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EFFECT OF AMILORIDE ON REGULATORY MECHANISMS OF VASCULAR SMOOTH MUSCLE CONTRACTION

MEETA CHATTERJEE,* PETER J. S. CHIU, RONALD J. DOLL and EDMUND J. SYBERTZ
Departments of Pharmacology and Chemistry, Schering-Plough Corp., Pharmaceutical Research Division, Bloomfield, NJ, U.S.A.

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Abstract—Experiments were conducted to characterize the effects of amiloride on the regulatory mechanisms of vascular smooth muscle contraction. Intact, saponin-skinned and A23187-treated strips of rabbit aorta were used for these studies. Amiloride significantly ($P < 0.05$) reduced the norepinephrine bitartrate (NE)-stimulated increase in intracellular Ca^{2+} in intact arteries. In saponin-skinned arteries, amiloride depressed both stress and concomitant levels of myosin light chain phosphorylation. This inhibition of stress appeared to be competitive with MgATP. In A23187-treated preparations, where the effects of amiloride were studied at physiological [MgATP] in the absence of functional membrane Ca^{2+}-channels, amiloride caused a reduction in both stress and myosin light chain phosphorylation. In other experiments on intact arteries, the contractile response to phorbol 12,13-dibutyrate, an activator of protein kinase C, was reduced by amiloride. We conclude that the vasorelaxant effects of amiloride are mediated via inhibition of myosin light chain kinase and protein kinase C, in addition to the inhibition of Ca^{2+} influx.

Amiloride, 3,5-diamino-*N*-(aminoiminomethyl)-6-chloropyrazinecarboxamide, is a K^+ sparing diuretic that inhibits Na^+ transport by the cells of the cortical collecting duct [1] and several nonrenal epithelia [2]. It also inhibits Na^+/H^+ exchange, Na^+/Ca^{2+} exchange and (Na^+-K^+) ATPase in various other tissues [3, 4]. Moreover, it has been shown to inhibit cAMP-protein kinase [5, 6], protein kinase C [7], and epidermal growth factor tyrosine kinase [8].

The crucial role of myosin light chain (MLC) kinase activation in smooth muscle contraction has been shown in different muscle types with several agonists [9]. Stress development in response to norepinephrine, histamine, high K^+ and electrical stimulation is a consequence of an increase in intracellular Ca^{2+} and subsequent phosphorylation of MLC by the activated MLC kinase. Recent evidence suggests that protein kinase C may also be involved in smooth muscle contraction [10–12]. Amiloride has been shown to relax K^+ depolarized and phenylephrine contracted arterial tissue [13–15]. The effects were interpreted to be due to α-adrenergic receptor antagonism and inhibition of Ca^{2+} influx. The objectives of the present study were to determine whether the vasorelaxant effects of amiloride were due to an inhibition of mechanisms beyond those associated with the plasma membrane, i.e. inhibition of intracellular regulatory mechanisms. In view of the inhibitory action that amiloride has on various membrane pumps, some of these studies were performed in vascular preparations in which membrane Ca^{2+} pumps were rendered non-functional by means of detergent or Ca^{2+} ionophore.

* Correspondence: Dr. Meeta Chatterjee, Schering Corporation, 60 Orange St., Bloomfield, NJ 07003.

METHODS

Tissue preparation

Male New Zealand white rabbits (2.0 to 2.5 kg) were killed by injecting air through the marginal ear vein. The thoracic aorta was removed within 5 min and stored in ice-cold physiological salt solution (PSS). The vessels were carefully dissected free of adhering tissues and cut into rings approx. 2 mm wide. Rings were cut to yield strips ~10 mm long. The strips were mounted vertically in 10 ml muscle baths with temperature maintained either at 37° (intact muscle studies, Ca^{2+} ionophore) or at 22° (skinned preparations). After 1-hr equilibration, they were stretched to their optimal length (L_0) for contraction by methods described by Aksoy *et al.* [16]. Control responses to phenylephrine (PE) or 110 mM K^+ were obtained.

