Effects of amiloride analogues on the production of prostacyclin by aortic endothelial cells

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- 1 The release of prostacyclin (PGI_2) from bovine aortic endothelial cells stimulated by adenosine 5'-triphosphate (ATP) was decreased by amiloride analogues bearing alkyl groups on the 5-amino nitrogen atom, like 5-(N-ethyl-N-isopropyl)amiloride (EIPA), which are inhibitors of the Na^+/H^+ exchanger. Analogues substituted on a terminal guanidino nitrogen atom were not inhibitory.
- 2 The release of PGI_2 induced by ATP was not significantly depressed in a Na^+ -poor medium or in a medium acidified to pH 6.9, two conditions known to inhibit the Na^+/H^+ exchanger.
- 3 Cytoplasmic alkalization by ammonium chloride did not suppress the inhibitory action of EIPA. By itself, ammonium chloride decreased the response of endothelial cells to ionophore A23187 and ATP, whereas sodium acetate had no effect.
- 4 EIPA did not decrease the mobilization of free arachidonic acid induced by ATP. It inhibited the conversion of exogenous arachidonate into PGI_2 and prostaglandin E_2 (PGE_2).
- 5 Although the intracellular pH was not measured in this study, it seems unlikely that cytoplasmic alkalization via the activation of the Na^+/H^+ exchanger plays a significant role in the stimulatory action of ATP on the release of PGI_2 from endothelial cells. The inhibition of that release by EIPA and other amiloride analogues might involve a direct effect on cyclo-oxygenase, although an action on the reacylation of free arachidonic acid cannot be excluded.

Introduction

The cytoplasmic pH, which is important for many cellular processes, is tightly controlled (Madhus, 1988). In mammalian cells, this control involves two separate mechanisms, the relative importance of which differs from one cell type to the other: an amiloride-sensitive Na^+/H^+ exchanger and a Na^+ -dependent $\text{Cl}^-/\text{HCO}_3^-$ exchanger inhibited by stilbene disulphonic acids (L'Allemain *et al.*, 1985; Jentsch *et al.*, 1985). Cell proliferation is the process whose dependency on cytoplasmic pH has been most studied in recent years. Optimal cell growth occurs at alkaline internal pH and several mitogens induce a cytoplasmic alkalization, via the activation of the Na^+/H^+ exchanger (Moolenaar *et al.*, 1983; Pouyssegur *et al.*, 1984; Lucas *et al.*, 1988; Perona & Serrano, 1988). In contrast, it was recently suggested that cytosolic acidification might constitute an early transducing signal in neutrophil chemotaxis (Yuli & Oplatka, 1987) and in inhibitory synaptic transmission (Kaila & Voipio, 1987).

The role of cytoplasmic pH in the control of arachidonic acid metabolism has been thoroughly investigated in human platelets. It was shown that various

procedures which inhibit the Na^+/H^+ exchanger decrease the mobilization of free arachidonic acid and the synthesis of thromboxane in response to adenosine 5'-diphosphate (ADP), adrenaline, low concentrations of thrombin and platelet-activating factor (Sweatt *et al.*, 1985; 1986; 1987). This suggests that changes in cytoplasmic pH and free calcium concentration might regulate phospholipase A_2 activity in a synergistic way.

Prostacyclin (PGI_2) is a potent inhibitor of platelet aggregation and *in vivo* its biosynthesis is increased in disease states involving the intravascular activation of platelets. This reflects a local compensatory response by the vascular endothelium (FitzGerald *et al.*, 1984; 1986). *In vitro*, the endothelial production of PGI_2 is indeed stimulated by factors released from activated platelets e.g. prostaglandin endoperoxides (Marcus *et al.*, 1980; Schafer *et al.*, 1984; Chesterman *et al.*, 1986) and adenine nucleotides, which act via P_{2y} -purinoceptors (Boeynaems & Galand, 1983; Pearson *et al.*, 1983; Van Coevorden & Boeynaems, 1984; Needham *et al.*, 1987). The biochemical events that mediate the

action of adenine nucleotides on aortic endothelial cells have been recently characterized as involving generation of inositol trisphosphate, increase of cytosolic calcium and activation of calcium-dependent protein kinases (Pirrotton *et al.*, 1987; Hallam & Pearson, 1986; Demolle *et al.*, 1988). In addition, Kitazono *et al.* (1988) have shown that adenosine 5'-triphosphate (ATP) and ADP induce a transient acidification followed by a sustained alkalization, due to Na^+/H^+ exchanger activation, in bovine aortic endothelial cells.

