Eplerenone Blocks Nongenomic Effects of Aldosterone on the Na^+/H^+ Exchanger, Intracellular Ca^{2+} Levels, and Vasoconstriction in Mesenteric Resistance Vessels

Luis Michea, Ana M. Delpiano, Catalina Hitschfeld, Lorena Lobos, Sergio Lavandero, and Elisa T. Marusic

Laboratory of Cellular and Molecular Physiology (L.M., A.M.D., C.H., L.L., E.T.M.), Faculty of Medicine, University Los Andes; and FONDAP Center for Molecular Studies of the Cell (S.L.), Faculties of Chemical and Pharmaceutical Sciences and Medicine, University of Chile, 6782468 Santiago, Chile

There is increasing evidence for rapid nongenomic effects of aldosterone. Aldosterone has been demonstrated to alter intracellular pH and calcium in isolated cells. However, few studies have correlated these effects with aldosterone-mediated physiological responses. Therefore, we studied rapid effects of aldosterone on vascular reactivity, intracellular Ca^{2+}, and pH in resistance vessels. Furthermore, we explored whether the new antimineralocorticoid drug eplerenone could effectively block nongenomic aldosterone-mediated effects. The vasoconstrictor action of aldosterone was examined directly by determining the diameter of small resistance mesenteric vessels (160–200 μm resting diameter), simultaneously with intracellular pH or Ca^{2+}. Aldosterone (10 nM) caused a rapid constriction of resistance vessels (8.1% ± 1.0% reduction in the diameter below control conditions, $P < 0.05$). Aldosterone potentiated phenylephrine-mediated constriction in small and large mesenteric vessels. Aldosterone induced a rapid increase of intracellular Ca^{2+} and cellular alkalinization. Vasoconstrictor action of aldosterone and nongenomic effects on the sodium-proton exchanger (NHE1) activity or intracellular Ca^{2+} responses was abolished by eplerenone. The vasoconstrictor response of aldosterone was related to phosphatidylinositol 3-kinase (PI3-K): the hormone decreased protein kinase B phosphorylation; pharmacological inhibition of PI3-K (10 μM LY294002 or 1 μM wortmannin) increased arterial contractility. Inhibitors of ERK 1/2 phosphorylation (15 μM PD98059) had no effect on aldosterone-mediated vasoconstriction. Inhibition of protein kinase C with 1 μM bisindolylmaleimide I and/or inhibition of NHE1 with 100 μM amiloride abolished aldosterone vasoconstrictor action of resistance mesenteric arteries. We conclude that aldosterone-mediated increase in vascular tone is related to a nongenomic mechanism that involves protein kinase C, PI3-K, and NHE1 activity. Eplerenone is an effective blocker of nongenomic effects of aldosterone in vascular tissue.

SEVERAL STUDIES HAVE shown deleterious effects of aldosterone in the cardiovascular system (1–3). In fact, following the pioneer work from Weber's laboratory (4), a series of studies on the direct action of aldosterone in the heart have been reported (5–7). The fact that the effects of aldosterone occur over a relatively extended time course (days/weeks) is indicative of a genomic action of the hormone (8).

Nongenomic actions of aldosterone have been studied *in vitro* in isolated human arteries (9), vascular smooth muscle cells (VSMCs) (10), lymphocytes (11), and endothelial cells (12) which include activation of the sodium-proton exchanger (NHE1) and increase in Ca^{2+} intracellular levels. Also, a biphasic effect of aldosterone on the Na pump has been described: an initial, nongenomic inhibition of the Na^+, K^+-ATPase activity, followed by the classical genomic action of aldosterone on the Na pump (13). Spironolactone, a nonspecific mineralocorticoid receptor antagonist, failed to block nongenomic aldosterone effects on NHE1 or intracellular Ca^{2+} (9, 14). However, eplerenone, a specific mineralocorticoid antagonist, completely blocked the nongenomic aldosterone action on the Na pump activity of isolated arteries (13).

Little is known about the physiological significance of nongenomic aldosterone action in resistance blood vessels. Recently Arima *et al.* (15) demonstrated that aldosterone caused nongenomic vasoconstiction in the glomerular microcirculation; but Uhrenholt *et al.* (16) concluded that aldosterone inhibits depolarization-induced vasoconstriction in renal efferent arterioles by a rapid nongenomic mechanism.

Based on the potential ability of aldosterone to acutely increase intracellular Ca^{2+} levels in vasculature smooth muscle cells (16), we postulated that aldosterone could favor vasoconstriction in blood vessels. We tested this hypothesis in rat mesenteric conduit and resistance arteries. The effects of aldosterone on vascular diameter of resistance vessels, simultaneously with measurements on intracellular calcium concentration and/or intracellular pH, were assessed in microperfused-pressurized arteries. To determine whether short-term aldosterone exposure might regulate different signaling pathways, we assessed the involvement of protein kinase C (PKC), ERK ½, and p38 MAPK as well as the phosphatidylinositol 3-kinase (PI3-K), which are related to aldosterone action in other tissues (16–19). In the present

Abbreviations: Bisindole I, Bisindolylmaleimide-I; KRB, Krebs-Ringer bicarbonate; MEK, MAPK kinase; NHE1, sodium-proton exchanger; PBST, PBS containing Tween 20; PI3-K, phosphatidylinositol 3-kinase; PKB, protein kinase B; VSMC, vascular smooth muscle cell.

work, we provide evidence of rapid aldosterone vasoconstrictor effect; a response that was blocked by eplerenone, a new mineralocorticoid receptor antagonist. We also provide evidence that nongenomic aldosterone actions on NHE1 and intracellular Ca^{2+} levels were blocked by eplerenone.

Materials and Methods

Animals

Male Sprague Dawley rats weighting between 180 and 220 g were allowed free access to tap water and standard rat chow. The Ethics Committee of the Faculty of Medicine of University Los Andes approved all protocols for animal experimentation, according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Vascular reactivity experiments

The superior mesenteric artery was removed and placed in cold (4 C) physiological Krebs-Ringer bicarbonate (KRB), containing (in millimoles per liter) 120 NaCl, 4.2 KCl, 1.18 KH_2PO_4, 1.2 $MgSO_4$, 1.3 $CaCl_2$, 25 Na_2HCO_3, 5 D-glucose, 5 HEPES, and 1.2 pyruvate, equilibrated with 5%CO_2-95%O_2 (pH 7.4). The tissue was cleaned of all adipose and connective tissue and cut into rings (each 3 mm long). Extreme care was taken during preparation of the rings to avoid stretching the tissue. Standard isometric tension measurements were performed, as previously described (20). Briefly, the rings were mounted on two 27-gauge stainless steel wires; the lower one was attached to a stationary glass rod, and the upper one was attached to a force-displacement transducer (Grass, Quincy, MA; FT-03C). The transducer was connected to a Grass polygraph (model 7) for continuous recording of blood vessel tension. After the equilibration period, the vascular rings were stabilized by two successive near-maximal contractions with KCl (60 mM). Phenylephrine-induced contraction (dose-response curve) was studied before and after incubation with 10 nM aldosterone for 10 min for each vascular ring.