$^{45}Ca^{2+}$ *Efflux measurements*

The protocol for measuring $^{45}Ca^{2+}$ efflux has been described by Chiu *et al.* [17]. Briefly, rabbit aortic strips were incubated in PSS containing $^{45}Ca^{2+}$ (2 μCi/ml), at 37° and gassed with 100% O_2, for 2 hr. Extracellular $^{45}Ca^{2+}$ was removed by rapidly washing tissues with 300 ml PSS. Aortic strips were transferred either at 2- or 5-min intervals into vials containing 4 ml PSS for 65 min. Amiloride (or sodium nitroprusside) and norepinephrine were added to PSS at 0 and 50 min respectively. At the end of efflux measurements, the tissues were rinsed for 60 min with ice-cold lanthanum solution in order to inhibit transmembrane Ca^{2+} fluxes and displace extracellular Ca^{2+} [18]. The tissues were blotted, weighed and digested in 0.5 ml Unisol (Isolab, OH).

The $^{45}Ca^{2+}$ activities in eluants and tissue were measured with a Tri-Carb liquid scintillation counter

(model 4530, United Technology-Packard). The $^{45}Ca^{2+}$ efflux data were expressed as rate coefficients which represent the rate of $^{45}Ca^{2+}$ lost as a percentage of average total tissue content for time intervals of 2 or 5 min [19].

A23187 *Studies*

After obtaining a control response in high K^+, strips were washed in 0 Ca^{2+}-PSS for 30 min by which time force had returned to basal levels. They were then incubated in 0 Ca^{2+}-PSS containing 5 μM A23187 for 1 hr. Control ionophore contractions were obtained with 2.5 mM $CaCl_2$ added directly to the bath. For experiments evaluating the effects of amiloride, muscles were preincubated with the compound for 45 min in solutions containing A23187 prior to addition of Ca^{2+}.

Skinning protocol

Tissues were skinned chemically with 300 μg/ml saponin for 15 min at pH 6.7 using a protocol modified from Somlyo *et al.* [20]. Subsequently, the detergent in the skinning solution was replaced with $MgCl_2$ for 20–30 min in a relaxing solution. Concentrations of free Mg^{2+} and MgATP were 0.8 and 5.0 mM respectively. Test solutions contained a calcium/5-ethyleneglycolbis - (β-aminoethylether)*N,N,N',N'*-tetraacetic acid (Ca^{2+}/EGTA) buffer solution or phorbol ester.

Solutions

The compositions of the solutions were as follows (in mM):

PSS: 140 NaCl, 4.7 KCl, 1.2 Na_2HPO_4, 2.0 morpholinopropanesulfonic acid (MOPS) (pH 7.4 at 22°), 0.02 sodium-ethylene diaminetetraacetate (EDTA), 1.2 $MgCl_2$, 1.6 $CaCl_2$, 5.6 D-glucose, bubbled with 100% O_2.

High K^+*:* The KCl in PSS was increased to 110 mM. NaCl was appropriately decreased to maintain osmolarity.

Phorbol ester solutions for intact muscles: Phorbol esters were stored in 1 mM stock solutions in 95% EtOH. Appropriate amounts of the esters were added to PSS containing 0.10 mg/ml bovine serum albumin (BSA).

Skinning solution: 5 EGTA, 30 piperazine-*N,N'*-bis[2-ethanesulfonic acid] (PIPES) (pH 6.7), 6 ATP, 6.0 $MgCl_2$, 24.75 disodium creatine phosphate, 300 μg/ml saponin, 1 μM leupeptin.

Relaxing solution: 5 EGTA, 30 PIPES (pH 6.7), 6 ATP, 6 $MgCl_2$, 24.75 disodium creatine phosphate, 1 μM leupeptin.

Ca^{2+} *Test solutions:* Variable amounts of Ca^{2+} were added to the relaxing solutions. The binding constants for the Ca^{2+}, Mg^{2+}, EGTA and ATP for calculations of free ion concentrations were obtained from Fabiato [21]. Free ion concentrations were calculated using a computer program written by Dr. Chi-Ming Hai, University of Virginia, Charlottesville, VA.

