Dependence of Na⁺/H⁺ Antiport Activation in Cultured Rat Aortic Smooth Muscle on Calmodulin, Calcium, and ATP

EVIDENCE FOR THE INVOLVEMENT OF CALMODULIN-DEPENDENT KINASES*

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The role of Ca^{2+}/calmodulin-dependent processes in the activation of the Na⁺/H⁺ antiport of primary cultures of rat aortic smooth muscle was studied using $^{22}Na^+$ uptake and measurement of intracellular pH (pH_i) with the fluorescent pH dye 2′,7′-bis-(2-carboxyethyl)-5(and 6)-carboxyfluorescein. Antiport activation following exposure to serum and by the induction of an intracellular acidosis could be markedly attenuated by calmodulin antagonists. Ionomycin also transiently elevated pH_i and 5-(N-ethyl-N-isopropyl) amiloride-sensitive $^{22}Na^+$ influx, effects consistent with activation of the antiport; these effects were abolished in cells exposed to calmodulin antagonists or [ethylenebis(oxyethylenenitrilo)]tetraacetic acid. Activation of the antiport following intracellular acidosis was markedly affected by cellular ATP depletion. A comparison of the abilities of control and 2-deoxy-D-glucose-treated cells to increase 5-(N-ethyl-N-isopropyl)amiloride-sensitive $^{22}Na^+$ influx in response to graded acidifications indicated that attenuation of Na⁺/H⁺ antiport activity was due to both a shift of its pH_i dependence and to a reduction in maximal activity. The results suggest that the Na⁺/H⁺ antiport of rat aortic smooth muscle is dependent on Ca^{2+}/calmodulin-dependent processes, presumably phosphorylation, which influences its activity by modulating (i) an intracellular proton dependent regulatory mechanism (allosteric site) and (ii) the maximum activity of the antiport.

An amiloride-sensitive Na⁺/H⁺ antiport has been detected in the plasma membranes of most mammalian cells (1, 2). Under normal circumstances this antiport catalyzes the electroneutral exchange of intracellular protons for extracellular sodium ions. In vascular smooth muscle we have shown that Na⁺/H⁺ antiport activity represents a major mechanism for Na⁺ influx (3). It is also the prime mechanism responsible for intracellular pH control (4). The antiport can be activated by intracellular acidosis and agents such as angiotensin II (5), growth factors (6), and phorbol esters (5). However, despite the ability of phorbol esters to activate the antiport in aortic

smooth muscle, its activation by angiotensin II (5) and serum (6) occurs by mechanisms independent of protein kinase C. Agents such as angiotensin II, serum, and platelet-derived growth factor also induce an increase in the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$).[1] This change in $[Ca^{2+}]_i$ usually precedes activation of the antiport (7, 8), suggesting a causal relationship. Moreover, the involvement of $[Ca^{2+}]_i$ in the activation of the Na⁺/H⁺ antiport of some cells is further supported by the inhibitory effects of calmodulin antagonists on activation of the antiport as well as the finding that the divalent cation ionophore A23187 activates the antiport in A_{10} smooth muscle cells (9) and human foreskin fibroblasts (10–12). However, this effect does not apply to all cells, because in fibroblasts (13) and in mouse neuroblastoma cells (14) A23187 does not increase antiport activity.

Thus, although intracellular acidosis and a number of agents activate the Na⁺/H⁺ antiport of vascular smooth muscle, the molecular mechanisms involved in this activation are unknown. In the present study we have carried out a detailed investigation of the effects of calmodulin antagonists, changes in $[Ca^{2+}]_i$ by ionomycin, and cellular ATP depletion on Na⁺/H⁺ antiport activation following exposure of the cells to serum and the induction of intracellular acidosis. Evidence is presented that, in aortic smooth muscle, activation of the antiport is dependent upon calmodulin, $[Ca^{2+}]_i$, and cellular ATP. The overall results suggest that these agents influence, probably via phosphorylation, the activity of the antiport at two regulatory sites. One involves the regulation of intracellular proton affinity and the other influences its maximal activity.

