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INHIBITION OF SODIUM-CALCIUM AND SODIUM-PROTON EXCHANGERS BY AMILORIDE CONGENERS IN ARTERIAL MUSCLE CELLS*

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Abstract—The inhibitory potencies of several amiloride congeners towards Na^+-Ca^{2+} and Na^+-H^+ exchange were compared in rat aortic myocytes. *N*-(2,4-Dimethylbenzyl)amiloride (DMB) was 10 times more potent towards Na^+-Ca^{2+} than Na^+-H^+ exchange. Amiloride and ethylisopropylamiloride were about 5,000 and 10,000 times more potent towards Na^+-H^+ than Na^+-Ca^{2+} exchange respectively. *N*-(3,4-Dichlorobenzyl)amiloride was almost equipotent towards both exchangers. About 40 nM ethylisopropylamiloride inhibited Na^+-H^+ exchange by 50%. Ethylisopropylamiloride (10 μM) had no effect on basal or angiotensin-evoked $^{45}Ca^{2+}$ efflux or net Ca^{2+} efflux. In contrast to ethylisopropylamiloride, 25–50 μM DMB, which strongly inhibits Na^+-Ca^{2+} exchange, markedly decreased both $^{45}Ca^{2+}$ efflux and net Ca^{2+} efflux produced by angiotensin. Replacing extracellular Na^+ with *N*-methyl-D-glucamine inhibited angiotensin-evoked $^{45}Ca^{2+}$ efflux similarly to DMB. Neither DMB nor Na^+ placement had any effect on basal or angiotensin-evoked production of [^{3}H]inositol phosphates. These findings suggest that Na^+-H^+ exchange has no major influence on short-term Ca^{2+} regulation and provide evidence that Na^+-Ca^{2+} exchange is a major pathway of rapid Ca^{2+} efflux in stimulated arterial muscle cells.

Na^+-Ca^{2+} exchange [1, 2] and Na^+-H^+ exchange [3, 4] are plentiful in smooth muscle cells from rat aorta. The abundance of Na^+-Ca^{2+} exchange is indicated by the multi-fold increases in $^{45}Ca^{2+}$ influx or cytosolic free Ca^{2+} that are produced by inverting the Na^+ gradient [1, 2]. The Na^+-Ca^{2+} exchanger makes a major contribution to decreasing cytosolic free Ca^{2+} after stimulation with a vasoconstrictor such as angiotensin II (ANG‡) [5]. The exchanger causes a rapid net efflux of Ca^{2+} after it has been released from the sarcoplasmic reticulum by ANG [5] or ionomycin [6]. In unstimulated cells with the normal Na^+ gradient, Na^+-Ca^{2+} exchange activity is latent, apparently because of the low degree of saturation of substrate and activator sites by Na^+ or Ca^{2+} [1, 5–7].

The lack of a specific inhibitor has hampered biochemical and physiological studies of Na^+-Ca^{2+} exchange. Amiloride is pyrazinoylguanidine with amino groups on the 3- and 5-positions and a chlorine atom on the 6-position of the pyrazine ring (see Table 1). Amiloride congeners inhibit Na^+ and Ca^{2+} channels and Na^+-H^+ and Na^+-Ca^{2+} exchangers in mammalian cells [7–11]. Appropriate derivatization of amiloride yields compounds with considerable selectivity for a particular transporter [12, 13]. The addition of hydrophobic substituents to the 5-amino group strikingly increases activity towards Na^+-H^+ exchange, whereas hydrophobic substitution of the terminal nitrogen of the guanidinium group increases potency towards Na^+ channels [12, 13]. Both of these substitutions increase activity towards Na^+-Ca^{2+} exchange and Ca^{2+} channels in membranes from brain and heart and a pituitary tumor cell line [10, 14, 15]. 2,4-DMB is a similarly potent inhibitor of Na^+-Ca^{2+} exchange in membrane vesicles [14] and rat aortic muscle cells [1, 16].

Although the Na^+-H^+ exchange is activated by ANG and other vasoconstrictors [4, 17–21], its role in Ca^{2+} regulation and contraction is unclear. First, Reynolds *et al.* [22] found that potent, selective inhibitors of Na^+-H^+ exchange have no effect on agonist-induced contraction of aortic rings. Second, ANG and other Ca^{2+}-mobilizing hormones that increase cytosolic pH by activating Na^+-H^+ exchange in the absence of bicarbonate either have no effect on or decrease cytosolic pH in the presence of bicarbonate [23–25]. It is not known if the inhibition of Na^+-H^+ exchange affects Ca^{2+} regulation in aortic myocytes. Here we compare the potencies of several amiloride congeners as inhibitors of Na^+-H^+ and Na^+-Ca^{2+} exchange in arterial myocytes and show that inhibition of Na^+-Ca^{2+} exchange, but not Na^+-H^+ exchange, markedly affected Ca^{2+} regulation by ANG.

