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Actions of amiloride analogues on prostacyclin synthesis by rat aortic rings

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1 Fresh rat aortic rings were incubated in HEPES-buffered salt solutions (pH 8.0) in the presence or absence of amiloride analogues. The effect of these drugs on prostacyclin (PGI_2) synthesis was determined by radioimmunoassay of the stable hydrolysis product 6-oxo-prostaglandin (PG)$F_{1\alpha}$.

2 Amiloride and phenamil (potent inhibitors of epithelial Na^+ transport) had no significant effect on basal or Ca^{2+}-stimulated PGI_2 synthesis.

3 Several analogues previously reported to inhibit Na^+/Ca^{2+} exchange caused a dose-related increase in 6-oxo-$PGF_{1\alpha}$ production in media containing NaCl 120 mM and $CaCl_2$ 2.5 mM. 2',3'-Benzobenzamil was the most potent analogue with a maximum stimulation of 4.51 ± 0.89 fold, and an EC_{50} of 3×10^{-5} M.

4 Amiloride analogues bearing substituents on the 5-amino group of the pyrazine ring have been reported to inhibit Na^+/H^+ exchange more potently than Na^+/Ca^{2+} exchange. Three of these compounds inhibited Ca^{2+}-stimulated 6-oxo-$PGF_{1\alpha}$ production at concentrations that did not significantly influence basal 6-oxo-$PGF_{1\alpha}$ production.

Introduction

Prostacyclin (PGI_2) is the principal cyclo-oxygenase product of vascular tissue (Moncada & Vane, 1979). It has potent actions on platelets and vascular smooth muscle and is synthesized by blood vessels in response to trauma (Bunting *et al.*, 1976). PGI_2 hydrolyses rapidly under physiological conditior. to the inactive product 6-oxo-prostaglandin (PG) $F_{1\alpha}$ (Johnson *et al.*, 1976b). PGI_2 synthesis is susceptible to physiological or pharmacological control in which Ca^{2+} and H^+ have been implicated. PGI_2 synthesis is increased by extracellular Ca^{2+} (Whorton *et al.*, 1984; Hassid & Oudinet, 1986; van de Velde *et al.*, 1986), and by the Ca^{2+} ionophore A23187 (Weksler *et al.*, 1978). PGI_2 synthesis is inhibited by 8-(N,N-diethylamino)octyl 3,4,5-trimethoxybenzoate (TMB8) (Brotherton & Hoak, 1982; Ritter, 1984) an antagonist of intracellular Ca^{2+} mobilisation (Malagodi & Chiou, 1974). In contrast, drugs that influence voltage-dependent Ca^{2+} channels have variable actions on PGI_2 synthesis in different preparations and are often only effective at high concentration (Whorton *et al.*, 1984; Jeremy *et al.*, 1985; 1986; Hassid & Oudinet, 1986; van de Velde *et al.*, 1986; Mehta *et al.*, 1986; Ritter *et al.*, 1987). In rat aortic rings, stimulation of PGI_2 synthesis by external Ca^{2+} is pH-dependent (Taylor *et al.*, 1986; Ritter *et al.*, 1987), raising the possibility that Ca^{2+} influx and/or intracellular Ca^{2+} action is inhibited by H^+.

Amiloride (3,5-diamino-6-chloro-N-(diaminomethylene) pyrazine-carboxamide) can influence intracellular H^+ and Ca^{2+} because of weak actions on Na^+/H^+ (Johnson *et al.*, 1976a; Aickin & Thomas, 1977) and Na^+/Ca^{2+} (Schellenberg *et al.*, 1983) exchange processes which it possesses in addition to its potent action on Na^+ transport (Bentley, 1968). Structure-activity studies with analogues of amiloride in various tissues have shown that specific substitutions give rise to compounds with greater relative potency for one or other of these processes (Cuthbert & Fanelli, 1978; Vigne *et al.*, 1984; Zhuang *et al.*, 1984; Schellenberg *et al.*, 1985; Kaczorowski *et al.*, 1985; Simchowitz & Cragoe, 1986). Table 1 shows the structures of some of these compounds and indicates their rank order of potency as inhibitors of ionic transport processes, summarized from the references cited above. Substitution on the 5-amino nitrogen atom of the pyrazine ring is generally associated with loss of activity on Na^+ transport by epithelia (Cuthbert & Fanelli, 1978, Kleyman *et al.*, 1986) but increased activity against Na^+/H^+ exchange (Vigne *et al.*, 1984; Simchowitz &