EXPERIMENTAL PROCEDURES

Culture of Vascular Smooth Muscle—Aortic smooth muscle cells from adult Sprague-Dawley rats (body weight 200–300 g) were isolated by collagenase and elastase digestion and grown in culture as previously described (3). Briefly, the cells were initially seeded at 1×10^6 cells/cm² onto 30-mm diameter plastic culture dishes (Sterilin Ltd., Feltham, Great Britain) and maintained in culture for 3–4 days in Medium 199 containing 5% fetal calf serum. For experiments involving the determination of intracellular pH (pH_i), the cells were cultured on glass coverslips which are placed into the culture dishes during the initial seeding (4). Vascular smooth muscle cells cultured in this manner stain positively with fluorescein isothiocyanate-labeled antibodies specific for smooth muscle myosin (3) and contain α-actin as the major actin isoform (15). Unless stated otherwise, all cells used in the experiments were "serum-deprived" by replacing the growth medium with Medium 199 containing 0.46% w/v bovine serum albumin 24 h prior to experimentation.

[1] The abbreviations used are: $[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; pH_i, intracellular pH; BCECF, 2′,7′-bis-(2-carboxyethyl)-5(and 6)-carboxyfluorescein; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; EIPA, 5-(N-ethyl-N-isopropyl)amiloride.

Salt Solutions—The standard salt solution (solution A) used in the experiments consisted of 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5.5 mM glucose in 10 mM HEPES-Tris adjusted to pH 7.4 at 37 °C. For experiments involving the uptake of $^{22}Na^+$ into cells, solution B was used. Its composition was as for solution A except that it contained 15 mM NaCl and 120 mM N-methyl-D-glucamine chloride instead of the 135 mM NaCl. Other chemicals were added as required to these solutions.

$^{22}Na^+$ *Uptake Measurements*—$^{22}Na^+$ influx in the cultured cells was measured as previously described (3). Briefly, in the experiments involving $^{22}Na^+$ uptake determinations, the cells were rinsed and equilibrated at 37 °C for 15 min in solution B (containing 15 mM Na^+). After this interval the solution was aspirated and replaced by a similar solution containing 2 mM ouabain and the compounds of interest. After 3 min, $^{22}Na^+$ (carrier-free, 10^6 cpm) was added to the incubations and isotope uptake measured at 37 °C over the ensuing 3-min period. $^{22}Na^+$ uptake was terminated by rapidly removing the incubation mix and washing (five times) the cells with ice-cold 0.1 M $MgCl_2$. The cells were harvested by scraping into 0.1 M nitric acid and centrifuged. Radioactivity due to liberated $^{22}Na^+$ was determined in the supernatant fraction using a liquid scintillation counter, and the cell pellets were assessed for protein using the method of Lowry et al. (16). Na^+/H^+ antiport activity was taken as the difference in $^{22}Na^+$ uptake measured in the absence and presence of EIPA (400 μM) following inhibition of the Na^+/K^+-pump with 2 mM ouabain. This concentration of EIPA induces maximal inhibition of Na^+/H^+ exchange in vascular smooth muscle (3).

Measurement of pH_i—The fluorescent pH indicator BCECF was used to monitor changes in cytosolic pH. Briefly, the method involved washing (three times) the smooth muscle attached to the coverslips with solution A followed by incubation at 37 °C in 1 ml of solution A containing 0.2 nmol of BCECF-AM. At the end of the incubation the coverslips were washed (three times) with solution A at 37 °C to remove any extracellular indicator. The coverslips were then loaded into a vertical coverslip holding device (17) which could be inserted into a standard (1 cm × 1 cm) fluorescence cuvette thermostated at 37 °C and perfused with a variety of media (see individual experiments). Fluorescence determinations were carried out using a Perkin Elmer LS-5 Luminescence spectrometer whose excitation wavelength was varied between 495 and 440 nm, but the emission wavelength remained at 530 nm. Under these conditions, the leakage of BCECF from cells on coverslips over a 15-min period is negligible (4). Interference due to autofluorescence and light scatter of unloaded cells represented less than 2% of the total 495 nm signal of BCECF-loaded cells. The ratio of the fluorescence signal from the two excitation wavelengths (495 nm and 440 nm) from BCECF-loaded cells was calibrated to pH_i using high (140 mM) potassium buffers of various pH levels containing 4 μM nigericin (4, 15).