MLC phosphorylation

For analysis of MLC phosphorylation, the strips were frozen in an acetone–dry ice slurry and analyzed by two-dimensional gel electrophoresis [16, 22]. Samples were frozen at steady-state stress.

Measurements of stress

Active stresses at L_0 were calculated in Newton/m^2 (N/m^2) smooth muscle cross-sectional area [23] taking into account that a significant fraction of the strip was composed of adventitial tissue. Approximately 60% of the fibrous proteins of rabbit aorta are collagen and elastin [24].

Data are expressed as mean ± SEM. Student's *t*-test ($P < 0.05$) was used to determine statistical differences between groups.

The drugs used in these studies were: amiloride, phenylephrine (PE), nifedipine, sodium-nitroprusside (SNP), trifluoperazine (TFP) (Sigma Chemical Co., St. Louis, MO), norepinephrine bitartrate (NE) (Calbiochem, Los Angeles, CA), A23187 (Behring Diagnostics, LaJolla, CA) and phorbol 12,13-dibutyrate (PDBu) (LC Services, Woburn, MA).

RESULTS

Intact strips of rabbit aorta generated stresses of $1.54 \pm 0.21 \times 10^5$ N/m^2 smooth muscle (N = 5) in response to 10^{-5} M phenylephrine at 37° and $9.55 \pm 1.55 \times 10^4$ N/m^2 smooth muscle (N = 6) at 22°. Maximum Ca^{2+}-dependent stresses developed by saponin-skinned preparations, in 7 μM Ca^{2+} at 22°, were $6.05 \pm 0.68 \times 10^4$ N/m^2 smooth muscle (N = 10). Maximum stresses in A23187-treated preparations with 4 mM $CaCl_2$ were $4.70 \pm 0.28 \times 10^4$ N/m^2 (N = 7).

Amiloride inhibited the contraction of rabbit aortic strips due to NE (3×10^{-7} M) (Fig. 1). Amiloride

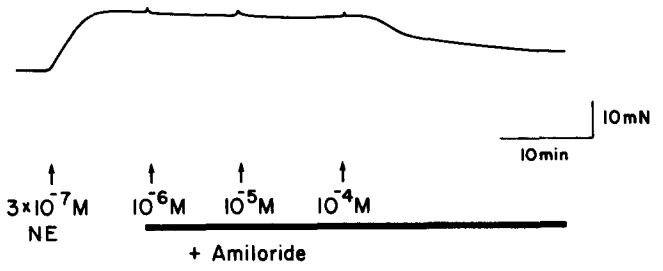

Fig. 1. Example of the vasorelaxant effect of amiloride on rabbit aortic strips contracted with 3×10^{-7} M NE.

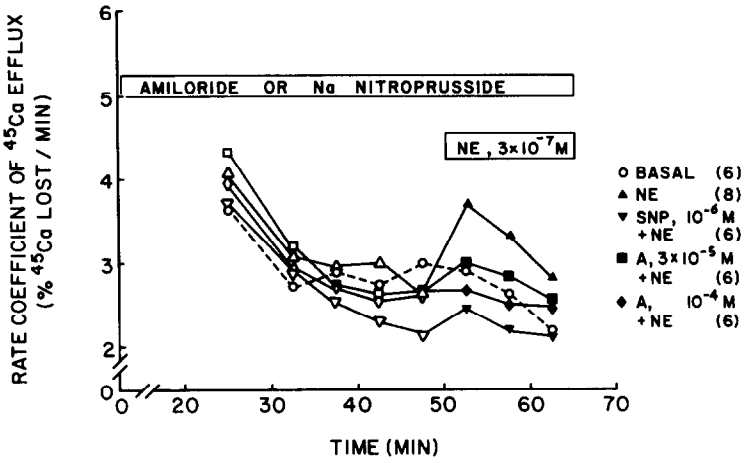

Fig. 2. Inhibitory effects of amiloride (A) and sodium nitroprusside (SNP) on NE-induced $^{45}Ca^{2+}$ efflux from rabbit aorta in physiological salt solution. Treatment with amiloride or sodium nitroprusside lasted through the entire efflux measurements (0–65 min). Tissues challenged with NE are represented by solid symbols (50–65 min).