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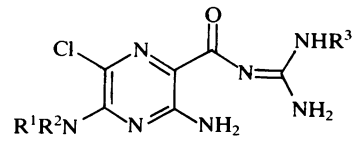
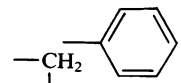
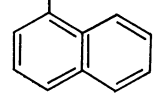
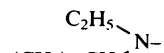
Table 1 Amiloride analogues: potencies as inhibitors of ion transport processes



Name	R^1R^2N–	R^3	Inhibitory potency*		
			Na^+	Na^+/Ca^{++}	Na^+/H^+
Amiloride	H_2N–	H	+ +	±	±
Phenamil	H_2N–		+++	±	–
Benzobenzamil	$(CH_3)_2N$–		++	+++	–
5–(N,N–dimethyl)–amiloride		H	–	±	+
5–(N–ethyl, N–iso–propyl) amiloride		H	–	+	++
5–(N,N-hexamethyl) amiloride		H	–	++	+++

* – inactive ± weakly active +, ++, +++ increasing activity

Cragoe, 1986). Certain of these 5-amino substituted compounds also inhibit Na^+/Ca^{2+} exchange, albeit generally less potently than their actions on Na^+/H^+ exchange (Kaczorowski *et al.*, 1985). Certain substituents on the terminal nitrogen atom of the guanidino moiety are associated with increased activity as inhibitors of Na^+ transport (Cuthbert & Fanelli, 1978; Kleyman *et al.*, 1986) and, in some compounds, disproportionately greater increase in inhibitory potency on Na^+/Ca^{2+} exchange (Kaczorowski *et al.*, 1985; Schellenberg *et al.*, 1985), without activity on Na^+/H^+ exchange (Simchowitz & Cragoe, 1986; 1987).

The object of the present study was to investigate the effects of these drugs on basal and Ca^{2+}-stimulated PGI_2 synthesis by fresh rat aortic rings, to determine whether amiloride-sensitive ion transport processes are implicated in the control of PGI_2 synthesis.

Methods

Aortic ring incubations

Aortic rings were prepared by methods similar to those described previously (Bunting *et al.*, 1976; Ritter *et al.*, 1987). Male CD rats (Charles River, Margate, U.K.) 200–300 g were anaesthetized with ether. The aorta was removed rapidly and rinsed with Hanks solution (Gibco, Uxbridge). It was cut into 1 mm rings with a McIlwain tissue chopper (Mickle Engineering Co. Guildford, Surrey). Rings were individually allocated to one of four groups so as to minimize differences between the groups. Each aorta yielded 4 groups of 12 rings. These were kept in Hanks solution on ice for less than 30 min before incubation. This was started by adding tissue to incubation fluid (1 ml) at 37°C and performed for 60 min with constant shaking. Incubations were terminated by removing medium which was stored at −20°C until assay for 6-oxo-$PGF_{1\alpha}$.