Materials—N-(4-Aminobutyl)-2-naphthalenesulfonamide HCl (W-12) was purchased from Seikagaku Kogyo, Tokyo, Japan. N-(4-Aminobutyl)-5-chloro-2-naphthalenesulfonamide HCl (W-13), N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide HCl (W-7), N-methyl-D-glucamine, EGTA, 2-deoxy-D-glucose, 3-O-methylglucose haloperidol, and rotenone were purchased from Sigma. Ionomycin and HEPES were purchased from Behring Diagnostics. Trifluoroperazine HCl was a gift from Smith, Kline, and French Labs (Australia) Ltd., Sydney, Australia. Diazepam was purchased from David Bull Laboratories, Melbourne, Australia. BCECF and BCECF-AM were purchased from Molecular Probes, Eugene, OR. 5-(N-Ethyl-N-isopropyl)-amiloride (EIPA) was synthesized as previously described (18). All other chemicals, biochemicals, and culture media were obtained from standard chemical suppliers.

RESULTS

Effect of Calmodulin Antagonists on Na^+/H^+ Antiport Activity—The role of calmodulin-dependent processes in the activation of the Na^+/H^+ antiport was initially investigated by assessing the effects of two closely related calmodulin antagonists, compounds W-12 and W-13, on EIPA-sensitive $^{22}Na^+$ influx rate, measured in the presence of 10% serum (Fig. 1). Preincubation of 4 h serum-deprived cells for 30 min with W-13 attenuated EIPA-sensitive $^{22}Na^+$ influx in a concentration-dependent manner (Fig. 1). This inhibition of EIPA-sensitive $^{22}Na^+$ influx by the highest concentration of W-13 (400 μM), approximately 95%, was due not only to inhibition of the
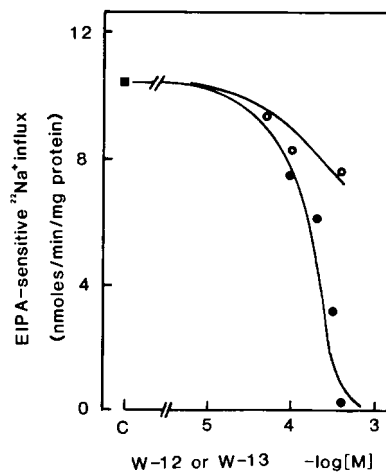

FIG. 1. **Inhibition by calmodulin antagonists of EIPA-sensitive $^{22}Na^+$ influx in vascular smooth muscle exposed to 10% serum.** Cells deprived of serum for 4 h were rinsed free of culture medium and incubated at 37 °C in solution B containing various concentrations of the calmodulin antagonists W-12 or W-13. After 30 min, the solution was aspirated and replaced by a similar solution containing 10% fetal calf serum (final Na^+, 15 mM), 2 mM ouabain, and the calmodulin antagonists W-12 (O) and W-13 (●) with and without EIPA. After 3 min incubation at 37 °C, $^{22}Na^+$ (10^6 cpm) was added and its uptake into cells measured over the next 3 min. Data are the means of three similar experiments.

serum-stimulated component of the influx (which under these conditions accounts for approximately 70% of the total EIPA-sensitive $^{22}Na^+$ influx), but also to near-complete inhibition of the basal EIPA-sensitive $^{22}Na^+$ influx. The IC_{50} for this inhibition by W-13 averaged 150 μM. In contrast to the potent effects of W-13 on EIPA-sensitive $^{22}Na^+$ influx, pre-incubation of the cells with the structurally related analogue W-12 (up to 400 μM), a compound approximately 5 times less potent than W-13 on calmodulin-dependent processes (19, 20), reduced EIPA-sensitive $^{22}Na^+$ uptake by only 33% at the highest concentration (Fig. 1).