MATERIALS AND METHODS

Smooth muscle cells were isolated from rat aorta and grown in culture dishes (35 mm diameter for

* The data on the inhibition of Na^+-Ca^{2+} and Na^+-H^+ exchange by the amiloride derivatives were presented at the 78th Annual Meeting of the American Society of Biological Chemists (Smith *et al.*, *Fed Proc* **46**: 2028, 1987).

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‡ Abbreviations: ANG, angiotensin II; DCB, *N*-(3,4-dichlorobenzyl)amiloride; DMB, *N*-(2,4-dimethylbenzyl)-amiloride; EIPA, 5-(*N*,*N*-ethylisopropyl)amiloride; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IC_{50}, drug concentration that causes 50% inhibition; NMG^+, *N*-methyl-D-glucamine; and PSS, physiological salts solution.

transport assays and 100 mm diameter for [^{3}H]inositol phosphates) in medium 199 containing 10% fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT) as previously described [5, 26]. The cultures were used between 5 and 15 passages.

Na^+-Ca^{2+} *exchange.* Reverse mode Na^+-Ca^{2+} exchange activity was assayed by measuring the initial rate of $^{45}Ca^{2+}$ influx that was Na^+ gradient dependent [1]. The cultures were incubated for 2 hr in culture medium containing 1 mM ouabain and 10 μg/mL nigericin to load the cells with Na^+. Then they were rinsed five times and incubated for 1 min in a buffer containing (in mM): 1 $CaCl_2$, 1 $MgCl_2$, 1 ouabain, 20 HEPES/Tris, pH 7.4, 4 μCi $^{45}Ca^{2+}$, and either 125 KCl or 120 NaCl plus 5 KCl. Extracellular $^{45}Ca^{2+}$ was removed and intracellular $^{45}Ca^{2+}$ was extracted and measured [1]. $^{45}Ca^{2+}$ uptake by cells that were not loaded with Na^+ was subtracted from uptake by the Na^+-loaded cells to obtain exchange activity. Na^+ gradient-dependent uptake increased linearly for at least 3 min.

Na^+-H^+ *exchange.* $^{22}Na^+$ influx was assayed in cells that were acid-loaded as described [27]. The cells were incubated for 1 hr in a buffer containing (in mM): 50 NH_4Cl, 70 KCl, 1 $MgCl_2$, 1 $CaCl_2$, and 20 HEPES/Tris, pH 7.4. Then they were rinsed 3 times (<15 sec) and incubated in assay buffer which contained (in mM): 120 KCl, 5 NaCl, 2 ouabain, 1 $CaCl_2$, 1 $MgCl_2$, 20 HEPES/Tris, pH 7.4, and 1 μCi $^{22}Na^+$. Uptake was stopped after 5 min by rinsing the cultures eight times with 0.1 M $MgCl_2$ containing 10 mM HEPES/Tris, pH 7.4. Intracellular $^{22}Na^+$ was extracted and measured by liquid scintillation spectrometry [1]. Uptake was linear from 0 to 10 min.

[^{3}H]*Inositol phosphates.* On the day after plating, the cultures were rinsed twice with 5 mL medium 199 before adding 5 mL medium 199 containing 2% dialyzed fetal bovine serum and 10 μCi [^{3}H]inositol. Two days later the cultures were rinsed five times with a physiological salts solution (PSS) which contained (in mM): 140 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, and 20 HEPES/Tris, pH 7.4. The cultures were incubated for 30 min with 5 mL PSS which contained 10 mM glucose. This solution was aspirated, the cultures were rinsed twice, and 5 mL of PSS containing the indicated additions was added. The incubation was stopped by aspiration and addition of 3 mL of 10% ice-cold trichloroacetic acid. After 20 min at 4° the extract was removed and the dish was rinsed three times with 2 mL of 5 mM *myo*-inositol. The inositol rinse and the acid extract were combined and washed four times with 3 ml ethyl ether. The pH of the extract was adjusted to 7.0 to 7.4 by adding 40 μL of 1 M Tris. [^{3}H]Inositol phosphates were separated on 0.8-mL AG 1-X8 columns as previously described [28]. [^{3}H]Inositol phosphate standards (Dupont-NEN, Boston, MA) were used to verify the separation of glycerol-phosphoinositol, inositol phosphate, and inositol bis-, tris-, and tetrakisphosphates.

Total cell Ca^{2+}, $^{45}Ca^{2+}$ efflux, and protein were measured as described [5, 29]. All incubations were at 37° unless otherwise indicated.