The purpose of the present study was to investigate the possible role of cytoplasmic pH changes in the control by ATP of PGI_2 release from bovine aortic endothelial cells. Analogues of amiloride, some of which are specific inhibitors of the Na^+/N^+ exchanger (Vigne *et al.*, 1984; Simchowicz & Cragoe, 1986), were the main tools used in this study.

Methods

Preparation, culture and incubation of endothelial cells

Bovine aortic endothelial cells were obtained by collagenase digestion of the aorta excised from a freshly slaughtered cow, as previously described (Booyse *et al.*, 1975; Van Coevorden & Boeynaems, 1984). The cells were seeded on 100 mm Petri dishes and incubated at 37°C under an atmosphere of 5% CO_2 /95% air in the following medium: MEM D-valine (80%, v/v), foetal calf serum (20%, v/v), 2 mM glutamine, 100 u ml^{-1} penicillin, $100 \mu\text{g ml}^{-1}$ streptomycin, $2.5 \mu\text{g ml}^{-1}$ amphotericin B. The medium was changed the following day and then every 3 days. After 4 or 5 days, the primary cultures formed confluent monolayers and could be sub-cultured. The cells were detached by a 5 min incubation in a Ca^{2+} - and Mg^{2+} -free Hanks buffer containing trypsin (0.1 mg ml^{-1}) and EDTA (1 mM). Thereafter, they were seeded on 35 mm Petri dishes and the culture was continued in the following medium: DMEM (60%, v/v), Ham's F-12 (20%, v/v), foetal calf serum (20%, v/v) with the same concentrations of penicillin, streptomycin, and amphotericin B as mentioned above.

Experiments were performed with confluent monolayers ($\pm 10^6$ cells per dish), between passage 2 and 5. The culture medium was removed and, after rinsing, the cells were incubated in 1 ml medium containing the tested agents, in general for 20 min. Amiloride analogues were dissolved in dimethyl sulphoxide (DMSO) and added to the cells 5 min before ATP and other stimuli. Control incubations contained the same amount of DMSO (0.5%, v/v). Incubation media of various compositions were used in this study: Dulbecco's MEM (DMEM); Krebs-

Ringer medium buffered with HEPES (composition, mM, NaCl 124, KCl 5, MgSO_4 1.25, CaCl_2 1.45, KH_2PO_4 1.25, HEPES buffer 25, pH 7.4 and 8 mM glucose), Krebs-Ringer medium as above, except that sodium chloride was replaced by an equal concentration of choline chloride.

Prostaglandin radioimmunoassay (RIA)

The production of PGI_2 was measured by the RIA of its stable degradation product, prostaglandin 6-keto- $\text{F}_{1\alpha}$ (6-K-PGF $_{1\alpha}$), performed directly in the incubation medium, without extraction and chromatography. A rabbit antiserum was raised against 6-K-PGF $_{1\alpha}$ coupled to BSA, as described by MacLough (1981). The limit of detection was 16 pg and the cross-reactivity was 1.2% with PGF $_{2\alpha}$, 0.3% with PGE $_2$ and <0.1% with thromboxane B $_2$. Aliquots (100 μl) of incubation media, [^3H]-6-K-PGF $_{1\alpha}$ (11,000 d.p.m.), anti-6-K-PGF $_{1\alpha}$ antiserum (final dilution 1:10,000) and bovine gamma globulins (2.5 mg ml^{-1}) in Tris buffer (50 mM, pH 7.4) were incubated in a total volume of 0.4 ml for 60 min at room temperature. Then 0.4 ml of a cold 25% (w/w) solution of polyethylene glycol was added to separate bound and free antigen. The various amiloride analogues tested did not interfere with the RIA of 6-K-PGF $_{1\alpha}$.

