine) were required to attain even less active force as compared to the control (Winquist *et al.*, 1985). Also, the greater degree of block by 3', 4'-dichlorobenzamil of the acetylcholine-mediated responses observed by Winquist *et al.* (1985) could have been due to its combined selective inhibitory effect against acetylcholine (see below and as suggested by Winquist *et al.*, 1985) and its EDRF releasing activity.

Thus, our results could be explained by Na^+/Ca^{2+} exchange being a sensitive way to extrude

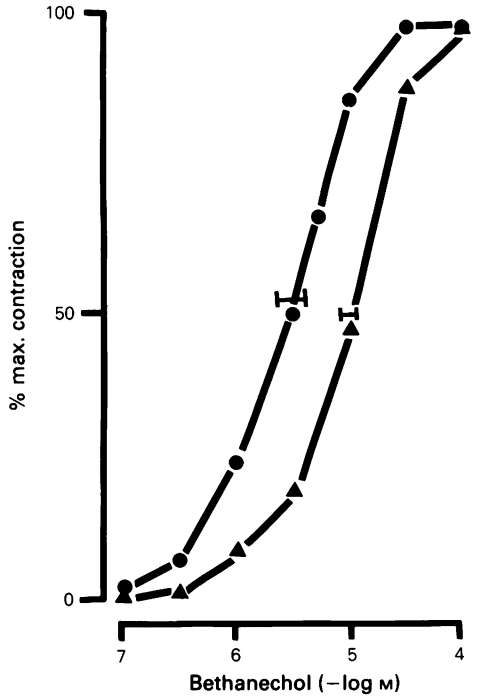


Figure 8 The effect of 2',3'-benzobenzamil (G1: 10 μM) on the contraction-concentration curve to bethanechol in the isolated longitudinal smooth muscle of the guinea-pig terminal ileum: (●) 0, (▲) 10 μM G. Horizontal error bars are s.e.means of the EC_{50} values derived from individually computer-fitted curves ($n = 6$).

Ca^{2+} from endothelial cells. Inhibition of this pathway with the amiloride analogues tested, as documented for other tissues would then cause cytoplasmic Ca^{2+} to increase, presumably above a threshold level for activation of the as yet unknown mechanism for EDRF release. Such a hypothesis remains to be tested, in particular by measuring changes in intracellular Ca^{2+} levels in response to the amiloride analogues used here.

The data with the 5-substituted amiloride analogues are complicated in as much as these compounds also possess anti Na^+/H^+ exchange activity. However, this antiporter is unlikely to be involved in the endothelium-dependent relaxation to the 5-substituted analogues since a more selective Na^+/H^+ exchange inhibitor, 5-(N-ethyl-N-isopropyl) -amiloride was 10–30 times less active in eliciting an endothelium-dependent relaxation in the dog coronary artery, although this may have been partly due to the endothelium-independent contraction of the smooth muscle observed with this compound. Also, analogues bearing substituents on the terminal guanidino-nitrogen atom, such as the ones used here, have been reported to be relatively inactive against Na^+/H^+ exchange in chick skeletal muscle cells (Vigne *et al.*, 1984), fibroblasts (L'Allemain *et al.*, 1984), A431 cells (Zhuang *et al.*, 1984), and human neutrophils (Simchowitz & Cragoe, 1986).

The three 5-substituted amiloride analogues used were approximately equi-active in eliciting endothelium-dependent relaxations (i.e. EC_{50}s = 1 μM), whereas the guanidino substituted derivates showed distinct structural requirements for activity. A study of six benzamil analogues showed that similar and maximal potency was achieved with the 2',3'-benzo, the 3',4'-dichloro and the 2',4'-dimethyl analogues. The 2',6'-dichloro analogue was 8 fold less active and the 4'-(methoxy-carbonyl) analogue was 24 fold less active than 2',3'-benzobenzamil whilst the 4'-aza analogue was inactive. A somewhat similar structure-activity relationship in anti Na^+/Ca^{2+} exchange activity has also been observed in plasma membrane vesicles from a number of tissues (see Kaczorowski *et al.*, 1985).

In contrast to endothelial cells, vascular smooth muscle cells, at least in the dog coronary artery, do not appear to have such an active Na^+/Ca^{2+} exchange; although it has been postulated to exist (Blaustein, 1977) it has not been demonstrated. Our data in endothelium-denuded rings of artery showed slow, weak relaxation reponses to the amiloride analogues which is opposite to what would be expected if Na^+/Ca^{2+} exchange operated to extrude Ca^{2+} from smooth muscle cells.

Acetylcholine

The other finding from this study was the selective block of acetylcholine-induced relaxation by the guanidino-substituted amiloride analogues. This is a separate action from their EDRF-releasing activity as shown by the lack of anti-acetylcholine activity of the more potent EDRF-releasing 5-substituted analogues. Also, atropine blocked the endothelial cell muscarinic receptors without affecting the relaxation response to 2',3'-benzobenzamil. This indicates that 2',3'-benzobenzamil does not act as a partial agonist at endothelial muscarinic receptors. Further, the absence or weak inhibitory activity of 2',3'-benzobenzamil at muscarinic receptors in the guinea-pig atrium and guinea-pig ileum respectively indicate that the block of acetylcholine-induced EDRF release in the coronary artery is probably not due to block of classic muscarinic receptors. It is perhaps of interest to note here that one other proposed antagonist of EDRF, quinacrine, also displays some selectivity against methacholine, in the greyhound coronary artery (Cocks & Angus, 1984). These phenomena may suggest the following: (1) there are at least two different EDRFs; (2) the transducing

mechanism for muscarinic receptors is different from other stimuli, or (3) the endothelial muscarinic receptor is distinct from either smooth muscle or cardiac muscarinic receptors.

In conclusion, we have demonstrated that various amiloride analogues bearing substituents on the 5-amino-nitrogen atom or the terminal guanidino nitrogen atom cause endothelium-dependent relaxations in the dog coronary artery possibly by blocking Na^+/Ca^{2+} exchange in endothelial cells. However, regardless of their mechanism of action it may be possible to use selective analogues clinically as antihypertensive drugs (see Haddy *et al.*, 1985) or to relieve spasm in certain arteries such as the coronary and cerebral artery.

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