Contractility of small-diameter mesenteric arteries and fluorometric determination of intracellular Ca^{2+} and pH

Arterial diameter was measured simultaneously with intracellular calcium or pH in perfused-pressurized mesenteric branches, according to previously described methods (21). Briefly, the rats were euthanized by decapitation and the mesenteric arcade was excised and placed in cold KRB. First- and second-order branches from the superior mesenteric artery (160–200 μm in diameter) from experimental animals were cleaned from adventitial tissue, transferred to a 2-ml vial, and incubated with the fluorescent dye FURA2-AM (1.0 μg/ml in KRB, Molecular Probes, Eugene, OR) for 75 min with gentle agitation at 37 C in a water-saturated atmosphere containing 95% O_2-5%CO_2. After the dye was loaded, tissue segments were cannulated at both ends (Microfilm, WPI, Sarasota, FL), and mounted in a thermostatically controlled 10-ml chamber. Temperature in the chamber was maintained at 37 C (BTC 9090 temperature controller, Brainchild Electronic Ltd., Taiwan). Tissues were superfused (flow rate 2.5 ml/min, peristaltic pump, Minipulse-2, Gilson, Villiers-Le-Bel, France) and microperfused (25 μl/min, syringe pump SP101i, WPI) with constantly gassed KRB for 30 min to remove extracellular dye. Intraluminal pressure was constantly monitored (pressure transducer P23XL, Spectramed, polygraph model 7, Grass) and set at 50 mm Hg by linking the distal end of the outflow cannula to a pressure column.

Fluorescent measurements were made with a dual-excitation wavelength imaging system using an Eclipse E400 epifluorescence microscope with a Fluor ×10 water immersion objective (Nikon, Tokyo, Japan) attached to an optical filter changer (λ 10–2, Sutter Instruments Co., Navato, CA). Emitted fluorescence was acquired by an intensified charge-coupled device video camera (IC-100, Photon Technology International, Lawrenceville, NJ) and processed by a scientific imaging software (IPLab Spectrum, Scanalytics, Fairfax, VA). Wavelengths for excitation were 340 and 380 nm; emission wavelength was 520 nm. Intracellular calcium levels are calculated as integral ratio from emission at 520 nm under variable excitation wavelengths 340/380 (21, 22). The images obtained at 380 nm excitation were used to estimate average vessel diameter of a 250-μm-long segment. In a separate set of experiments, intracellular pH was determined by monitoring the fluorescence of acetoxymethyl ester of the pH-sensitive dye 2′, 7′-bis(2-carboxyethyl)-5,6-carboxyfluorescein (5 μM) as previously described (9). Arteries were prepared and loaded with the dye as indicated for FURA2 experiments. Wavelengths for excitation were 495 and 440 nm; emission wavelength was 530 nm. The ratio of the 495/440-nm fluorescence values was used to estimate intracellular pH.

Drug treatments

The effect of aldosterone (10 nM) on arterial contractility was measured in the presence of hormone alone and in arteries pretreated with bisindolylmaleimide-I (Bisindole I; 1 μM), LY294002 (10 μM), PD98059 (15 μM), or wortmannin (100 nM and 1 μM) as indicated in the figures. All drugs were added 10 min before the addition of the hormone, and they were present throughout the study. Aldosterone was dissolved in ethanol and diluted in KRB (10^{-6} M) and finally added (1:100 vol/vol) into the superfusion chamber (0.01% methanol final concentration). Drugs were prepared as stock solutions and diluted in KRB to the desired concentration before use. To test drug effects, all the superfusion volume was quickly changed (<10 sec), and solutions of identical composition were used in the new superfusion media. Bisindole I was dissolved in dimethylsulfoxide (final concentration in KRB 0.005%). Identical aliquots of solvents used for the drugs were added to the control and hormone-treated paired experiments.

To study the effects of aldosterone, eplerenone, and LY294002 on ERK 1/2 and protein kinase B (PKB) phosphorylation, intact rat mesenteric arteries were carefully dissected, cleaned from adventitial tissue, separated into five segments, and incubated in vials containing 2 ml KRB constantly gassed with 95% O_2-5% CO_2 at 37 C (water saturated atmosphere) with gentle agitation (Dubnoff, Chicago, IL). After 45–60 min of washing, arterial segments were transferred to a new vial and incubated with the corresponding hormone, inhibitor, and/or solvent in a paired fashion, as indicated in *Results*. After the incubation period, mesenteric first- and second-order branches were quickly removed and used to prepare tissue extracts.

Preparation of tissue extracts

Soluble protein fractions from mesenteric arteries were prepared. Small-diameter arteries were homogenized in an ice-cold glass-glass homogenizer (122, VWR United, Strasbourg, France) into 150 μl of ice-cold lysis buffer: 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 1 mM sodium pyrophosphate, 1 mM sodium vanadate, 140 mM NaCl, and complete mini-protease inhibitor cocktail minitablets (1 tablet per 10 ml, Roche, Stockholm, Sweden), 10% (vol/vol) glycerol and 1% (vol/vol) Triton X-100. Samples were centrifuged at 14,000 × *g* for 20 min at 4 C, and supernatants were divided in aliquots. One aliquot was stored at −70 C for protein concentration determination (BCA method using BSA as a standard), and the other aliquot was heated at 95 C with 0.33 volumes of 4× SDS-PAGE sample buffer and stored at −20 C.

Western blot analysis for ERK, p38-MAPK, and PKB

Tissue extracts (15 μg protein for ERK and p38MAPK and 35 μg protein for PKB) were separated by SDS-PAGE on 12% (wt/vol) polyacrylamide gels and electrotransferred to nitrocellulose (ERK and p38-MAPK) or polyvinylidene di fluoride (for PKB) membranes using a Trans-blot unit (Bio-Rad Laboratories, Hercules, CA) for 1.5 h at 100 V. Protein gels stained with Coomassie blue were used for densitometry to check for protein abundance in each sample. Membranes were blocked with 3% (wt/vol) BSA in PBS (pH 7.4) containing 0.1% (vol/vol) Tween 20 (PBST) overnight at 4 C. Then membranes were incubated for 1 h with the primary antibody (25 C). After washing in PBST (4 × 15 min each), blots were incubated for 1 h at room temperature with horseradish peroxidase-linked secondary antibody (1:5000, 3% wt/vol BSA in PBST). Finally, membranes were washed again in PBST (4 × 15 min each). Specific binding was detected using enhanced chemiluminescence with exposure to film (Kodak, Rochester, NY) for 10–90 sec. After stripping the membranes were subjected to immunoblot analysis with anti-phospho-ERK antibody or anti-phospho-p38-MAPK antibody for 1 h at 25 C.