Assay of free arachidonic acid release

Arachidonic acid was measured by gas liquid chromatography (g.l.c.) with electron capture detection (ECD), as described previously (Boeynaems *et al.*, 1985). Incubations were performed in a medium containing BSA (1 mg ml^{-1}) and indomethacin ($1 \mu\text{g ml}^{-1}$). After the addition of $1 \mu\text{g}$ docosahexaenoic acid as an internal standard, the incubation medium was extracted with 1 volume of ethyl acetate. The free fatty acids were converted into pentafluorobenzyl esters by a modification of the method of Wickramasinghe *et al.* (1973). To the dry residue of the extract were added 290 μg pentafluorobenzylbromide (in 5 μl acetonitrile) and 44 μg diisopropylethylamine (in 5 μl acetonitrile). After 5 min at 40°C and evaporation of the solvent under nitrogen, the samples were redissolved in 50 μl hexane. The 20 fold reduction in the amounts of reagents used as compared with the original procedure made it possible to analyze the samples directly without prior purification. Analysis was performed in a Varian model 3700 chromatograph (Varian Associates, Palo Alto, CA, U.S.A.) equipped with a ^{63}Ni ECD and a 2 m column of 3% OV-1 on Gas chrom Q (Applied Science Laboratories) operated isothermally at 235°C.

Figure 1 Effects of amiloride analogues, substituted by alkyl groups on the 5-amino nitrogen atom, on the release of prostacyclin (PGI₂) from bovine aortic endothelial cells stimulated by ATP. Incubation was performed in DMEM: the drugs were added to the cells 5 min prior to ATP (10 μM). Results represent the amount of 6-K-PGF_{1α} accumulated in the medium at the end of a 20 min incubation (mean ± s.d. (vertical bars) of triplicate determinations in 1 representative experiment out of 3 (a) or 2 (b)). (a) Hatched columns, control; open columns, ATP (10 μM). (b) (○) Control; (●) ATP (10 μM) + 5-(N-ethyl-N-isopropyl)amiloride (EIPA); (Δ) ATP (10 μM) + 5-(N,N-dimethyl)amiloride (DMA).

Metabolism of radiolabelled arachidonic acid

Confluent monolayers of bovine aortic endothelial cells were incubated in DMEM, with or without 5-(N-ethyl-N-isopropyl)-amiloride (50 μM). After

Table 1 Effect of 5-(N-ethyl-N-isopropyl)amiloride (EIPA) on the release of prostacyclin (PGI₂) from bovine aortic endothelial cells: comparison between ATP and ionophore A23187

	6-K-PGF _{1α} (ng per dish)	
—	0.5 ± 0.2	0.6 ± 0.3
ATP (10 μM)	3.3 ± 0.6	0.7 ± 0.2
A23187 (0.1 μM)	9.3 ± 2.1	3.1 ± 0.6
	—	EIPA (50 μM)

The cells were incubated for 20 min in DMEM containing the tested agents. EIPA was added 5 min before ATP or A23187. Results represent the amount of 6-K-PGF_{1α} accumulated in the medium at the end of the incubation (mean ± s.d. of triplicate determinations in 1 representative experiment out of 3).

5 min, [³H]-arachidonic acid (0.8 μCi ml⁻¹; 21 ng ml⁻¹) was added to all dishes and the incubation stopped after 60 min. The media were collected and 200 μl aliquots were directly analyzed by reversed-phase high performance liquid chromatography (r.p.h.p.l.c.) on a C₁₈ μBondapak column (3.9 × 300 mm, 10 μm particles; Waters Associates, Millipore Corp., Milford, MA, U.S.A.). The injector (model U6K) and the pump (model 600) were from Waters Associates. Elution was performed sequentially with acetonitrile-water-acetic acid (26:74:0.1, v/v/v; 40 min) and methanol (15 min) at a flow rate of 2 ml min⁻¹. Fractions of 2 ml volume were collected for liquid scintillation counting.