Materials. The reagents and their sources were: $^{45}CaCl_2$ (20–30 Ci/g in water) and $^{22}NaCl$ (~1000 Ci/g in water) from Dupont-NEN, Boston, MA; *myo*[2-^{3}H]inositol (100 Ci/g) from Amersham, Arlington Heights, IL; and nigericin was from the Sigma Chemical Co., St. Louis, MO. The amiloride derivatives were obtained from Merck, Sharp & Dohme Research Laboratories, West Point, PA. They were dissolved in dimethyl sulfoxide. The concentration of dimethyl sulfoxide in the assay solutions was 0.1% or less and had no effect on Na^+-Ca^{2+} or Na^+-H^+ exchange, $^{45}Ca^{2+}$ efflux, or total cell Ca^{2+}.

RESULTS

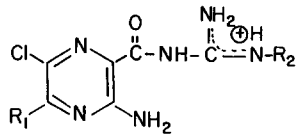
Inhibition of Na^+-Ca^{2+} *exchange by amiloride analogs.* Replacing extracellular Na^+ with K^+, choline, or NMG^+ only slightly increases Ca^{2+} influx in rat aortic myocytes [1, 2]. In contrast to membrane depolarization and/or the withdrawal of external Na^+, inverting the normal Na^+ gradient (i.e. loading the cells with Na^+ and withdrawing extracellular Na^+) markedly increases Ca^{2+} influx in these cells [1, 2]. Na^+-gradient dependent $^{45}Ca^{2+}$ uptake approximately equals DMB-inhibitable uptake regardless of the osmotic substitute for external Na^+ [1]. These findings indicate that $^{45}Ca^{2+}$ influx produced by inverting the Na^+ gradient occurs via the Na^+-Ca^{2+} exchanger.

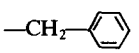
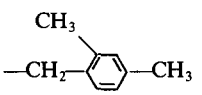
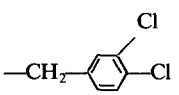
The amiloride derivatives differed markedly in inhibitory potency towards Na^+-Ca^{2+} exchange. Amiloride itself was the least potent compound tested. 3,4-DCB and 2,4-DMB were 100 and 530 times more potent than amiloride respectively. EIPA, 5-(*N*-*t*-butyl)amiloride, phenamil, and benzamil were 15–22 times more potent than amiloride (Table 1). Therefore, derivatization of either the 5-amino-pyrazine-nitrogen or the guanidino-nitrogen increased potency towards Na^+-Ca^{2+} exchange (Fig. 1 and Table 1) as reported by Kaczorowski *et al.* [14] for Na^+-Ca^{2+} exchange in membrane vesicles from brain and heart tissues and GH_3 cells.

Inhibition of Na^+-H^+ *exchange by amiloride analogs.* EIPA was the most potent inhibitor of Na^+-H^+ exchange among the analogs tested (Fig. 2). EIPA was 1.8 times more potent than 5-(*N*-*t*-butyl)amiloride, 63 times more potent than amiloride, and 3000–5000 times more potent than phenamil, benzamil, DMB, and DCB (Fig. 2 and Table 1). DCB was almost equipotent towards Na^+-H^+ and Na^+-Ca^{2+} exchange. Clearly hydrophobic substitution of the 5-amino-pyrazine nitrogen strikingly increased potency towards Na^+-H^+ exchange, whereas derivatization of the guanidino-nitrogen markedly decreased potency towards this exchanger (Table 1). Addition of benzyl or phenyl groups to the terminal guanidino nitrogen decreased potency towards Na^+-H^+ exchange (Table 1) as previously observed in other cell types [13].

The concentration of Na^+ in the assay medium markedly affects the potency of amiloride towards Na^+-H^+ exchange [30]. Because most physiological experiments are done at high external Na^+, we compared the potency of EIPA at 5 and 120 mM Na^+. Increasing external Na^+ from 5 to 120 mM

Table 1. Summary of inhibitory potencies of amiloride congeners towards Na^+-Ca^{2+} and Na^+-H^+ exchange



Compound	R_1	R_2	IC_{50} (μM) Na^+/Ca^{2+}	IC_{50} (μM) Na^+/H^+
Amiloride	$-NH_2$	H	5880	1.2
EIPA	$-N(CH_2CH_3)(CH(CH_3)_2)$	H	261	0.019
Butylamiloride	$-NH-C(CH_3)_3$	H	361	0.034
Phenamil	$-NH_2$		268	78
Benzamil	$-NH_2$		386	90
DMB	$-NH_2$		11	98
DCB	$-NH_2$		99	59

Drug concentrations that inhibited exchange by 50% (IC_{50}) were obtained from log–log plots of the data using "Dose–Effect Analysis with Microcomputers" (Elsevier-BIOSOFT, Cambridge, U.K.). The linear regression coefficients for the Na^+-Ca^{2+} exchange data were 0.992, 0.999, 0.916, 0.998, 0.978, 0.981, and 0.968 for amiloride to DCB respectively. For Na^+-H^+ exchange the regression coefficients were 0.996, 0.967, 0.975, 0.984, 0.985, 0.964, and 0.988 for amiloride to DCB respectively.

increased the IC_{50} of EIPA from 19 to 41 nM (Fig. 3 and Table 1).