In the case of phospho-PKB and total PKB detection, polyvinylidene di fluoride membranes were blocked with 5% nonfat milk in Tris-buffered saline (20 mM Tris/HCl, 137 mM NaCl) plus 0.1% (vol/vol) Tween 20 (TTBS) overnight. They were later incubated as described for nitrocellulose membranes but using Tris-buffered saline (20 mM Tris/HCl, 137 mM NaCl) plus 0.1% (vol/vol) Tween 20. Blots were quantified by laser-scanning densitometry, and results were expressed as the ratio of phosphorylated protein kinase to total protein kinase levels. The ratio of phosphorylated protein kinase to the total protein kinase was calculated in each experiment, and results were expressed as the relative band ratio of each experimental condition, compared with the control-paired sample.

Materials

All biochemicals and other reagents were purchased from Sigma (St. Louis, MO) unless stated otherwise. Enhanced chemiluminescence was obtained from PerkinElmer Life Sciences (Chemiluminescence Reagent Plus, MA). Autoradiographic films were from Kodak. Prestained molecular mass standard proteins were purchased from Invitrogen (Carlsbad, CA). Protein assay reagents (BCA protein assay kit) were from Pierce (Rockford, IL). Bisindole-I, LY294002, PD98059, and wortmannin were from Calbiochem-Novabiochem Corp. (San Diego, CA). Polyclonal antibodies against phosphorylated and total ERK, p38-MAPK, and PKB used in Western blot analysis were purchased from Cell Signaling Technology Inc. (Beverly, MA). FURA2-acetoxymethylester was from Molecular Probes Inc. Eplerenone was kindly provided by Pharmacia.

Statistical analysis

Values are reported as mean ± SEM. Differences between mean values were assessed by ANOVA or Student's *t* test (paired). Values were considered significant for $P < 0.05$.

Results

Vasoconstrictor response and intracellular Ca^{2+}

Phenylephrine-elicited concentration-dependent constrictions were measured in isolated superior mesenteric rings in the presence and absence of aldosterone. As shown in Fig. 1, aldosterone (10 nM) significantly increased phenylephrine response. No changes were observed in the threshold of phenylephrine-contractile dose. Aldosterone itself had no effect in vascular tone, as measured by isometric tension studies, in large mesenteric vessels. However, in small branches of the mesenteric arteries (160–200 μm), aldosterone elicited a significant vasoconstrictor response (8.1% ±1.0% reduction of resting diameter after 10 min of hormone addition, Fig. 2). Eplerenone (2 μM) completely blocked aldosterone-vasoconstrictor response. As shown in Fig. 2A, aldosterone induced a rapid rise of intracellular Ca^{2+}. Eplerenone suppressed the rapid aldosterone-mediated rise in intracellular Ca^{2+}. Figure 2B depicts the changes of arterial diameter in the presence of 10 nM aldosterone and in the measure of phenylephrine dose response. Eplerenone blocked aldosterone-mediated vasoconstriction. Eplerenone alone had no effect on intracellular Ca^{2+} levels or in phenylephrine-induced responses.

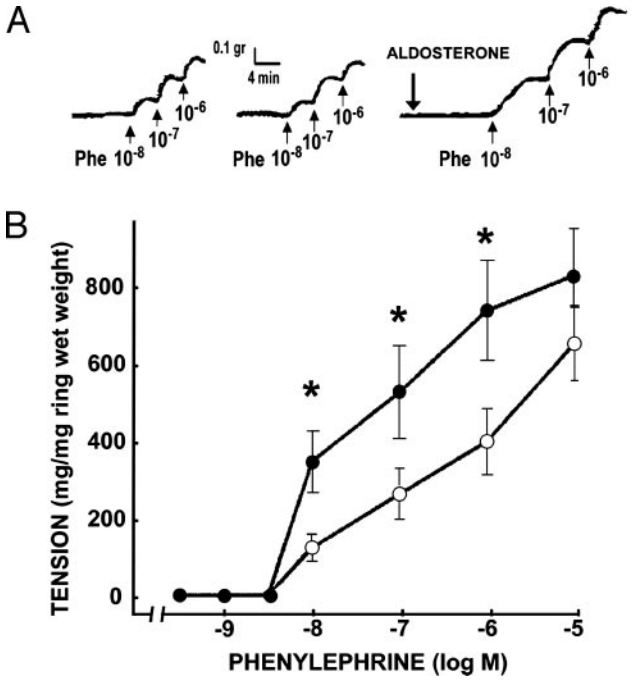

FIG. 1. Effect of aldosterone (10 nM) on phenylephrine-mediated vasoconstriction in large mesenteric vessels. A, Representative original polygraph recordings of the dose-dependent constrictor response to phenylephrine (Phe) in the absence and presence of 10 nM aldosterone. The first two traces correspond to consecutive phenylephrine dose-response curves; the third trace was obtained in the same artery 10 min after the addition of aldosterone. B, Mean values ± SE of six independent experiments. *, $P < 0.05$, compared with contraction in absence of aldosterone. *Open circles* represent constrictor responses in the absence of aldosterone; *filled circles* correspond to phenylephrine dose-response curve in the presence of 10 nM aldosterone.

Intracellular signals and rapid aldosterone action in mesenteric arteries

The involvement of PKC on aldosterone-mediated vasoconstriction in resistance mesenteric vessels was evaluated in the presence of 1 μM Bisindole I. As shown in Fig. 3, this inhibitor completely blocked the effect of aldosterone on the vascular tone, without affecting the phenylephrine dose-response curve.

To determine whether ERK 1/2 or p38 MAPK were activated by aldosterone, mesenteric arteries were incubated with 10 nM aldosterone for different periods of time. Extracts were then prepared and assayed for the phosphorylated forms of ERK 1/2 and p38 MAPK by Western blot analysis. As shown in Fig. 4A, aldosterone had no effect on ERK phosphorylation, measured up to 20 min after hormone addition. ERK activity was also measured when eplerenone was added in addition to aldosterone (Fig. 4B). These findings are consistent with the lack of effect of PD98059 [MAPK kinase (MEK)-dependent signal inhibitor] on the aldosterone-vasoconstrictor response (Fig. 4C).