Materials

All the amiloride analogues tested were prepared as previously published (Cragoe *et al.*, 1967; Cragoe & Woltersdorf, 1978).

DMEM, Ham's F-12, MEM-D-val, foetal calf serum, penicillin, streptomycin, amphotericin B and glutamine were from Gibco. Collagenase type I was from Cooper (Worthington). Trypsin was purchased from Flow Laboratories. Arachidonic acid and ATP were obtained from Sigma Chem. Co., ionophore A23187 from Boehringer-Mannheim, BSA (fatty acid-poor) from Calbiochem-Behring and 6-K-PGF_{1α} from Upjohn Diagnostics. [³H]-arachidonic acid and [³H]-6-K-PGF_{1α} were purchased from Amersham Corp.

Results

The presence of 50 μM 5-(N-ethyl-N-isopropyl)amiloride (EIPA) had no significant effect on the basal

Table 2 Effects of amiloride analogues, substituted by alkyl groups on the 5-amino nitrogen atom, on the release of prostacyclin (PGI₂) from bovine aortic endothelial cells stimulated by bradykinin

Drugs	6-K-PGF _{1α} (ng per dish)	
	0.2 ± 0.1	3.6 ± 0.4
EIPA (10 μM)		1.7 ± 0.6
EIPA (50 μM)		0.3 ± 0.1
MIBA (10 μM)		2.0 ± 0.2
MIBA (50 μM)		0.3 ± 0.1
HMA (10 μM)		1.0 ± 0.4
HMA (50 μM)		0.2 ± 0.1
	—	Bradykinin (0.1 μM)

Incubation was performed in DMEM, the drugs being added to the cells 5 min before bradykinin (0.1 μM). Results represent the amount of 6-K-PGF_{1α} accumulated in the medium at the end of a 20 min incubation (mean ± s.d. of triplicate determinations in 1 representative experiment out of 3).

EIPA = 5-(N-ethyl-N-isopropyl)amiloride;

MIBA = 5-(N-methyl-N-isobutyl)amiloride;

HMA = 5-(N,N-hexamethylene)amiloride.

release of PGI₂ from bovine aortic endothelial cells, with release of 103 ± 13% of the control (mean ± s.e.mean, *n* = 13). However, EIPA inhibited the stimulation of that release by ATP, in a concentration-dependent way (Figure 1). At 10 μM, EIPA produced a partial inhibition in some but not all experiments; at 50 μM, the release of PGI₂ in response to ATP (10 μM) was decreased to 19 ± 5% of the control value (mean ± s.e.mean, *n* = 12).

Likewise, the stimulatory effect of ionophore A23187 (0.1 μM) was decreased to a mean 39% of the control value (*n* = 3) by EIPA (50 μM) (Table 1). 5-(N-methyl-N-isobutyl)amiloride (MIBA) and 5-(N,N-hexamethylene)amiloride (HMA) produced the same degree of inhibition as EIPA (Figure 1a). These various compounds also inhibited the release of PGI₂ induced by bradykinin (Table 2). The ATP effect was only partially decreased by 5-(N,N-dimethyl)amiloride (DMA) at a concentration as high as 500 μM (Figure 1b), whereas amiloride itself did not produce a significant inhibition at 1 mM (116% of the control, mean of 3 experiments). 5-N-(4-chlorobenzyl)-2',4'-dimethylbenzamil (CBDMB) slightly increased the basal release of PGI₂ and did not inhibit the response to ATP (Figure 2). 2',4'-Dimethylbenzamil (DMB) itself increased the production of PGI₂, but this effect was not additive to that of ATP (Figure 2).