at 100 μM achieved 78.8 ± 3.71% (N = 5) inhibition of the NE control at 37° and 61.7 ± 8.12% inhibition (N = 6) at 22°. NE at 3×10^{-7} M caused a significant increase in $^{45}Ca^{2+}$ efflux from aortic tissues during a 15-min exposure (3.69 ± 0.27 (N = 8) vs 2.91 ± 0.28 in the control (N = 6) percent of $^{45}Ca^{2+}$ lost/min measured during the first 5 min after exposure to NE ($P < 0.05$). This stimulated $^{45}Ca^{2+}$ efflux, consequent to an increase in cytosolic Ca^{2+}, was inhibited significantly ($P < 0.05$) by amiloride at 3×10^{-5} M (2.96 ± 0.12% $^{45}Ca^{2+}$ lost/min, N = 6) and at 10^{-4} M (2.69 ± 0.12% $^{45}Ca^{2+}$ lost/min measured during the same time interval, N = 6) (Fig. 2). Sodium-nitroprusside (10^{-6} M), used as a reference standard, also inhibited NE-induced Ca^{2+} efflux (2.47 ± 0.11% $^{45}Ca^{2+}$ lost/min, N = 6; $P < 0.05$).

We utilized a saponin-treated rabbit aorta preparation, in which membrane Ca^{2+}-channels were rendered non-functional, to ascertain whether the vasorelaxant effects of amiloride were partly mediated by a mechanism other than inhibition of Ca^{2+} mobilization (Fig. 3). Both steady-state stress and myosin light chain phosphorylation in the presence of 100 and 300 μM amiloride were reduced significantly ($P < 0.05$) from control values obtained using 5.9×10^{-7} M Ca^{2+} alone (an EC_{50} concentration for stress development). In view of reports that the inhibition of several protein kinases (other than MLC kinase) by amiloride is competitive with ATP [6–8], we undertook experiments with the skinned fiber preparation to determine whether the observed inhibition of stress, presumably via an inhibition of MLC kinase, was competitive with MgATP (Fig. 4). The dependence of force on MgATP was determined in the absence and presence of 100 μM amiloride. Free Ca^{2+} and Mg^{2+} were constant at 3.0 μM and 0.8 mM. The MgATP dependence of steady-state force was depressed and shifted to higher [MgATP] in the presence of amiloride.

The inhibitory effects of amiloride on force development at physiological intracellular concentrations of MgATP were studied in A23187-treated preparations (Fig. 5). Contractions elicited in A23187-treated muscles with 2.5 mM $CaCl_2$ (an EC_{50} concentration for force development, unpublished observations) were not affected by the Ca^{2+} channel blocker verapamil but were reduced by TFP (Table 1). Amiloride significantly inhibited ($P < 0.05$) both stress and MLC phosphorylation.

In light of recent evidence that protein kinase C is involved in regulation of vascular smooth muscle contraction [10–12], we investigated the effects of amiloride on PDBu-dependent muscle activation.

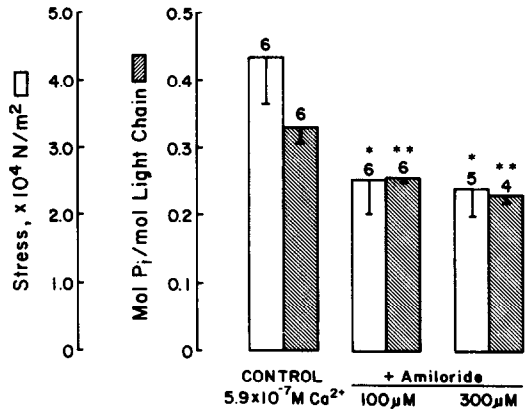

Fig. 3. Effects of 100 and 300 μM amiloride on steady-state stress and MLC phosphorylation in saponin-skinned rabbit aorta. [MgATP] was 5.0 mM. Free Ca^{2+} and Mg^{2+} were 5.9×10^{-7} M and 8×10^{-4} M respectively. The muscles were pretreated with amiloride for 45 min before addition of Ca^{2+}. Basal value of myosin light chain phosphorylation, obtained in solutions containing 5 mM EGTA and zero added Ca^{2+}, was 0.21 ± 0.05 mol P_i/mol light chain (mean ± SEM, N = 7). Key: (*) significant difference from force in 5.9×10^{-7} M Ca^{2+} alone ($P < 0.05$); and (**) significant difference from myosin light chain phosphorylation in 5.9×10^{-7} M Ca^{2+} alone ($P < 0.05$).