In many tissues, activation of p38 MAPK is associated with hormonal action. However, aldosterone had not effect on p38 MAPK phosphorylation in resistance mesenteric arteries after 5, 10, or 20 min, as measured by Western blot analysis (data not shown).

The PI3-K signaling pathway has been involved in many

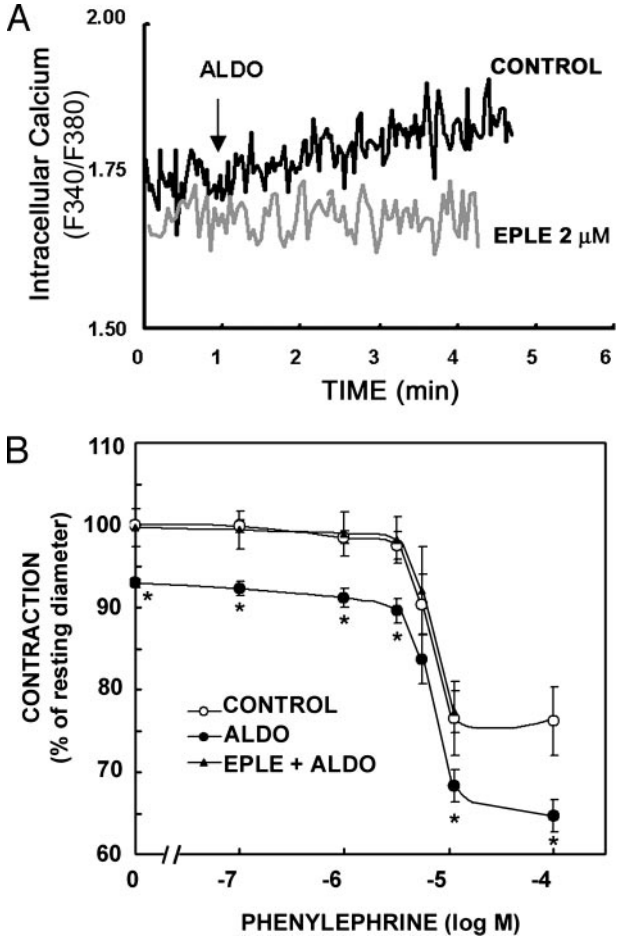

FIG. 2. Effect of aldosterone on intracellular Ca^{2+} and vascular diameter in resistance mesenteric vessels. Intracellular Ca^{2+} was measured by FURA-2 imaging in isolated small vessels, simultaneously with the arterial diameter. A, Representative original recordings of six independent experiments: 10 nM aldosterone (ALDO) promote rapid calcium increase in mesenteric arteries; 2 μM eplerenone (EPLE), added 10 min in advance of aldosterone inhibited the rise in intracellular-free Ca^{+} caused by aldosterone. B, Effect of 10 nM aldosterone on the arterial diameter and after phenylephrine dose-response curve and/or in the presence of 2 μM eplerenone. *, $P < 0.05$, compared with contractile response in absence of aldosterone.

aldosterone-mediated effects. PKB is activated by hormones and growth factors in a wortmannin-sensitive pathway involving PI3-K. Therefore, to evaluate PI3-K, we measured PKB phosphorylation. Figure 5, A and B, includes the results of total and phospho-PKB levels after incubation of mesenteric arteries with 10 nM aldosterone for 10 min. As shown in the Fig. 5, aldosterone had a significant inhibitory effect on protein phosphorylation, compared with control arteries. Vessels incubated in the presence of aldosterone plus 5 μM eplerenone were not different from controls. As expected, the treatment with 10 μM LY294002 completely blocked the phosphorylation of PKB. Consistent with these observations, LY294002 increased the vasoconstrictor response of mesenteric arteries, and no additional effect of aldosterone was observed (Fig. 5C). Similar results were obtained with the use of 0.1 or 1 μM wortmannin, another inhibitor of the PI3-K pathway (data not shown).

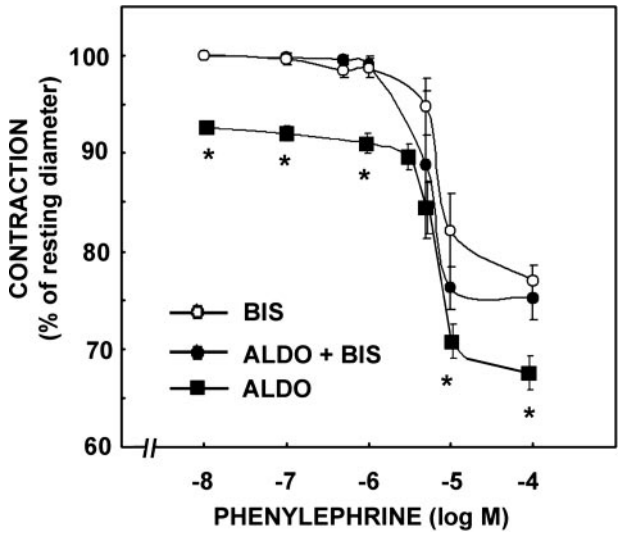

FIG. 3. Effect of PKC inhibition on aldosterone-mediated vasoconstriction responses. Dose-response curves to phenylephrine were measured in the presence of hormone alone (ALDO) and in mesenteric arteries pretreated with 1 μM Bisindole I (BIS); n = 4. *, $P < 0.05$ aldosterone alone *vs.* aldosterone plus Bisindole I.

Aldosterone vasoconstrictor response and the Na^{+}/H^{+} exchanger

Figure 6A depicts the rapid aldosterone-mediated rise of intracellular pH in isolated resistance mesenteric arteries. The aldosterone activation of NHE1 was completely blocked by 2 μM eplerenone or 100 μM amiloride (not shown). Eplerenone alone had no significant effect on intracellular pH.

To determine whether NHE1 is involved in the vasoconstrictor response to aldosterone, we assessed the effect of amiloride in resistance mesenteric vessels. As shown in Fig. 6B, 100 μM amiloride completely abolished aldosterone-mediated vasoconstriction. A partial inhibition of phenylephrine response was present.

Discussion

Depending on the cell type, aldosterone can elicit numerous rapid responses. In human mononuclear blood cells, aldosterone rapidly increases cellular volume as a consequence of an increment in intracellular Na^{+} and Ca^{2+} levels. Aldosterone activation of NHE1 has been described in VSMCs, endothelial cells, and human uterine and chorionic arteries. Spironolactone, a nonspecific mineralocorticoid receptor antagonist, had no effect on the above-mentioned nongenomic aldosterone actions (16); even though another aldosterone antagonist, RU28136, was an effective blocker of the aldosterone-mediated rise in intracellular pH in isolated uterine arteries (9). Here we present data that eplerenone, a new mineralocorticoid receptor antagonist, blocks rapid aldosterone effects on intracellular Ca^{2+} and pH.