Because antiport activity in smooth muscle appeared dependent on calmodulin-dependent processes, we next examined whether the activation of the Na^+/H^+ antiport by intracellular protons was also influenced by calmodulin-dependent processes. The ability of the cells to recover from an acid load was examined after preexposing the cells for 20 min to two calmodulin antagonists W-7 (200 μM) and trifluoroperazine (50 μM) (21, 22). At these concentrations, W-7 and trifluoroperazine inhibit myosin light chain phosphorylation in vascular smooth muscle by approximately 70–80% (23). In these experiments the NH_4Cl withdrawal technique was used to acidify the cytoplasm. This procedure involves loading the cells with ammonium ions by a 4-min incubation in the presence of 15 mM NH_4Cl, either in the absence or presence of the calmodulin antagonists. Subsequent removal of NH_4Cl results in a large decrease in pH_i as free ammonia rapidly leaves the cytoplasm (see Fig. 6 for complete time course of pH changes). The subsequent recovery of pH_i is dependent upon activation of the Na^+/H^+ antiport (4). In control cells recovery of pH_i was rapid and complete within 4 min of introducing the acid load (Fig. 2). However, in the cells preincubated and then continuously exposed to either W-7 or trifluoroperazine, recovery of pH_i was markedly attenuated (Fig. 2). Neither calmodulin antagonist had any appreciable inhibitory effect on the recovery of pH_i when introduced at the time of NH_4Cl withdrawal (results not shown).

Intracellular Calcium and Activation of the Na^+/H^+ Antiport—Because Ca^{2+}/calmodulin-dependent processes ap-
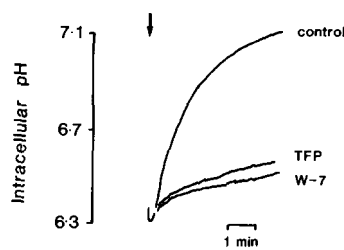
FIG. 2. **Effect of calmodulin antagonists on the Na⁺/H⁺ antiport-dependent recovery from acidosis induced by the withdrawal of an NH₄Cl preload.** Cells cultured on glass coverslips were loaded with the fluorescent intracellular pH indicator BCECF during a 30-min incubation at 37 °C with BCECF-AM dissolved in solution A. The calmodulin antagonists (W-7, 200 μM, trifluoperazine (*TFP*), 50 μM) were present during the final 15 min of the loading period and throughout the subsequent procedures. Intracellular acidosis was induced by exposing the cells at 37 °C to 15 mM NH₄Cl in solution A for 4 min prior to its rapid withdrawal (*arrow*). Trace is a composite of three experiments on the same cell culture. Data are typical of four similar experiments with each inhibitor.
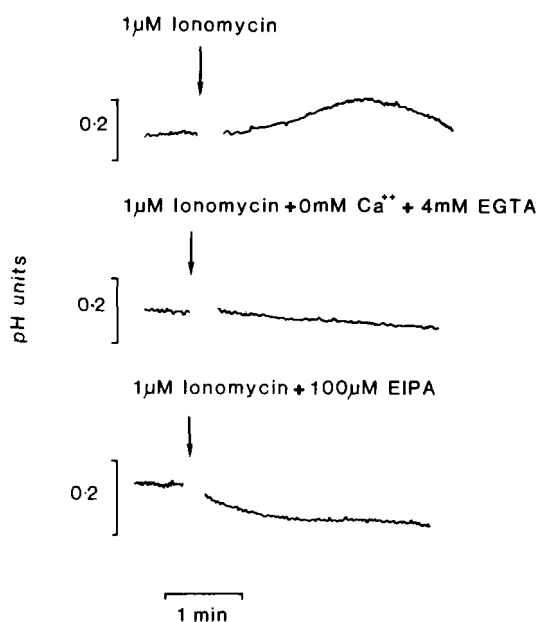

FIG. 3. **Effect of ionomycin on pH$_i$ in aortic smooth muscle.** Cells cultured on glass coverslips were loaded with the fluorescent pH indicator BCECF as indicated under "Experimental Procedures." Traces depict fluorescence measurements from three successive coverslips from the same primary culture. *Top tracing* depicts the initial alkalinization which occurs upon exposing the cells to 1 μM ionomycin. *Middle tracing* demonstrates the complete attenuation of the alkalinization by removal by extracellular calcium. *Bottom tracing* demonstrates the attenuation of the alkalinization by the Na⁺/H⁺ antiport inhibitor EIPA. Results are typical of four similar experiments.