Effects of DMB and EIPA on $^{45}Ca^{2+}$ efflux and net Ca^{2+} efflux produced by ANG. ANG decreased total cell Ca^{2+} by 41.9% in 1 min (Fig. 4) as previously reported [5]. DMB strongly inhibited the effect of ANG on total cell Ca^{2+} (Fig. 4). In the presence of 50 μM DMB, ANG decreased total Ca^{2+} by 12.4% (Fig. 4). A 50 μM concentration of DMB almost completely inhibited reverse mode Na^+-Ca^{2+} exchange (Fig. 1). DMB (10–50 μM) also inhibited ANG-evoked $^{45}Ca^{2+}$ efflux but had no effect on basal efflux (Fig. 5). In contrast to DMB, 10 μM EIPA had no effect on ANG-evoked $^{45}Ca^{2+}$ efflux (Fig. 5). EIPA (10 μM), like 50 μM DMB, had no effect on total cell Ca^{2+} or $^{45}Ca^{2+}$ efflux in the absence of ANG (Figs. 4 and 5).

Effect of replacing external Na^+ with NMG^+ on basal and ANG-evoked $^{45}Ca^{2+}$ efflux. Replacing external Na^+ with NMG^+ inhibited ANG-evoked efflux (Fig. 5B) similarly to 25–50 μM DMB (Fig. 5A). External Na^+ replacement had no effect on basal $^{45}Ca^{2+}$ efflux (Fig. 5B). In the presence of NMG^+ instead of Na^+, DMB (25 μM) caused no further inhibition of ANG-stimulated efflux (N = 6, data not shown). This finding is consistent with the hypothesis that DMB and the removal of external Na^+ inhibit ANG-evoked efflux by the same mechanism.

Another active Ca^{2+} transporter, presumably the

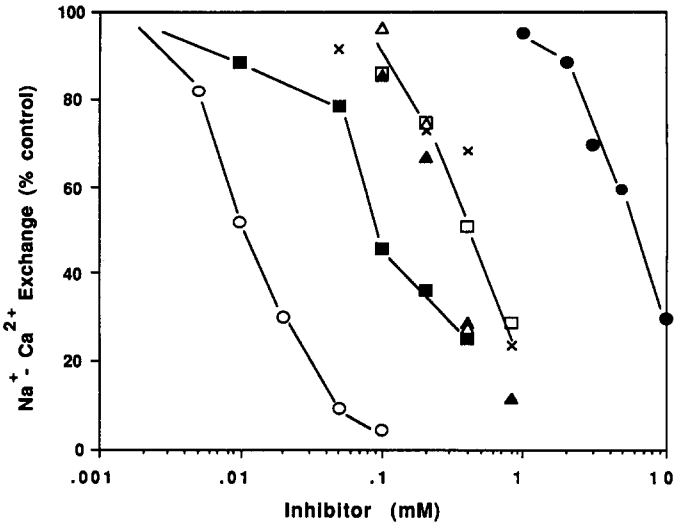


Fig. 1. Inhibition of Na^+-Ca^{2+} antiport activity by seven amiloride congeners. Values are means of 2 or 3 experiments on duplicate cultures. Control exchange activity ranged from 8.8 to 9.0 nmol/min/mg. All SE values were less than 10% of control. Key: amiloride (●), DMB (○), DCB (■), benzamil (□), phenamil (▲), EIPA (△), and butylamiloride (×).