Eplerenone binds specifically to the cytosolic receptor (23–26), and it is possible that the same receptor is related to both genomic and nongenomic action of aldosterone. In this regard, previous work from our group (9) indicated that when the protective mineralocorticoid receptor enzyme was blocked with carbenoxolone, cortisol mimicked the nongenomic aldo-

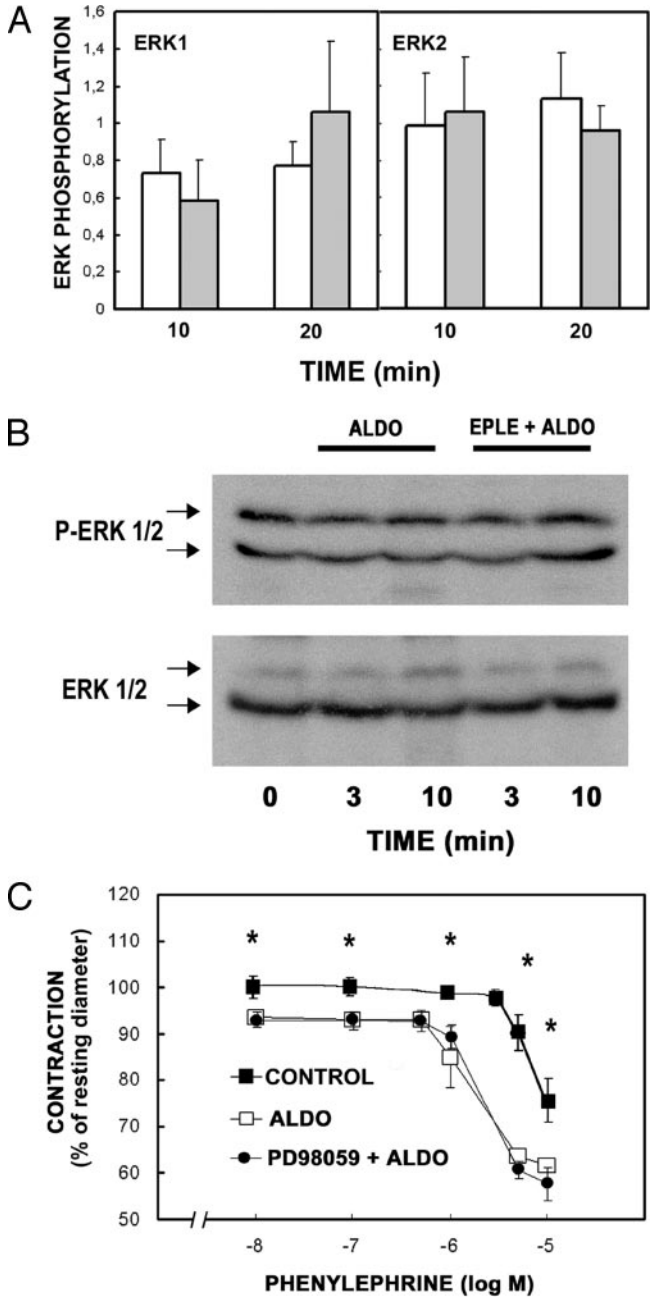

FIG. 4. Aldosterone and ERK 1/2 phosphorylation in mesenteric arteries. A, 10 nM aldosterone had no significant effect on ERK 1/2 phosphorylation at 10 and 20 min after the addition of the hormone (n = 4). *Open bars*, control; *gray bars*, aldosterone. B, Western blot analysis of the protein isolated from resistance arteries at 3 and 10 min in the presence of 10 nM aldosterone (ALDO) or aldosterone plus 2 μM eplerenone (EPLE); n = 3. C, Phenylephrine dose-response curves of aldosterone or aldosterone plus 15 μM PD98059, an inhibitor of MEK I. No significant differences were observed in the presence of the inhibitor, compared with aldosterone alone.

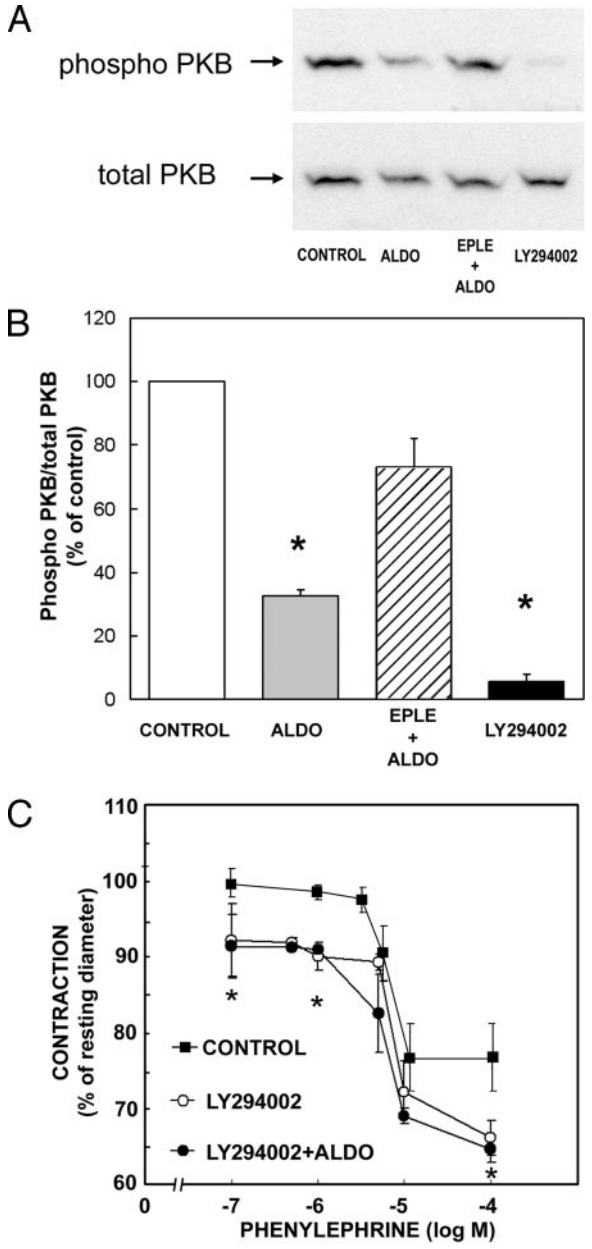

FIG. 5. Aldosterone and PI3-K pathway in mesenteric arteries. A, Representative Western blot analysis of phospho-PKB: incubation for 10 min in the presence of 10 nM aldosterone (ALDO) or 10 μM LY294002 significantly inhibited PKB phosphorylation; time 10 min. Eplerenone (2 μM, EPLE) suppressed the inhibitory effect of aldosterone. B, Bar graph represents mean ± SE (n = 3). C, LY294002 alone potentiated phenylephrine-mediated constriction (*, $P < 0.05$); no further vasoconstrictor effect of aldosterone plus LY294002 was observed.