Concentration-effect relationships on PGI_2 synthesis were studied in incubation fluid containing (mM): NaCl 120, KCl 4, $CaCl_2$ 2.5, glucose 5 and N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) buffer 25 (pH 8.0). One group of rings from each aorta was incubated without drug (control). Each of the other 3 groups of rings was incubated with a different concentration of amiloride or one of its analogues. In experiments in which the effect of drugs

on Ca^{2+}-stimulated PGI_2 synthesis was determined, one group of aortic rings from each animal was incubated with no added $CaCl_2$ (basal), one with $CaCl_2$ 20 mM (Ca^{2+}-stimulated), one with no $CaCl_2$ but with drug (to determine if the analogue affected basal PGI_2 synthesis) and one with $CaCl_2$ 20 mM and drug (to determine if it influenced Ca^{2+}-stimulated PGI_2 synthesis). All experiments on a single drug were performed on the same batch of rats within a 2 day period. The concentrations of $CaCl_2$, KCl, glucose and HEPES were the same as in the other protocol.

Amiloride and the analogues studied were prepared specially for this study by previously published methods (Cragoe *et al.*, 1967; 1981). They were dissolved in dimethyl sulphoxide on the day of each experiment. Equal volumes of dimethyl sulphoxide were added to the control tubes. Chemicals were AnalaR grade (BDH Chemicals, Poole Dorset, U.K.).

Analysis of 6-oxo-$PGF_{1\alpha}$

6-oxo-$PGF_{1\alpha}$ was determined by radioimmunoassay, using a previously described antibody (Hensby *et al.*, 1981; Orchard *et al.*, 1982), a generous gift from Dr L. Myatt (Institute of Obstetrics, Hammersmith Hospital, London). Briefly, assays were performed in triplicate on unextracted samples, using approximately 5 nCi (160 Ci mmol^{-1}) of [^{3}H]-6-oxo-$PGF_{1\alpha}$ (Amersham International, Amersham), per tube, and a final dilution of antiserum of 1:30,000. Unbound ligand was separated with activated charcoal; 50% displacement of tritiated ligand was caused by 25.4 ± 2.3 pg (mean ± s.e.mean) of standard 6-oxo-$PGF_{1\alpha}$. Standard 6-oxo-$PGF_{1\alpha}$ was a gift from Dr John Pike (Upjohn Co., Kalamazoo, MI, USA). Samples were diluted with phosphate buffered gelatin saline so that 0.1 ml caused 20–80% displacement of [^{3}H]-6-oxo-$PGF_{1\alpha}$. Triplicate assays were performed at different dilutions. Controls were performed that showed that, in the absence of unlabelled 6-oxo-$PGF_{1\alpha}$, relevant concentrations of the drugs and solvents did not affect the binding of [^{3}H]-6-oxo-$PGF_{1\alpha}$ to antibody.

Analysis

Results are shown as mean ± s.e.mean, $n = 8$ unless stated otherwise. Comparisons were made by Student's paired t test on untransformed data and considered significant when $2P < 0.05$.

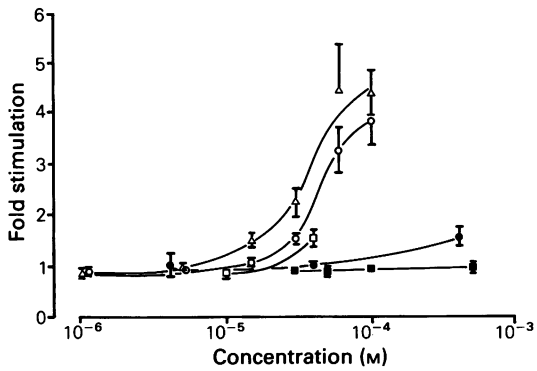


Figure 1 Concentration-response curves for phenamil (■), 5-(N,N-dimethyl)amiloride (●), 5-(N-ethyl-N-isopropyl)amiloride (□), 5-(N,N-hexamethylene) amiloride (○) and 2'3'-benzobenzamil (△) on prostacyclin (PGI_2) synthesis by rat aorta. Responses ('fold stimulation') expressed as ratios of 6-oxo-$PGF_{1\alpha}$ production in the presence of drug to that in its absence in matched incubations (mean values are shown; s.e.mean indicated by vertical lines; $n = 8$ at each point). Concentrations are plotted on a logarithmic scale.