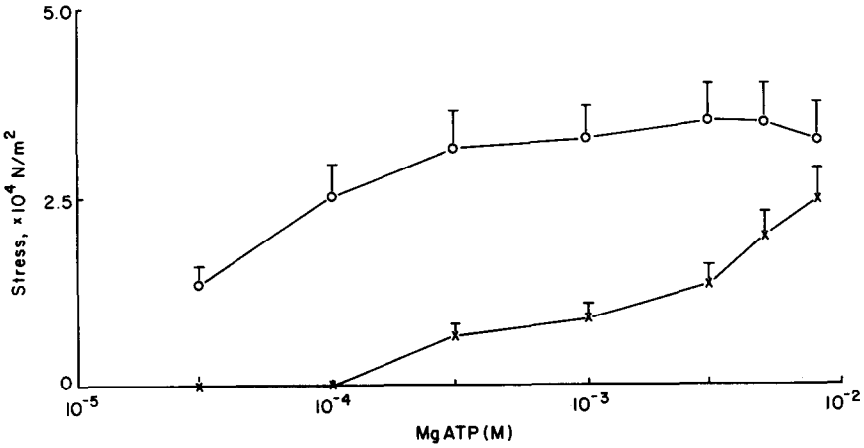

Fig. 4. MgATP requirement for steady-state stress development of saponin-treated muscles in the absence (○) and presence (×) of 100 μM amiloride. Free Ca^{2+} and Mg^{2+} were maintained at 3×10^{-6} M and 8×10^{-4} M respectively. Solutions contained an ATP-regenerating system of 5 mM creatine phosphate and 5 units/ml creatine kinase. Values are means ± SEM, N = 6.

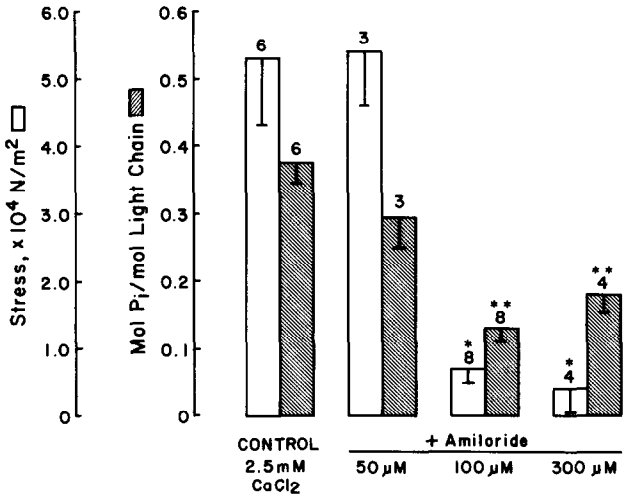

Fig. 5. Inhibition of steady-state stress and concomitant myosin light chain phosphorylation by amiloride in response to 2.5 mM $CaCl_2$ in strips of A23187-treated rabbit aorta. Muscles were treated with amiloride for 45 min prior to addition of $CaCl_2$. Values are means ± SEM. Key: (*) significant reduction in stress ($P < 0.05$), and (**) significant reduction in MLC phosphorylation ($P < 0.05$) from control values.