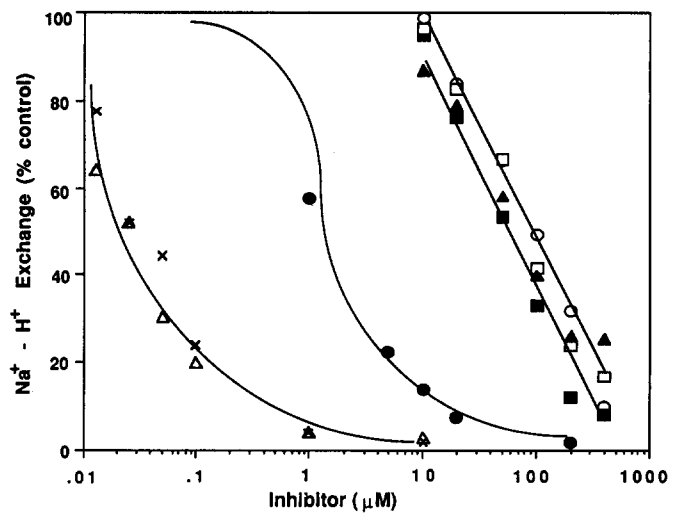


Fig. 2. Inhibition of Na^+-H^+ antiport activity by seven amiloride congeners. Values are means of 3–5 experiments on duplicate cultures. Control exchange activity ranged from 18.9 to 22.7 nmol/min/mg. All SE values were less than 10% of control. Key: amiloride (●), DMB (○), DCB (■), benzamil (□), phenamil (▲), EIPA (△), and butylamiloride (×).

sarcolemmal Ca^{2+}-ATPase, probably caused the 12.4% decrease in total Ca^{2+} produced by ANG in the presence of 50 μM DMB because removing extracellular Na^+ also strongly inhibited, but did not block ANG-evoked $^{45}Ca^{2+}$ efflux (Fig. 5 and [5]). Thus, it seems likely that the Ca^{2+}-ATPase contributes significantly to net Ca^{2+} efflux, at least when Na^+-Ca^{2+} exchange is blocked by DMB or the removal of external Na^+, as previously suggested [6, 31].

Lack of effects of DMB or removal of external Na^+ *on ANG-evoked production of* [^{3}H]*inositol phosphates.* Because ANG evokes the production of [^{3}H]inositol trisphosphate [32] which releases Ca^{2+} from the sarcoplasmic reticulum, it is possible that DMB inhibits ANG-stimulated Ca^{2+} efflux by decreasing the production of inositol trisphosphate. A 1-min incubation of the cells with ANG evoked multi-fold increases in [^{3}H]inositol mono-, bis-, and trisphosphates and had little or no effect on cellular [^{3}H]glycerophosphoryl inositol or [^{3}H]inositol tetrakisphosphate (Fig. 6). DMB (50 μM) or replacing external Na^+ with NMG^+ had no effect on basal [^{3}H]inositol phosphates or the increases in

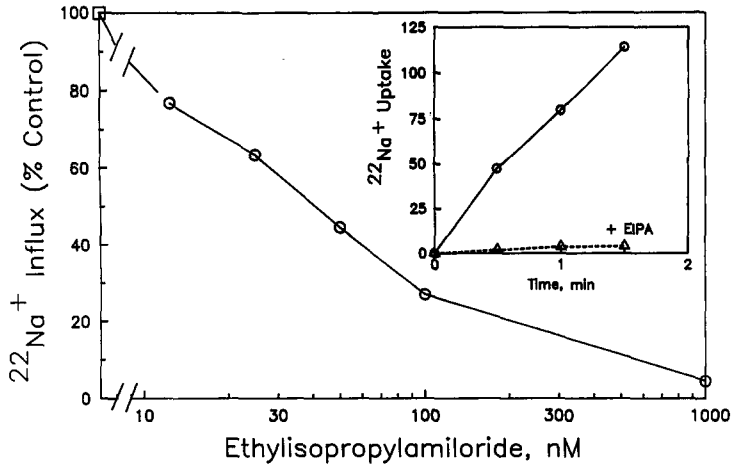


Fig. 3. Effect of EIPA concentration on $^{22}Na^+$ influx assayed at 120 mM extracellular Na^+. The cells were incubated in the NH_4Cl equilibration buffer (see Materials and Methods) for 30 min, rinsed and incubated for 1 min in assay buffer which contained (in mM): 120 NaCl, 5 KCl, 2 ouabain, 1 $CaCl_2$, 1 $MgCl_2$, 20 HEPES/Tris, pH 7.4, and 2 μCi $^{22}Na^+$. Control influx (100%) was 227 nmol/min/mg. Inset: time course of $^{22}Na^+$ uptake (nmol/culture) in the absence (○) and presence (△) of 20 μM EIPA. Each culture contained 0.34 mg protein. The IC_{50} of 41 nM was obtained as indicated in the legend to Table 1. The linear regression coefficient of the log–log plot was 0.9988. Values are means of duplicates that differed by <10%. An additional experiment gave similar results.