sterone effect on human vascular tissue. Also, eplerenone blocked the biphasic effect of aldosterone on the Na pump activity in isolated rat arteries, as evidenced by a rapid nongenomic inhibition of Na^+-K^+-ATPase activity followed by the classical aldosterone genomic response on the number of Na pumps (13). The inhibitory effect on the Na pump was maximal 10 min after the addition of 10 nM aldosterone and was not inhibited by actinomycin D or cycloheximide. Therefore, we selected a 10-min period to measure most of aldosterone vasoconstrictor effects on rat mesenteric arteries; in fact, a significant increase in vascular tone was observed 10 min after the addition of the hormone. The effect of aldosterone on vascular tone was clearly evident in small resistance mesenteric vessels, whereas aldosterone potentiated phenylephrine-induced vasoconstriction in large mesenteric arteries. Our data are consistent

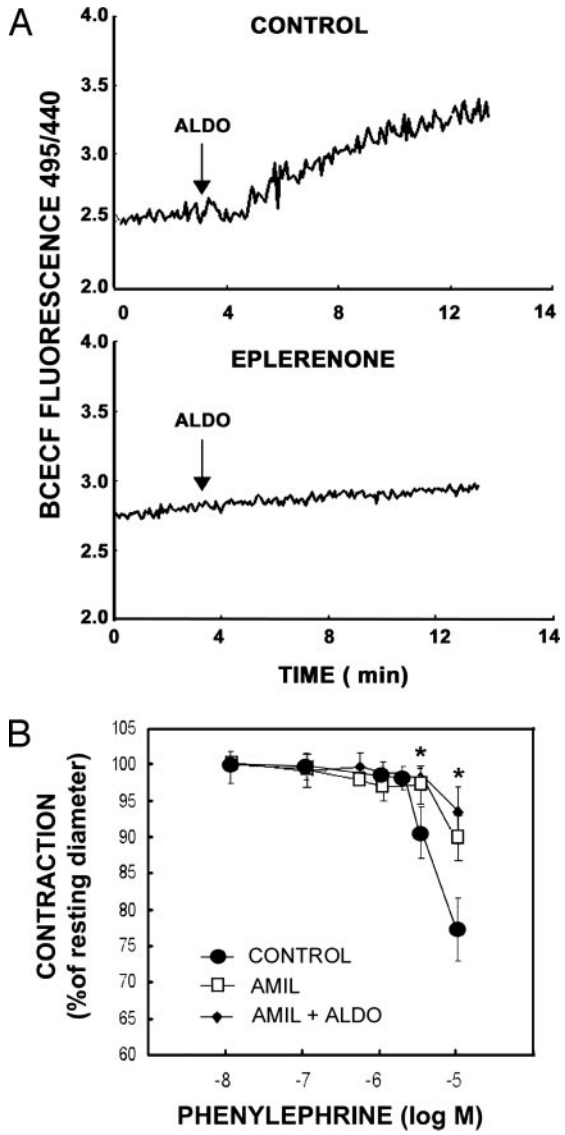

FIG. 6. Aldosterone and intracellular pH levels in mesenteric arteries. A, Representative 2′, 7′-bis(2-carboxyethyl)-5,6-carboxyfluorescein fluorescence ratio time course in the presence of 10 nM aldosterone (ALDO) alone or aldosterone plus 2 μM eplerenone. B, Contractile responses to phenylephrine in the presence of 100 μM amiloride (AMIL) or aldosterone plus amiloride. *, $P < 0.05$, compared with contractile response of phenylephrine alone.

with recent studies of Arima *et al.* (14) on the vasoconstrictor action of aldosterone in the afferent and efferent arterioles; but they differ from the work of Uhrenholt *et al.* (15), which reported that aldosterone had no direct effect in internal diameter of rabbit renal afferent arterioles; they concluded that aldosterone inhibits depolarization-induced vasoconstriction. Furthermore, Liu *et al.* (17) found that aldosterone attenuated phenylephrine-mediated constriction in endothelium-intact aortic ring preparations of normotensive Wistar rats. The reasons for this discrepancy with our results are not clear, but it may be due to differences between resistance vessels and conduit arteries used in the above work. It is known that in humans, the iv application of aldosterone significantly increases systemic vascular resistance within a few minutes (27, 28).

To study the mechanism(s) for aldosterone-induced vasoconstriction, we first examined the contribution of PKC because several studies demonstrated the role of PKC in the intracellular signaling for nongenomic aldosterone action on the VSMCs (29–33). As expected, we found that pretreatment with Bisindole I completely abolished aldosterone-induced constriction in small mesenteric arteries, demonstrating the participation of PKC and the phosphoinositide pathway in the intracellular signaling for aldosterone-induced constriction of resistance arteries. Mihailidou *et al.* (18) recently showed that rapid nongenomic effects of aldosterone in the heart are mediated by PKCϵ. However, the precise mechanism of post-PKC activation for rapid aldosterone constriction remains unclear. Long-term aldosterone action caused a significant increase in the activities of ERK 1/2, c-Jun N-terminal kinase, and big mitogen-activated protein kinase-1 in renal cortical tissue, whereas p38 MAPK activity remained unchanged (34). We now found that aldosterone inhibited PKB phosphorylation; furthermore, inhibition of PI3-K with either LY294002 or wortmannin also induced vasoconstriction. These observations are consistent with the involvement of PI3-K in aldosterone-mediated effects on vasomotion. However, Uhrenholt *et al.* (15) and Liu *et al.* (17) in different vascular territories found that short-term aldosterone decreases vascular reactivity simultaneously with PI3-K-activation.