Replacement of sodium chloride by choline chloride in the incubation medium slightly decreased the release of PGI₂ induced by ATP (33% inhibition at

Figure 2 Effects of amiloride analogues on the release of prostacyclin (PGI₂) from bovine aortic endothelial cells and its stimulation by ATP: comparison between 5-(N-ethyl-N-isopropyl)amiloride (EIPA) and compounds bearing substituents on a terminal guanidino nitrogen. Incubation was performed in DMEM and the drugs were added to the cells 5 min before ATP (10 μM). Results represent the amount of 6-K-PGF_{1α} accumulated in the medium at the end of a 20 min incubation (mean ± s.d. (vertical bars) of triplicate determinations in 1 representative experiment out of 4): control; hatched columns, EIPA (50 μM); stippled columns, 5-N-(4-chlorobenzyl)-2',4'-dimethylbenzamil (CBDMB) (50 μM); open columns, 2',4'-dimethylbenzamil (DMB) (50 μM).

10 μM ATP, *n* = 4). In a medium acidified to pH 6.9, the stimulatory effect of ATP (10 or 100 μM) was not decreased (118% of the control, *n* = 3), whereas the PGI₂ response to a low concentration of ATP (1 μM) was partially inhibited (77% of the control, *n* = 3) (Figure 3).

The inhibitory action of EIPA was not suppressed by the addition to the medium of ammonium chloride, which induces a cytoplasmic alkalinization (Figure 4). The action of EIPA at low concentration was not potentiated by incubating the endothelial cells in a Krebs-Ringer medium buffered with HEPES and containing no bicarbonate, nor by the substitution of choline chloride for sodium chloride in such medium (data not shown).

Figure 3 Effects of experimental conditions known to inhibit the activity of the Na⁺/H⁺ exchanger on the release of prostacyclin (PGI₂) from bovine aortic endothelial cells stimulated by ATP. The cells were incubated for 20 min in a medium containing various concentrations of ATP. This medium was either normal Krebs-Ringer-HEPES, or contained choline chloride instead of sodium chloride (a) or DMEM, normal or acidified to pH 6.9 by the addition of hydrochloric acid (b). Results represent the amount of 6-K-PGF_{1α} accumulated in the medium at the end of the incubation (mean ± s.d. (vertical bars) of triplicate determinations in 1 representative experiment out of 4 (a) or 3(b)). (a) (●) Normal medium; (○) Na⁺-poor medium; (b) (●) normal medium; (○) medium acidified to pH 6.9.

Figure 4 Inhibition by 5-(N-ethyl-N-isopropyl)amiloride (EIPA) of prostacyclin (PGI₂) release from bovine aortic endothelial cells stimulated by ATP: insensitivity to cytoplasmic alkalization by ammonium chloride. The cells were incubated for 20 min in DMEM containing various concentrations of ammonium chloride. EIPA (50 μM) was added 5 min before ATP (10 μM). Results represent the amount of 6-K-PGF_{1α} accumulated in the medium at the end of the incubation (mean ± s.d. (vertical bars) of triplicate determinations in one representative experiment out of 2). Open columns, control no NH₄Cl; hatched columns, NH₄Cl 5 mM; stippled columns, NH₄Cl 10 mM.

Cytoplasmic alkalization by ammonium chloride partially inhibited the release of PGI₂ in response to ATP (63 ± 15% of the control, mean ± s.d. of 3 experiments: Figure 4) or ionophore A23187 (49 ± 12%, of the control, mean ± s.d. of 5 experiments: Figure 5). Acidification with sodium acetate had no significant effect on the response to either agonist (Figure 5).