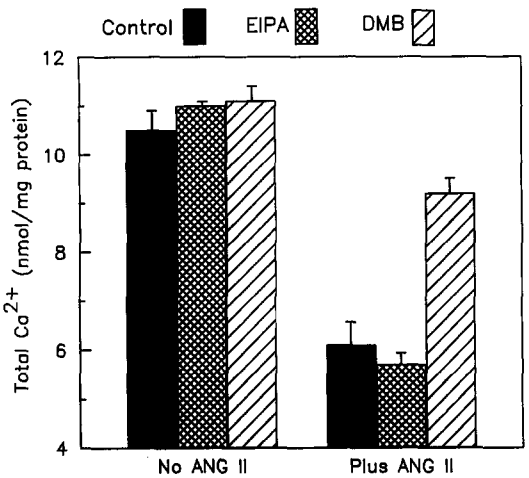


Fig. 4. Effects of DMB and EIPA on the decrease in total Ca^{2+} produced by ANG. Total cell Ca^{2+} was measured after labeling the cells for 24 hr in 2 mL medium 199 containing 10 μCi $^{45}Ca^{2+}$ [1]. Additions were made directly to the labeling medium. The concentrations of ANG, EIPA, and DMB were 40 nM, 10 μM, and 50 μM respectively. The amilorides were added 60 sec before ANG. One minute after adding ANG, the cultures were rinsed and extracted [1]. Values are means ± SE for 2 experiments (N = 7).

[^{3}H]inositol mono-, bis-, or trisphosphates produced by ANG (Fig. 6). Therefore, DMB or the removal of external Na^+ does not inhibit ANG-stimulated Ca^{2+} efflux by decreasing the production of inositol trisphosphate.

DISCUSSION

The present findings support the view that Na^+-Ca^{2+} exchange, but not Na^+-H^+ exchange, makes an important contribution to rapid changes in Ca^{2+} homeostasis in aortic myocytes. DMB may inhibit ANG-evoked Ca^{2+} efflux by blocking Na^+-Ca^{2+} exchange because 25–50 μM 2,4-DMB blocked Na^+-Ca^{2+} exchange (Fig. 1 and [1]) and inhibited ANG-evoked net Ca^{2+} efflux (Fig. 4) and $^{45}Ca^{2+}$ efflux (Fig. 5) similarly to the replacement of external Na^+ with NMG^+ [5]. Although the present results do not exclude the possibility that DMB inhibits Ca^{2+} release from the sarcoplasmic reticulum, this is unlikely. First, DMB had no effect on the production of [^{3}H]inositol phosphates evoked by ANG (Fig. 6). Second, DMB strongly inhibits net Ca^{2+} efflux produced by ionomycin, a Ca^{2+} selective ionophore [6].

At low concentrations ionomycin mimics the effects of ANG on Ca^{2+} regulation in aortic myocytes [6]. A 1 μM concentration of ionomycin selectively releases stored Ca^{2+} rather than producing net Ca^{2+} influx, apparently because ionomycin has a low affinity for Ca^{2+} (K_m = 8–10 mM) [33, 34] and the concentration of ionized Ca^{2+} is considerably higher in the sarcoplasmic reticulum than in the external medium. Because DMB similarly inhibits net Ca^{2+} efflux produced by ANG or ionomycin, it seems likely that DMB inhibits Na^+-Ca^{2+} exchange rather than Ca^{2+} release from the sarcoplasmic reticulum. Na^+-Ca^{2+} exchange is the only known common component in ANG- or ionomycin-evoked net Ca^{2+} efflux. Replacing extracellular Na^+ with NMG^+ inhibited $^{45}Ca^{2+}$ efflux (Fig. 5) and net Ca^{2+} efflux produced by ANG [5] or ionomycin [6] similarly to 50 μM DMB (Figs. 4 and 5). Replacing external Na^+