peared to be involved in activating the Na⁺/H⁺ antiport, we next examined the effect of increasing intracellular calcium on antiport activity. When cells equilibrated in solution A were exposed to 1 μM calcium ionophore ionomycin, the intracellular pH of the cells gradually rose (Fig. 3). Approximately 2.5–3 min after exposure to ionomycin the elevation in pH averaged 0.15 pH units and thereafter declined to acidic levels (not shown). This initial alkalinization could be prevented by removal of extracellular calcium and was not dependent on any electroneutral exchange of Ca²⁺ for intracellular protons, because it could be attenuated by 100 μM EIPA (Fig. 3). Confirmation of the ability of ionomycin to activate the antiport in aortic smooth muscle by a calcium-dependent mechanism was made by assessing the effects of ionomycin

on ²²Na⁺ entry. In cells equilibrated in solution B, ionomycin initiated a concentration-dependent increase in EIPA-sensitive ²²Na⁺ influx (Fig. 4). The ionomycin-induced elevation in EIPA-sensitive ²²Na⁺ influx appeared also dependent upon extracellular calcium and calmodulin. EIPA-sensitive ²²Na⁺ influx measured in the presence of 1 μM ionomycin averaged 25.0 ± 4.3 nmol of ²²Na⁺/min/mg of protein and was reduced to 7.8 ± 4.3 nmol of ²²Na⁺/min/mg of protein when EGTA (4 mM) was present to reduce extracellular calcium to below 200 nM. Furthermore, a 30-min exposure to the calmodulin antagonist W-13 reduced ionomycin-stimulated EIPA-sensitive ²²Na⁺ influx to 4.4 ± 1.8 nmol of ²²Na⁺/min/mg of protein. This apparent dependence of antiport activity on calmodulin was further evaluated by examining the effects, in a concentration-dependent manner, of two structurally unrelated calmodulin antagonists, haloperidol and diazepam (22). Preincubation of cells for 20 min with haloperidol and diazepam caused a concentration-dependent inhibition of EIPA-sensitive ²²Na⁺ influx measured in the presence of 1 μM ionomycin (Fig. 5). The IC₅₀ values for haloperidol and diazepam averaged 55 and 115 μM, respectively, which are in good agreement with the IC₅₀ values of 60 and 140 μM reported for the inhibition of the calmodulin-dependent activation of rat brain phosphodiesterase (22).

ATP and Activation of the Na⁺/H⁺ Antiport—Phosphorylation of the Na⁺/H⁺ antiport has been suggested as a potential mechanism by which agents such as phorbol esters and a variety of growth factors elevate pH$_i$ (24, 25). Because in aortic smooth muscle activation of the antiport by a variety of experimental manipulations involves Ca²⁺/calmodulin-dependent processes, it was of interest to investigate the effects of graded reductions in cellular ATP on antiport activation. Reductions in cellular ATP were produced by incubating cells for a total of 30–40 min in solution A (containing 5.5 mM glucose) or solution A in which the glucose had been replaced with an equimolar amount of either of the nonmetabolizable sugars 3-O-methylglucose or 2-deoxy-D-glucose. The latter sugar also consumes ATP during its initial phosphorylation by hexokinase, leading to rapid ATP depletion (26). Intracellular pH of cells incubated in solution A containing glucose, 3-O-methylglucose, and 2-deoxy-D-glucose averaged 7.06 ±
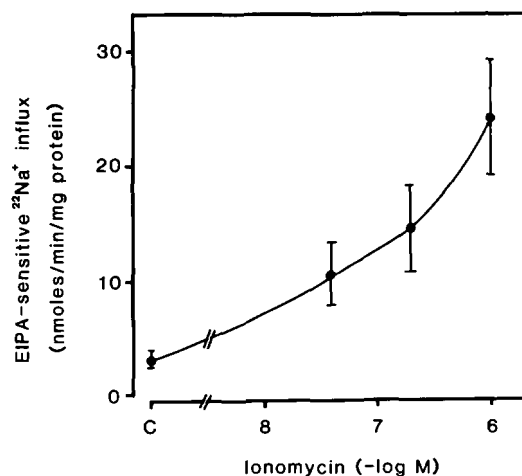