In contrast to its inhibitory effect on the release of PGI₂, EIPA (50 μM) did not decrease the mobilization of free arachidonic acid induced by ATP, but had a slight potentiating effect (169% ± 27% of the control value, mean ± s.e. mean of 6 experiments: Figure 6). EIPA decreased the conversion of exogenous arachidonic acid into PGI₂ and PGE₂, evaluated either by RIA or by h.p.l.c. analysis of the

Figure 5 Modulation by cytoplasmic alkalinization or acidification of prostacyclin (PGI_2) release from bovine aortic endothelial cells stimulated by ionophore A23187. The cells were incubated in DMEM, containing either ammonium chloride (solid columns), sodium acetate (stippled columns) or additional sodium chloride (hatched columns) as control (20 mM for each). Results represent the amount of 6-K-PGF $_{1\alpha}$ accumulated in the medium at the end of a 20 min incubation (mean $\pm$ s.d. (vertical bars) of triplicate determinations in one representative experiment out of 5).

incubation fluid, following the addition of radio-labelled substrate (Tables 3 and 4).

Discussion

The release of PGI_2 from bovine aortic endothelial cells stimulated by ATP was inhibited by amiloride analogues substituted by alkyl groups on the 5-amino nitrogen atom, which are well-known blockers of the Na^+/H^+ exchanger (Vigne *et al.*, 1984; Simchowicz & Cragoe, 1986). This inhibition was not mimicked by analogues bearing substituents on a terminal guanidino nitrogen atom, which are inactive on the Na^+/H^+ exchanger, but inhibit the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (Kaczorowski *et al.*, 1985). DMB itself actually induced PGI_2 release.

Although the concentrations of amiloride analogues required to inhibit PGI_2 release were higher than those needed to inhibit the Na^+/H^+ exchanger in the bovine aortic endothelial cells (IC_{50} of HMA was less than $1 \mu\text{M}$ according to Kitazono *et al.*, 1988), this specificity would be consistent with a role of the Na^+/H^+ exchanger and of cytoplasmic alkalinization in the action of ATP on endothelial PGI_2

Figure 6 Comparison between the effects of 5-(N-ethyl-N-isopropyl)amiloride (EIPA) on prostacyclin (PGI_2) release and arachidonic acid mobilization in bovine aortic endothelial cells stimulated by ATP. EIPA ($50 \mu\text{M}$) was added 5 min before ATP ($50 \mu\text{M}$): the incubations were performed in Krebs-Ringer medium buffered with HEPES. Results represent the amounts of 6-K-PGF $_{1\alpha}$ or arachidonate (20:4) accumulated in the medium at the end of a 20 min incubation (mean $\pm$ s.d. (vertical bars) of triplicate determinations in one representative experiment out of 6).

production. However several other arguments do not support this hypothesis. The ATP-induced release of PGI_2 was minimally affected by two other procedures classically used to inhibit the Na^+/H^+ exchanger: reduction of the Na^+ concentration or

Table 3 Effect of 5-(N-ethyl-N-isopropyl)amiloride (EIPA) on the conversion of exogenous arachidonic acid into prostacyclin (PGI_2) by bovine aortic endothelial cells

Arachidonic acid ($\mu\text{g ml}^{-1}$)	6-K-PGF $_{1\alpha}$ (ng per dish)	
0	1.0 ± 0.2	0.5 ± 0.2
0.3	2.7 ± 0.6	1.3 ± 0.4
1.0	5.2 ± 1.1	2.9 ± 0.5
	—	EIPA ($50 \mu\text{M}$)

The cells were incubated for 20 min in DMEM containing different concentrations of arachidonic acid and EIPA; EIPA was added 5 min before arachidonic acid. Results represent the amount of 6-K-PGF $_{1\alpha}$ accumulated in the medium at the end of the incubation (mean $\pm$ s.d. of triplicate determinations in 1 representative experiment out of 4).