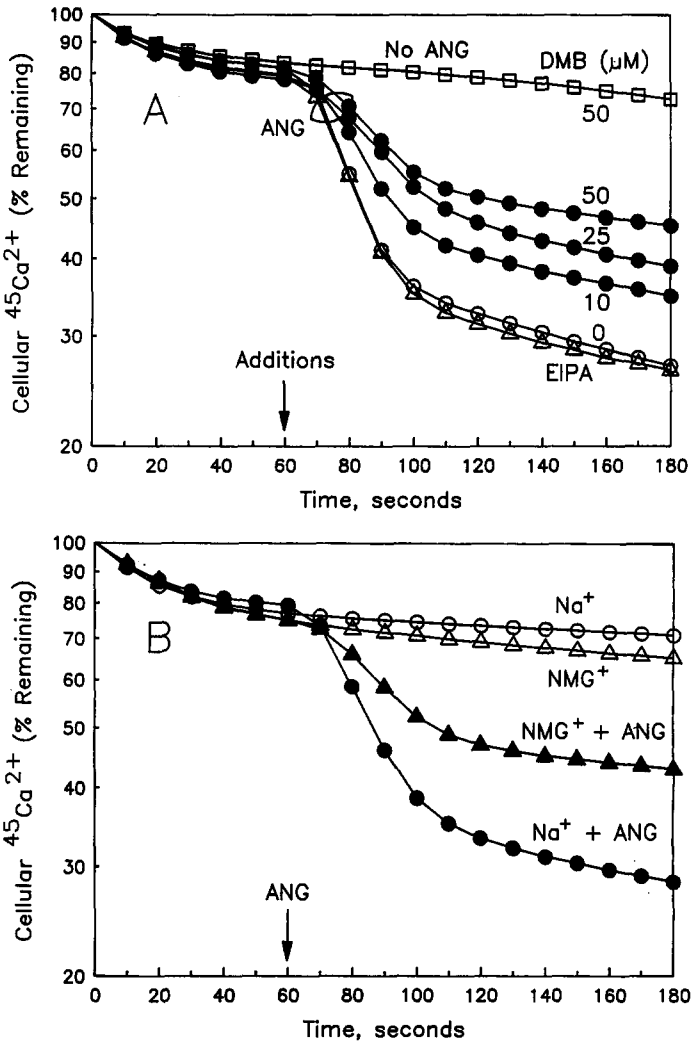


Fig. 5. Effects of DMB or the replacement of external Na^+ with NMG^+ on ANG-evoked $^{45}Ca^{2+}$ efflux. $^{45}Ca^{2+}$ efflux was measured after labeling the cultures for 2 hr with 1 mL of PSS containing 10 mM glucose and 20 μCi $^{45}Ca^{2+}$. (A) Efflux was started by rinsing the cultures eight times with PSS and adding 1 mL of PSS. From 60 to 180 sec some cultures received only 100 nM ANG (○), or 50 μM DMB (□). Some cultures (●) received DMB (10, 25, or 50 μM) and 100 nM ANG from 60 to 180 sec. Other cultures (△) received 10 μM EIPA and 100 nM ANG from 60 to 180 sec. (B) To assay efflux in the absence of external Na^+ the cultures were rinsed with PSS containing NMG^+ (△, ▲) instead of Na^+ (○, ●). ANG (100 nM) was present from 60 to 180 sec to the cultures indicated (▲, ●). Values are means of duplicates that differed by less than 2% remaining. Two additional $^{45}Ca^{2+}$ efflux experiments gave similar results.

with NMG^+ had no effect on basal or ANG-evoked increases in [^{3}H]inositol phosphates (Fig. 6) or the peak increase in cytosolic free Ca^{2+} produced by ANG, but potentiates the plateau phase of Ca^{2+} response to ANG [5]. Therefore, it appears that the removal of extracellular Na^+ or the addition of 25–50 μM DMB inhibits ANG-stimulated Ca^{2+} efflux by blocking Na^+-Ca^{2+} exchange in aortic myocytes.

Although amiloride compounds are potent inhibitors of Ca^{2+} channels [9, 10], it is unlikely that Ca^{2+} channels contribute significantly to $^{45}Ca^{2+}$ uptake in the present experiments. First, in rat aortic myocytes $^{45}Ca^{2+}$ influx produced by membrane depolarization [1] or ANG stimulation [5] is much less than $^{45}Ca^{2+}$ influx produced by inverting the Na^+ gradient [1]. Second, in Na^+-loaded myocytes, the replacement of extracellular Na^+ with choline, NMG^+, or K^+ produces similar increases in DMB-inhibitable $^{45}Ca^{2+}$ influx. Therefore, probably little or no $^{45}Ca^{2+}$ influx occurs by Ca^{2+} channels under the conditions used to assay Na^+-Ca^{2+} exchange in these cells. Third, DMB (Fig. 4) or external Na^+ withdrawal [5] similarly inhibits net Ca^{2+} efflux evoked by ANG. Net Ca^{2+} efflux against a large electrochemical Ca^{2+} gradient can occur only via an active transporter, not via channels.