FIG. 4. **Concentration dependence of Na⁺/H⁺ antiport activation by ionomycin.** Cells were rinsed and then equilibrated in solution B for 15 min. The solution was then aspirated and replaced by a similar solution containing ²²Na⁺ (10⁶ cpm) and ionomycin in the absence and presence of EIPA. ²²Na⁺ uptake was measured over 3 min, the period of time normally associated with cytosolic alkalinization by ionomycin (Fig. 3). Results are the means ± S.E. of four experiments.
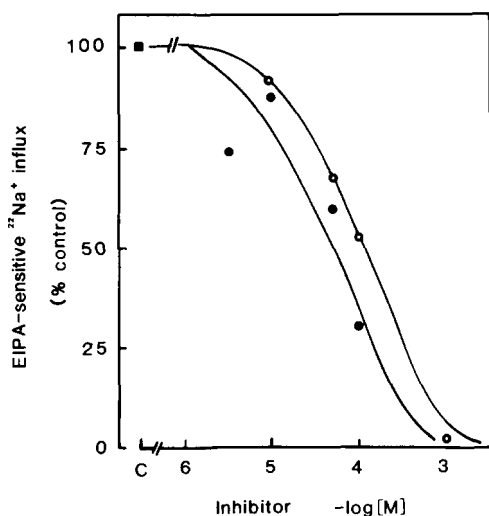
FIG. 5. **Inhibition by calmodulin antagonists of EIPA-sensitive ^{22}Na⁺ influx in ionomycin-treated vascular smooth muscle.** Cells were incubated at 37 °C in solution B containing various concentrations of the calmodulin antagonists haloperidol or diazepam. After 20 min the solution was aspirated and replaced by a similar solution containing 1 μM ionomycin (final Na⁺, 15 mM), 2 mM ouabain and haloperidol (●) and diazepam (○) with and without EIPA. After 3 min incubation at 37 °C, ^{22}Na⁺ (10^6 cpm) was added and its uptake into cells measured over the next 3 min. Data are the means of three similar experiments.

0.16, 6.97 ± 0.21, and 6.71 ± 0.10, respectively. The ability of the Na⁺/H⁺ antiport in cells exposed to 3-O-methylglucose or 2-deoxy-D-glucose to be activated by an acid load was attenuated (Fig. 6). When the cells were preexposed to 15 mM NH₄Cl for 3 min to induce an acid load, the subsequent acidosis in all three groups of cells was similar, with pH$_i$ averaging 6.58 ± 0.18. The initial rates of recovery in the 3-O-methylglucose- and 2-deoxy-D-glucose-exposed cells were reduced by 46.1 ± 8.7 and 95 ± 5%, respectively, compared with those incubated in solution A (Fig. 6).

The effects of ATP depletion on the elevation in EIPA-sensitive ^{22}Na⁺ uptake induced by exposing the cells to 1 μM ionomycin were also investigated. In these experiments ATP depletion was induced by exposing the cells for 15 min to solution A in which an equimolar amount of 2-deoxy-D-glucose was substituted for the glucose or by exposing the cells to solution A containing 25 μM rotenone. This latter procedure has been shown to reduce ATP levels during this period by values in excess of 80% (27). Under these conditions, EIPA-sensitive ^{22}Na⁺ uptake, measured in solution B containing 1 μM ionomycin, was reduced from 29.8 ± 1.5 nmol of ^{22}Na⁺/min/mg of protein to 3.1 ± 1.3 and 7.5 ± 2.9 nmol of ^{22}Na⁺/min/mg of protein in cells incubated with 2-deoxy-D-glucose and rotenone, respectively.