Table 1. Stress and myosin light chain phosphorylation in A23187-treated rabbit aorta

	Stress ($\times 10^4$ N/m^2)	MLC phosphorylation (mol P_i/mol LC)
2.5 mM $CaCl_2$	5.33 ± 1.00 (6)	0.37 ± 0.03 (6)
+ 10^{-5} M Verapamil	5.19 ± 1.43 (5)	0.38 ± 0.02 (4)
+ 10^{-4} M TFP	0.81 ± 0.27* (5)	0.24 ± 0.03* (4)

Values are mean ± SE (N = 4–6). Strips of rabbit aorta were bathed in solutions containing 10^{-5} M verapamil or 10^{-4} M TFP for 30 min prior to the addition of 2.5 mM $CaCl_2$. Muscles were frozen at steady-state stress (30 min) for analysis of 20,000 Da myosin light chain phosphorylation. The basal value of myosin light chain phosphorylation, obtained in solutions containing 5 μM A23187 and zero added Ca^{2+}, was 0.21 ± 0.02 mol P_i/mol LC (N = 4).

* Significant difference from value at 2.5 mM $CaCl_2$ alone ($P < 0.05$).

Table 2. Inhibition by amiloride of PDBu-dependent stress in intact rabbit aorta

	Stress ($\times 10^4$ N/m^2)
10^{-6} M PDBu	10.45 ± 1.27 (9)
+ 50 μM Amiloride	13.13 ± 1.80 (4)
+ 100 μM Amiloride	3.57 ± 0.27* (6)
+ 300 μM Amiloride	4.0 ± 0.65* (5)

Values are mean ± SE (N = 4–9). Control strips of rabbit aorta were contracted with 10^{-6} M PDBu. Other muscle strips were pretreated with 50, 100 or 300 μM amiloride for 45 min.

* Significant inhibition of PDBu-dependent stress ($P < 0.05$).

Amiloride at 100 and 300 μM concentrations caused a modest inhibition of PDBu-dependent contraction (Table 2).

DISCUSSION

Relaxation of arterial smooth muscle by amiloride has been reported by others [13–15]. The studies have indicated an interaction of amiloride with the α-adrenergic receptor. Other mechanisms of vasorelaxation have not yet been elucidated fully. We have investigated the effects of amiloride on known intracellular regulatory processes of smooth muscle contraction. The data in the present study suggest that the vasorelaxant effect of amiloride may be mediated through reduction of cytosolic [Ca^{2+}] as well as inhibition of MLC kinase and protein kinase C.

We have expressed the force generated by the muscles as stress (force/cross-sectional area) which takes into account the weight and length of the strips. However, the calculation of stress for the rabbit aorta is difficult since adventitial tissue constitutes a significant fraction of the strip cross-sectional area. Based on the work of Leung *et al.* [24], we assumed in our calculations that the smooth muscle fraction constituted 40% of the total strip weight. The calculated values of maximum stress for the intact and skinned rabbit aorta at 22° were comparable to stresses generated under similar conditions by strips of porcine carotid artery from which the adventitia had been removed [25].

NE causes an increase in cytosolic Ca^{2+} [26] through activation of receptor-operated Ca^{2+} channels and release from intracellular storage sites [27]. The resultant phosphorylation of the 20,000 dalton myosin light chain has been shown to be crucial for development of contraction. An inhibition of this rise in Ca^{2+} would result in a predictable inhibition of contraction. The observed increase in $^{45}Ca^{2+}$ efflux in response to NE (Fig. 2) reflects an increase in intracellular [Ca^{2+}]. In the presence of amiloride, $^{45}Ca^{2+}$ efflux in response to NE was reduced significantly ($P < 0.05$). These findings are similar to those reported by others [14, 15]. The data are consistent with either an α-adrenergic receptor antagonism or inhibition of membrane mechanisms regulating Ca^{2+} transport mediating vasorelaxation [13–15].

The observed reduction of stress and concomitant MLC phosphorylation in saponin- and A23187-treated rabbit aortic strips by amiloride suggest that the inhibition of MLC kinase is one mechanism of vasorelaxation. This is further supported by observations that amiloride inhibited MLC kinase isolated from chicken gizzard in a manner competitive with ATP*. Amiloride has been shown to inhibit a list of other purified serine-specific protein kinases including cAMP-dependent protein kinase [5, 6]. It also inhibited a purified serine/threonine-specific kinase protein kinase C [7] and growth factor receptor tyrosine kinase [8]. The inhibition of cAMP-dependent protein kinase by amiloride was competitive with ATP. The observed shift in the MgATP dependence of force to higher substrate concentrations in our skinned muscle preparations (Fig. 4) suggests that amiloride inhibition of contraction is also competitive with ATP.