Many studies in cultured cells have found ERK activation by aldosterone, but it is not known whether short-term aldosterone action implicates ERK activity in intact vessels, as seen in VSMCs. Recently Mazak *et al.* demonstrated that aldosterone potentiates angiotensin II-induced signaling in vascular smooth muscle cells (19). Masset *et al.* (29) noted that there are important tissue and/or stimulus-specific differences in the effect of the PKC and MAPK pathway. For example, Watts (35) reported that PD98059 reduced serotonin but not angiotensin II-induced contraction of aortic rings. In contrast, at the arteriolar level, ERK 1/2 play only a minor role, if any, in norepinephrine-induced vascular response (29). Also, arteriolar response to norepinephrine was insensitive to inhibition of p38 MAPK. We found that p38 MAPK is not activated by short-term aldosterone exposure in mesenteric arteries. Also, no rapid changes were observed in ERK 1/2 phosphorylation. Therefore, we conclude that aldosterone has no rapid activation on the above-mentioned kinases in resistance mesenteric arteries. This possibility was further evaluated with the studies on vasoconstriction: PD98059, a known inhibitor of the ERK/MAPK activator kinase MEK-1, was unable to block aldosterone-mediated increase of vascular tone.

The last part of our study aimed to explore whether activation of NHE1 by aldosterone was involved in the effect of the hormone on vascular tone. Young and Funder (5) suggested that cardiac fibrosis may involve coronary VSMCs and that NHE1 activity is a possible contributor to the cascade of transcriptional events that contribute to the deleterious effect of aldosterone in the heart. Tepel *et al.* (36) showed that activation of NHE1 produces vasoconstriction of renal resistance vessels. The exchanger is quiescent at physiological intracellular pH levels, but it is activated rapidly in response to intracellular acidosis. Several stimuli,

including growth factors and hormones, increase NHE1 activity through various signaling pathways (37). These include direct phosphorylation of the cytoplasmic regulatory domain (38), binding of calmodulin (39), and interaction with accessory proteins (40). Although a regulatory role of NHE1 has been suggested for several pathways (37, 41), the most extensively studied kinase mechanism of NHE1 activation involves PKC (37, 42). Here we found that aldosterone increased NHE1 activity, an effect that was blocked by 100 μM amiloride as well as by eplerenone; both drugs also inhibited aldosterone action on vascular tone. Also, amiloride had a partial inhibitory effect on phenylephrine-vasoconstriction response, as previously suggested (43).

The present study highlights the nongenomic effects that aldosterone exerts on NHE1 activity, intracellular Ca^{2+}, and the contractile performance of systemic resistance arteries. Eplerenone, a new specific mineralocorticoid receptor antagonist, blocks both rapid aldosterone-mediated vasoconstriction and the nongenomic effects on intracellular Ca^{2+} and pH levels. Further studies are necessary to resolve whether eplerenone blockage of rapid aldosterone effects on resistance arteries are mediated by the classic cytosolic mineralocorticoid receptor or a new membrane receptor present in the vascular tissue.

Acknowledgments

Address all correspondence and requests for reprints to: Luis Michea, M.D., Ph.D., Faculty of Medicine, University Los Andes, S. Carlos Apoquindo 2200, Las Condes, 6782468 Santiago, Chile. E-mail: lmichea@uandes.cl.

This work was supported by Grant 1010185 from Fondo Nacional de Investigación Científica y Tecnológica (tto L.M.) and in part by Fondo Investigacíon Avanzada Grant 15010006 (to S.L.).