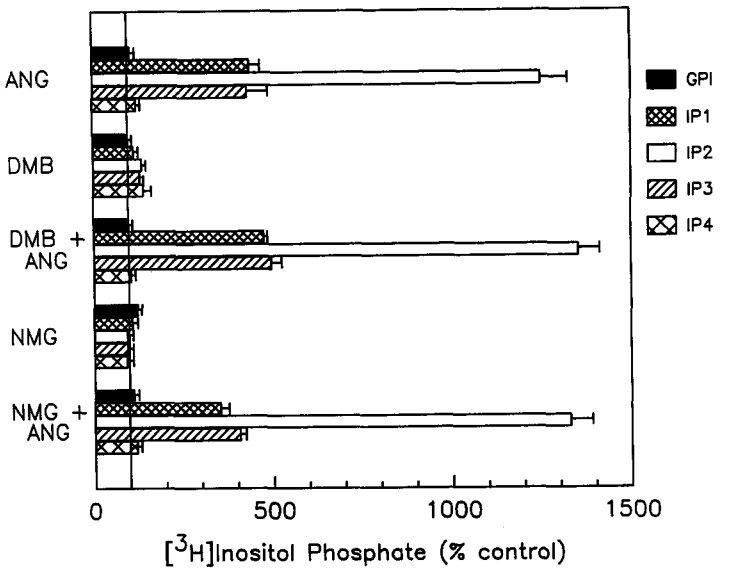


Fig. 6. Lack of effects of DMB or the replacement of external Na^+ with NMG^+ on ANG-evoked [^{3}H]inositol phosphate production. The cultures (1.8 mg protein/100 mm diameter dish) were rinsed twice with PSS or PSS containing NMG^+ instead of Na^+ and incubated for 1 min with 100 nM ANG or 50 μM DMB or both as indicated. Control values (counts/min/culture) were: glycerophosphoinositol (GPI) 11,144 ± 810; inositol phosphate (IP_1) 4,155 ± 874; inositol bisphosphate (IP_2) 839 ± 119; inositol trisphosphate (IP_3) 744 ± 107; inositol tetrakisphosphate (IP_4) 4289 ± 324 (mean ± SE, N = 6). Values are means ± SE (N = 4–8).

The lack of effect of EIPA on ANG-evoked net Ca^{2+} efflux (Fig. 4) or $^{45}Ca^{2+}$ efflux (Fig. 5) suggests that Na^+-H^+ exchange has no major role in short-term Ca^{2+} regulation in these cells. The IC_{50} of EIPA towards Na^+-H^+ exchange was 19 and 41 nM at 5 and 120 mM external Na^+ respectively (Fig. 3 and Table 1). These IC_{50} values are similar to those reported for a variety of other cultured cells including chick skeletal and cardiac muscle [35, 36], porcine kidney epithelial cells [39], and human epidermoid carcinoma (A431) cells [38]. Haggerty *et al.* [39] have suggested that Na^+-H^+ exchangers with this high affinity for EIPA is a "housekeeping" form of the exchanger that contributes to cytosolic pH homeostasis rather than transepithelial transport. Reynolds *et al.* [22] observed that two amiloride derivatives that potently inhibit Na^+-H^+ exchange did not decrease the contraction of rat aortic rings by phenylephrine. The present findings on aortic myocytes are consistent with the contraction data.

DCB appears to be a mechanism-based inhibitor of Na^+-Ca^{2+} exchange in sarcolemmal vesicles [37]. The protonated acylguanidinium moiety appears to bind mostly to a Na^+ specific site because the inhibition of exchange by lower concentrations of DCB is competitive with Na^+ [37]. At higher concentrations DCB also interacts with a site that is common for Na^+, K^+, and Ca^{2+} [37]. DCB and DMB are equipotent inhibitors of exchange in membrane vesicles, bovine brain, and guinea pig and porcine heart [14, 40]. In cultured aortic muscle cells, however, DMB was 10 times more potent than DCB towards Na^+-Ca^{2+} exchange (Table 1). Differences in assay conditions or in exchanger properties between intact cells versus isolated membranes apparently do not cause the relative differences in the potencies of DMB and DCB. First, DMB was almost equipotent towards exchange activity in intact smooth muscle cells (Table 1) or sarcolemmal vesicles from these cells (Lyu R-M and Smith JB, unpublished data) and membranes from heart, brain, or a rat pituitary tumor cell line (GH_3) [14]. Second, Kaczorowski *et al.* [14] found that DMB is three times more potent than DCB towards exchange activity in membrane vesicles from GH_3 cells. Species or cell type differences in exchanger structure may account for the differences in the relative potency of DMB and DCB. Further structure–activity studies with amiloride derivatives may reveal distinct features of the cardiac and smooth muscle Na^+-Ca^{2+} exchangers.

In conclusion, we have compared the potency of seven different amiloride compounds towards Na^+-H^+ and Na^+-Ca^{2+} exchange in rat aortic myocytes. Selective inhibition of Na^+-Ca^{2+} exchange with 2,4-DMB markedly inhibited Ca^{2+} efflux evoked by angiotensin II, whereas selective inhibition of Na^+-H^+ exchange with EIPA had no effect on angiotensin evoked Ca^{2+} efflux. These findings suggest that Na^+-H^+ exchange plays no major role in acute Ca^{2+} regulation by vasoconstrictors and corroborate the evidence from Na^+ substitution experiments that Na^+-Ca^{2+} exchange is an important pathway of Ca^{2+} efflux in vascular smooth muscle.

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