Table 2 Effect of amiloride analogues on basal and Ca^{2+}-stimulated prostacyclin (PGI_2) synthesis by rat aortic rings

Drug (M)	*6-oxo-$PGF_{1\alpha}$* (ng mg^{-1} h^{-1}))			
Amiloride (10^{-3})	11.3 ± 1.6	12.4 ± 2.1	21.0 ± 2.8	18.7 ± 3.0
Phenamil (3×10^{-5})	10.5 ± 0.7	10.5 ± 1.0	25.6 ± 1.5	25.0 ± 1.2
2'3'-Benzobenzamil (1.5×10^{-5})	5.4 ± 0.9	8.3 ± 1.1	21.4 ± 2.6	26.1 ± 2.7
5-(N,N-dimethyl)amiloride (4×10^{-5})	23.9 ± 1.9	24.9 ± 1.5	41.7 ± 2.1	34.9 ± 2.1***
5-(N-ethyl-N-isopropyl)amiloride (10^{-5})	21.0 ± 1.4	18.1 ± 1.6	38.0 ± 2.0	30.8 ± 1.1*
5-(N,N-hexamethylene)amiloride (10^{-6})	19.7 ± 1.6	17.9 ± 1.2	75.1 ± 5.4	56.1 ± 4.8**
Ca^{2+}	0	0	+	+
Amiloride analogue	0	+	0	+

Prostacyclin production was measured as 6-oxo-$PGF_{1\alpha}$ in the presence (+) and absence (0) of amiloride analogues under basal (0) and 20 mM Ca^{2+}-stimulated (+) conditions ($n = 8$ for each analogue). The 5-N-substitued analogues each significantly inhibited Ca^{2+}-stimulated 6-oxo-$PGF_{1\alpha}$ production.
*$2P < 0.05$; **$2P < 0.025$, ***$2P < 0.005$.

Results

Fresh aortic rings were incubated in balanced salt solutions in the presence or absence of amiloride or its analogues to determine their effects on PGI_2 production as described in the methods. Neither amiloride (10^{-3} M) nor phenamil (3×10^{-5}–5×10^{-4} M) influenced 6-oxo-$PGF_{1\alpha}$ production, while the other analogues increased it in a dose-related manner (Figure 1). The order of potency was 2',3'-benzobenzamil > 5-(N,N-hexamethylene)amiloride > 5-(N-ethyl-N-isopropyl)amiloride > 5-(N,N-dimethyl)amiloride. 2',3'-Benzobenzamil caused the greatest stimulation ($4.51 \pm 0.89 \times$ control, $n = 8$), with the half-maximal effect occurring at approximately 3×10^{-5} M. 5-(N,N-hexamethylene)amiloride was only slightly less potent than 2',3'-benzobenzamil. 5-(N-ethyl-N-isopropyl)amiloride and 5-(N,N-dimethyl)amiloride also caused significant stimulation ($2P < 0.01$, for each drug) at the highest concentrations studied (4×10^{-5} M and 4×10^{-4} M respectively).

To determine the effect of amiloride analogues on Ca^{2+}-stimulated PGI_2 synthesis, concentrations were selected that did not significantly increase basal 6-oxo-$PGF_{1\alpha}$ production. The results are shown in Table 2. Ca^{2+} (20 mM) stimulated 6-oxo-$PGF_{1\alpha}$ production ($2P < 0.005$, $n = 8$ in each of the 6 experiments). At the doses used, none of the drugs significantly influenced basal 6-oxo-$PGF_{1\alpha}$ ($2P > 0.05$), but the 5-N substituted analogues each significantly inhibited Ca^{2+}-stimulated 6-oxo-$PGF_{1\alpha}$ ($2P < 0.05$–0.005: Table 2). Amiloride and its analogues bearing substituents on the guanidino moiety, i.e. 2',3'-benzobenzamil and phenamil, did not significantly alter Ca^{2+}-stimulated 6-oxo-$PGF_{1\alpha}$ ($2P > 0.05$).