References

1. **Rocha R, Funder JW** 2002 The pathophysiology of aldosterone in the cardiovascular system. Ann NY Acad Sci 970:89–100
2. **Weber KT** 2001 Aldosterone in congestive heart failure. N Engl J Med. 345: 1689–1697
3. **Funder JW** 2002 New biology of aldosterone, and experimental studies on the selective aldosterone blocker eplerenone. Am Heart J 144:S8–S11
4. **Brilla CG, Weber KT** 1992 Mineralocorticoid excess, dietary sodium, and myocardial fibrosis. J Lab Clin Med 120:893–901
5. **Young M, Funder J** 2003 Mineralocorticoid action and sodium-hydrogen exchange: studies in experimental cardiac fibrosis. Endocrinology 144:3848–3851
6. **Rocha R, Stier Jr CT** 2001 Pathophysiological effects of aldosterone in cardiovascular tissues. Trends Endocrinol Metab 12:308–314
7. **Weber KT, Sun Y, Wodi LA, Munir A, Jahangir E, Ahokas RA, Gerling IC, Postlethwaite AE, Warrington KJ** 2003 Toward a broader understanding of aldosterone in congestive heart failure. J Renin Angiotensin Aldosterone Syst 4:155–163
8. **Fujisawa G, Dilley R, Fullerton MJ, Funder JW** 2001 Experimental cardiac fibrosis: differential time course of responses to mineralocorticoid-salt administration. Endocrinology 142:3625–3631
9. **Alzamora R, Michea L, Marusic ET** 2000 Role of 11β-hydroxysteroid dehydrogenase in nongenomic aldosterone effects in human arteries. Hypertension 35:1099–1104
10. **Christ M, Douwes K, Eisen C, Bechtner G, Theisen K, Wehling M** 1995 Rapid effects of aldosterone on sodium transport in vascular smooth muscle cells. Hypertension 25:117–123
11. **Wehling M** 1995 Aldosterone specific membrane receptors, rapid activation of the sodium-hydrogen exchanger, and cardiovascular implications. Cardiovasc Res 29:167–171
12. **Schneider SW, Yano Y, Sumpio BE, Jena BP, Geibel JP, Gekle M, Oberleithner H** 1997 Rapid aldosterone-induced cell volume increase of endothelial cells measured by the atomic force microscope. Cell Biol Int 21: 759–768
13. **Alzamora R, Marusic ET, Gonzalez M, Michea L** 2003 Nongenomic effect of aldosterone on Na+, K+-adenosine triphosphatase in arterial vessels. Endocrinology 144:1266–1272
14. **Losel RM, Falkenstein E, Feuring M, Schultz A, Tillmann HC, Rossol-Haseroth K, Wehling M** 2003 Nongenomic steroid action: controversies, questions, and answers. Physiol Rev 83:965–1016
15. **Arima S, Kohagura K, Xu HL, Sugawara A, Abe T, Satoh F, Takeuchi K, Ito S** 2003 Nongenomic vascular action of aldosterone in the glomerular microcirculation. J Am Soc Nephrol 14:2255–2263
16. **Uhrenholt TR, Schjerning J, Hansen PB, Norregaard R, Jensen BL, Sorensen GL, Skott O** 2003 Rapid inhibition of vasoconstriction in renal afferent arterioles by aldosterone. Circ Res 93:1258–1266
17. **Liu SL, Schmuck S, Chorazcyzewski JZ, Gros R, Feldman RD** 2003 Aldosterone regulates vascular reactivity: short-term effects mediated by phosphatidylinositol 3-kinase-dependent nitric oxide synthase activation. Circulation 108:2400–2406
18. **Mihailidou AS, Mardini M, Funder JW** 2004 Rapid, nongenomic effects of aldosterone in the heart mediated by epsilon protein kinase C. Endocrinology 145:773–780
19. **Mazak I, Fiebeler A, Muller DN, Park JK, Shagdarsuren E, Lindschau C, Dechend R, Viedt C, Pilz B, Haller H, Luft FC** 2004 Aldosterone potentiates angiotensin II-induced signaling in vascular smooth muscle cells. Circulation 109:2792–2800
20. **Palacios J, Marusic ET, Lopez NC, Gonzalez M, Michea L** 2004 Estradiol-induced expression of N(+)-K(+)-ATPase catalytic isoforms in rat arteries: gender differences in activity mediated by nitric oxide donors. Am J Physiol Heart Circ Physiol 286:H1793–H1800
21. **Schuster A, Oishi H, Beny JL, Stergiopulos N, Meister JJ** 2001 Simultaneous arterial calcium dynamics and diameter measurements: application to myoendothelial communication. Am J Physiol Heart Circ Physiol 280:H1088–H1096
22. **Arnon A, Hamlyn JM, Blaustein MP** 2000 Ouabain augments Ca(2+) transients in arterial smooth muscle without raising cytosolic Na(+). Am J Physiol Heart Circ Physiol 279:H679–H691
23. **Cook CS, Berry LM, Burton E** 2004 Prediction of *in vivo* drug interactions with eplerenone in man from in vitro metabolic inhibition data. Xenobiotica 34:215–228
24. **Baxter JD, Funder JW, Apriletti JW, Webb P** 2004 Towards selectively modulating mineralocorticoid receptor function: lessons from other systems. Mol Cell Endocrinol 217:151–165
25. **Menard J** 2004 The 45-year story of the development of an anti-aldosterone more specific than spironolactone. Mol Cell Endocrinol 217:45–52
26. **Delyani JA, Rocha R, Cook CS, Tobert DS, Levin S, Roniker B, Workman DL, Sing YL, Whelihan B** 2001 Eplerenone: a selective aldosterone receptor antagonist (SARA). Cardiovasc Drug Rev 19:185–200
27. **Schmidt BM, Montealegre A, Janson CP, Martin N, Stein-Kemmesies C, Scherhag A, Feuring M, Christ M, Wehling M** 1999 Short term cardiovascular effects of aldosterone in healthy male volunteers. J Clin Endocrinol Metab 84:3528–3533
28. **Wehling M, Spes CH, Win N, Janson CP, Schmidt BM, Theisen K, Christ M** 1998 Rapid cardiovascular action of aldosterone in man. J Clin Endocrinol Metab 83:3517–3522
29. **Massett MP, Ungvari Z, Csiszar A, Kaley G, Koller A** 2002 Different roles of PKC and MAP kinases in arteriolar constrictions to pressure and agonists. Am J Physiol Heart Circ Physiol 283:H2282–H2287
30. **Gokina NI, Knot HJ, Nelson MT, Osol G** 1999 Increased Ca2+ sensitivity as a key mechanism of PKC-induced constriction in pressurized cerebral arteries. Am J Physiol 277:H1178–H1188
31. **Hill MA, Falcone JC, Meininger GA** 1990 Evidence for protein kinase C involvement in arteriolar myogenic reactivity. Am J Physiol 259:H1586–H1594
32. **Hill MA, Zou H, Potocnik SJ, Meininger GA, Davis MJ** 2001 Invited review: arteriolar smooth muscle mechanotransduction: Ca(2+) signaling pathways underlying myogenic reactivity. J Appl Physiol 91:973–983
33. **Ungvari Z, Pacher P, Kecskemeti V, Papp G, Szollar L, Koller A** 1999 Increased myogenic tone in skeletal muscle arterioles of diabetic rats. Possible role of increased activity of smooth muscle Ca2+ channels and protein kinase C. Cardiovasc Res 43:1018–1028
34. **Nishiyama A, Yao L, Nagai Y, Miyata K, Yoshizumi M, Kagami S, Kondo S, Kiyomoto H, Shokoji T, Kimura S, Kohno M, Abe Y, Nishiyama A, Yao L, Nagai Y, Miyata K, Yoshizumi M, Kagami S, Kondo S, Kiyomoto H, Shokoji T, Kimura S, Kohno M, Abe Y** 2004 Possible contributions of reactive oxygen species and mitogen-activated protein kinase to renal injury in aldosterone/salt-induced hypertensive rats. Hypertension 43:841–848
35. **Watts SW** 1998 Activation of the mitogen-activated protein kinase pathway via the 5-HT2A receptor. Ann NY Acad Sci 861:162–168
36. **Tepel M, Jankowski J, Ruess C, Steinmetz M, van der Giet M, Zidek W** 1998 Activation of Na+, H+ exchanger produces vasoconstriction of renal resistance vessels. Am J Hypertens 11:1214–1221
37. **Wakabayashi S, Shigekawa M, Pouyssegur J** 1997 Molecular physiology of vertebrate Na+/H+ exchangers. Physiol Rev 77:51–74

38. **Sardet C, Counillon L, Franchi A, Pouysségur J** 1990 Growth factors induce phosphorylation of the Na^+/H^+ antiporter, a glycoprotein of 110 kDa. Science 247:723–726
39. **Bertrand B, Wakabayashi S, Ikeda T, Pouyssegur J, Shigekawa M** 1994 The Na+/H+ exchanger isoform 1 (NHE1) is a novel member of the calmodulin-binding proteins. Identification and characterization of calmodulin-binding sites. J Biol Chem 269:13703–13709
40. **Lin X, Barber DL** 1996 A calcineurin homologous protein inhibits GTPase-stimulated Na^+/H^+ exchange. Proc Natl Acad Sci USA 93:12631–12636
41. **Bianchini L, L'Allemain G, Pouyssegur J** 1997 The p42/p44 mitogen-activated protein kinase cascade is determinant in mediating activation of the Na+/H+ exchanger (NHE1 isoform) in response to growth factors. J Biol Chem 272:271–279
42. **Phan VN, Kusuhara M, Lucchesi PA, Berk BC** 1997 A 90-kD Na(+)-H+ exchanger kinase has increased activity in spontaneously hypertensive rat vascular smooth muscle cells. Hypertension 29:1265–1272
43. **Daniel EE, Shi AG, Wang ZL, Guan YY, Hoo K, Cragoe EJ, Kwan CY** 1991 α-Adrenoceptors in vascular smooth muscle: all is not well. Blood Vessels 28:104–114T