We have demonstrated an inhibition of MLC phosphorylation by amiloride in skinned or permeabilized preparations. This raises the question of whether this direct inhibition of MLC kinase is a relevant mechanism mediating vasorelaxation in intact smooth muscle. Although we have not measured intracellular concentrations of amiloride in our studies of intact muscle, others have demonstrated that amiloride can be taken up and concentrated in murine erythroleukemia cells [28]. This suggests that amiloride can cross the plasma membrane. Thus, we assumed that amiloride could also enter vascular smooth muscle cells. This was supported by the observations that amiloride blocked both MLC phosphorylation and force in Ca^{2+} ionophore-treated cells (Fig. 5). Therefore, it is possible that amiloride can have a direct effect on MLC kinase in intact vascular smooth muscle.

Chatterjee and Tejada [12] reported that slow stress development in response to various concentrations of PDBu is associated with very little parallel change in MLC phosphorylation in Triton X-100 skinned medial strips of porcine carotid artery. This suggested that contraction under these conditions was mediated by a mechanism other than MLC kinase. Phorbol esters are known activators of protein kinase C. These findings together with other reports on activation of intact smooth muscle contraction by other phorbol esters [10, 11] implicate protein kinase C as a regulator of vascular smooth muscle contraction. The observed reduction of PDBu-dependent contractions by amiloride (Table 2) suggests that the compound can functionally inhibit protein kinase C. This inhibition of PDBu-dependent contractions could be a consequence of an inhibition of Ca^{2+} release from intracellular or extracellular sources. However, evidence that PDBu-induced contractions in intact rabbit aorta are not associated with Ca^{2+} mobilization as monitored by $^{45}Ca^{2+}$ efflux and influx [29] argues against this possibility.

The activation of cAMP-dependent protein kinase

* C. Foster, Schering Corp., unpublished observations; cited with permission.

has been shown to result in phosphorylation of MLC kinase and subsequent decrease in the affinity of the kinase for Ca^{2+}-calmodulin in purified protein systems [30]. This was proposed to be a mechanism for the observed relaxation of smooth muscle in response to agonists causing an increase in intracellular cAMP [30], in addition to changes in intracellular Ca^{2+} [31]. Thus, the reported inhibition of cAMP-dependent protein kinase by amiloride [6] could potentially have affected the overall response in our muscle preparations. Several lines of evidence made this an unlikely possibility. First, intact tracheal muscles contracted with carbachol show no change in MLC kinase activity upon relaxation with isoproterenol [32]. Also, skinned coronary arteries incubated with cAMP-protein kinase do not exhibit any change in calcium sensitivity [33]. It must also be pointed out that, although there have been reports of a reduction in Ca^{2+} sensitivity of skinned guinea pig *Taenia coli* incubated with cAMP [34], we have not observed such changes in skinned porcine carotid artery under similar conditions (unpublished observations). Hence, we feel that the vasorelaxant activity of cAMP, under physiological conditions, is most likely mediated via changes in intracellular Ca^{2+}, which is controlled in preparations treated with A23187 or saponin. Also, it is unlikely that cAMP-protein kinase was activated under our experimental conditions. Therefore, we conclude that interactions of amiloride with a partially activated cAMP-dependent protein kinase in our preparations did not contribute to the results shown.

We have also not ruled out the possibility that amiloride may activate cellular phosphatases leading to a reduction in measured MLC phosphorylation. However, the lack of effects by amiloride on protein phosphatases 1 and 2A purified from rabbit skeletal muscle or protein phosphatase 2C isolated from rat liver [6] speaks against this possibility.

We conclude that the vasorelaxant properties of amiloride that we demonstrated were due to an inhibition of Ca^{2+} mobilization as well as inhibition of MLC kinase and protein kinase C. These enzymes are involved in the regulation of muscle contraction. In light of the reported inhibition of many other kinases and the high concentrations required, amiloride appears to be a nonspecific inhibitor of protein kinases and other ATP-linked processes.

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