Table 4 Effect of 5-(N-ethyl-N-isopropyl)amiloride (EIPA) on the transformation of radiolabelled arachidonic acid by bovine aortic endothelial cells

	$^3\text{H} \times 10^{-3}$ (c.p.m.)	
6-K-PGF _{1α}	32 ± 1	8 ± 1
PGE ₂	33 ± 9	17 ± 1
Arachidonate	663 ± 103	790 ± 6
	—	EIPA (50 μM)

The cells were incubated in DMEM with or without EIPA, [^3H]-arachidonic acid ($0.8 \mu\text{Ci ml}^{-1}$) was added 5 min later and the incubation was stopped after 60 min. The incubation media were analyzed by h.p.l.c. as described in Methods. Results represent the total radioactivity in the fractions where 6-K-PGF_{1α}, PGE₂ and arachidonate were eluted (mean ± s.d. of triplicate determinations in 1 representative experiment out of 2).

the pH of the incubation medium. The inhibition by EIPA was not suppressed by ammonium chloride, which increases the cytoplasmic pH in bovine aortic endothelial cells (Kitazono *et al.*, 1988), independently from the Na^+/H^+ exchanger activity. The potency of EIPA was not increased in a medium with a reduced concentration of Na^+ , which is known to compete with amiloride analogues for binding to the Na^+/H^+ exchanger. Kitazono *et al.* (1988) have shown that the action of ATP on the cytoplasmic pH is biphasic, with an initial acidification followed after 3 min by a lasting alkalinization. On the other hand, ATP induces a transient burst of PGI₂ release which lasts less than 5 min (Pearson *et al.*, 1983; Van Coevorden & Boeynaems, 1984); this release being coincident with the acidification phase and not with the alkalinization. Finally, we have observed that EIPA does not inhibit the mobilization of free arachidonic acid in ATP-stimulated endothelial cells and that it decreases the conversion of exogenous arachidonic acid into PGI₂ and PGE₂. These data suggest that EIPA might have a direct inhibitory effect on cyclo-oxygenase, although an effect on arachidonate reacylation into phospholipids cannot be excluded.

It is known that besides its three main targets, the epithelial Na^+ channel, the Na^+/H^+ exchanger and the $\text{Na}^+/\text{Ca}^{2+}$ exchanger, amiloride inhibits protein synthesis (Lubin *et al.*, 1982), protein kinase C (Besterman *et al.*, 1985), the (Na^+ , K^+) ATPase

(Soltoff & Mandel, 1982) and the T calcium channel (Tang *et al.*, 1988). The inhibition of endothelial PGI₂ observed in this study might thus correspond to an additional side-effect of this class of drugs.

Sweatt *et al.* (1985, 1986, 1987) have shown that EIPA and related amiloride analogues, as well as the reduction of extracellular Na^+ concentration or pH, inhibit the activation of a phospholipase A₂ and the synthesis of thromboxane B₂, induced in human platelets by ADP, adrenaline, platelet-activating factor and low concentrations of thrombin. Dieter *et al.* (1987) made similar observations in rat Kupffer cells stimulated by zymosan. Sweatt *et al.* (1986) proposed that changes in cytoplasmic pH and free calcium concentration might produce a synergistic activation of phospholipase A₂. Our results indicate that such a synergism does not operate in aortic endothelial cells.

Results described in this study are consistent with those of Ritter *et al.* (1987) who showed that amiloride analogues substituted on the 5-amino nitrogen inhibit the calcium-induced release of PGI₂ from rat aortic rings, while analogues bearing substituents on the terminal nitrogen atom of the guanidino moiety were able to stimulate the basal production of PGI₂. Since the releases of PGI₂ and endothelial-dependent relaxing factor (EDRF) are often coupled and are both calcium-dependent, a stimulatory effect of this class of analogues on EDRF might be expected. Cocks *et al.* (1988) recently detected such a stimulation in canine coronary arteries. It is difficult to reconcile these last data with the observation that the same analogues inhibit the release of EDRF induced by acetylcholine or ionophore A23187 in the rat and rabbit aorta (Winqvist *et al.*, 1985; Schoeffter & Miller, 1986).

In conclusion, cytoplasmic alkalinization via Na^+/H^+ exchanger activation does not appear to play a significant role in the stimulatory action of ATP on the release of PGI₂ from endothelial cells. The inhibition of that release by EIPA and other amiloride analogues might involve a direct effect on cyclo-oxygenase.

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