Discussion

The most striking finding of this study is the dose-dependent stimulation of 6-oxo-$PGF_{1\alpha}$ production by some, but not all, amiloride analogues (Figure 1). It is noteworthy that the drugs that caused stimulation are those previously found to inhibit Na^+/Ca^{2+} exchange in other systems (Schellenberg *et al.*, 1983; 1985; Kaczorowski *et al.*, 1985). In contrast, drugs with relative selectivity for epithelial Na^+ transport (amiloride and phenamil) were inactive. It is thus possible that Na^+/Ca^{2+} exchange in freshly prepared aortic rings in the conditions of the present experiments maintains a low intracellular Ca^{2+} concentration. When Na^+/Ca^{2+} exchange is inhibited, intracellular Ca^{2+} may therefore increase, stimulating PGI_2 synthesis. It is not possible to test this directly in this preparation and stimulation of 6-oxo-$PGF_{1\alpha}$ production by these drugs could be due to some quite different pharmacological action. The rank order of potency of the analogues studied is, however, consistent with an action mediated by inhibition of Na^+/Ca^{2+} exchange, and it may be possible to test this directly in cultured cells using a fluorescent indicator of intracellular Ca^{2+} (cf. Hallam, 1986).

The second finding of note is that while high concentrations of 5-N substituted analogues stimulated 6-oxo-$PGF_{1\alpha}$ production (Figure 1), lower concentrations which had no effect on basal 6-oxo-$PGF_{1\alpha}$ significantly inhibited Ca^{2+}-stimulated 6-oxo-$PGF_{1\alpha}$ (Table 2). As noted previously (Ritter *et al.*, 1982) there was considerable variation of 6-oxo-$PGF_{1\alpha}$ production by aortic rings obtained from different animals but consistent effects were observed within groups of rings obtained from a single animal. The variation between animals was minimized by performing all experiments with each drug within 2 days. This is reflected in standard errors of 5–17% of the respective means (Table 2), despite substantial differences between the control means in the different experiments which were performed several weeks to months apart. Amiloride, phenamil and 2',3'-benzobenzamil did not share the inhibitory action of the 5-N-substituted analogues, so it is possible that this effect relates to inhibition of Na^+/H^+ exchange, though again we cannot rule out the possibility of another underlying pharmacological action. Inhibition of Na^+/H^+ exchange resulting in intracellular acidification could however account for the findings if H^+ inhibits the intracellular action of Ca^{2+} on PGI_2 synthesis as it inhibits cellular Ca^{2+} entry (Ritter *et al.*, 1987). Such an intracellular interaction of Ca^{2+} with H^+ has been inferred from studies on human platelets (Sweatt *et al.*, 1986a,b; Siffert & Akkerman, 1987).

The observations that under different conditions 5-amino substituted amiloride analogues may either stimulate (Figure 1) or inhibit (Table 2) PGI_2 synthesis by rat aorta, limits the usefulness of the preparation. If the tissue contains both Na^+/Ca^{2+} and Na^+/H^+ exchangers, then the effects of altering Na^+ will be complex and unpredictable: lowering external Na^+ may cause stimulation of PGI_2 synthesis by reducing Ca^{2+} efflux, but inhibition of PGI_2 synthesis by reducing H^+ efflux. Indeed we have found that substitution of Na^+ by choline causes only small changes in PGI_2 synthesis (Cockcroft, Aksoy & Ritter: unpublished observations). Similarly, drugs like amiloride itself, with weak actions on both Na^+/H^+ and Na^+/Ca^{2+} exchangers, have little effect on 6-oxo-$PGF_{1\alpha}$ production.

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