Mechanism of Modulation of Na⁺/H⁺ Antiport Activity by 2-Deoxy-D-glucose—Previous studies in vascular smooth muscle indicated that intracellular protons play an important regulatory role in activating the Na⁺/H⁺ antiport (4). Our finding that the metabolic inhibitor 2-deoxy-D-glucose virtually abolishes the ability of the Na⁺/H⁺ antiport to be activated by high intracellular concentrations of protons (pH$_i$ ~ 6.6) (Fig. 6) led us to examine its effects on intracellular proton sensitivity and the maximal activity of the antiport. In these experiments we compared the effects of preincubating cells in solution A or solution A containing 5.5 mM 2-deoxy-D-glucose (substituted for glucose) on their ability to activate the Na⁺/H⁺ antiport in response to graded reductions in pH$_i$. Reductions in pH$_i$ were induced by preincubating the cells
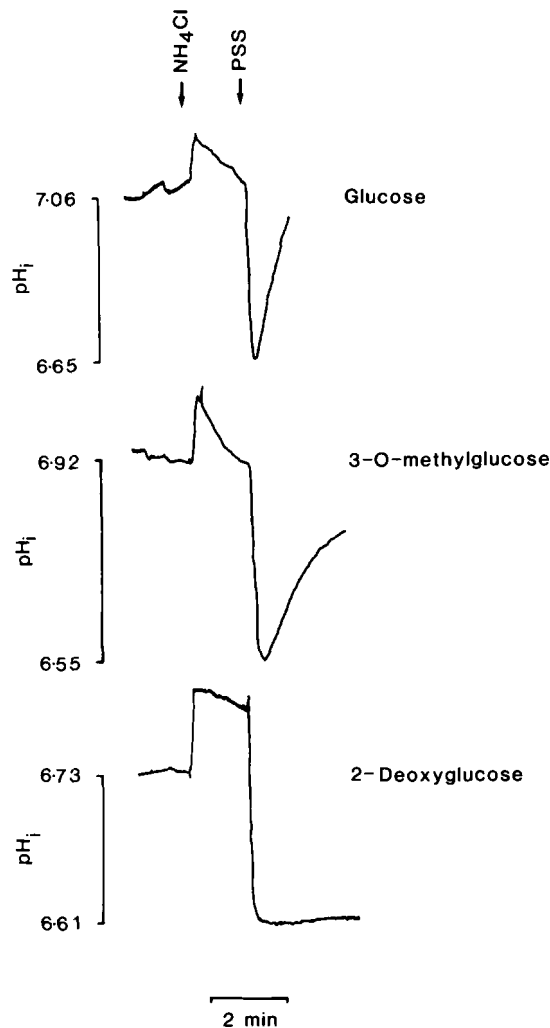

FIG. 6. **Effect of cellular ATP depletion on Na⁺/H⁺ antiport-mediated recovery from intracellular acidosis induced by NH₄Cl preloads.** Cells were loaded with the pH fluorescent indicator BCECF as described under "Experimental Procedures" in solution A (*top panel*), solution A in which glucose had been replaced by 5.5 mM 3-O-methylglucose (*middle panel*), and solution A containing 5.5 mM 2-deoxy-D-glucose (substituted for the glucose). Tracings depict the abilities of these cells to recover from intracellular acidosis induced by a 15 mM NH₄Cl preload when the cells are continuously maintained in the above-mentioned solutions. *Arrows* represent the times at which NH₄Cl was added to and removed from the cuvette of the fluorimeter. Results are typical of three or four similar experiments with each sugar.

with increasing concentrations of NH₄Cl (up to 40 mM) for 15 min prior to its rapid removal. EIPA-sensitive ^{22}Na⁺ uptake was measured in solution B containing 2-deoxy-D-glucose (substituted for glucose) over a 2.5-min period immediately following withdrawal of NH₄Cl. In agreement with previous reports (24, 26), EIPA-sensitive ^{22}Na⁺ uptake increased with increasing acid loads induced by NH₄Cl pre-exposure (Fig. 7). In control cells, half-maximal effects on EIPA-sensitive ^{22}Na⁺ uptake occurred at approximately 12 mM NH₄Cl. By contrast, in the 2-deoxy-D-glucose-treated cells, half maximal effect on ^{22}Na⁺ uptake occurred at approximately 25 mM NH₄Cl, indicating a reduction in the sensitivity of the antiport to increasing concentrations of intracellular protons. This effect on intracellular proton sensitivity was accompanied by a marked reduction in the activity of the antiport. Under conditions which caused maximal stimulation of the antiport (30 and 40 mM NH₄Cl), the activity of the antiport in the 2-deoxy-D-
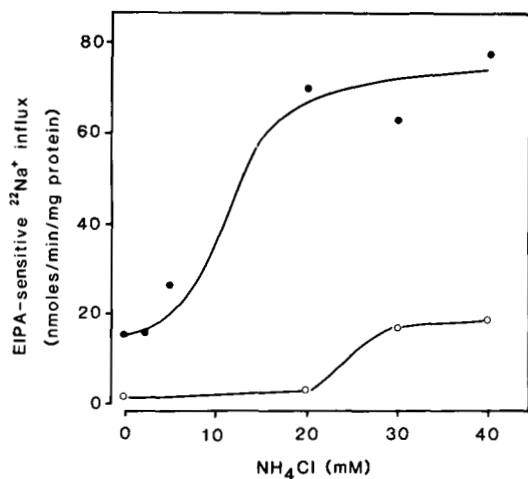
FIG. 7. **Effect of preloading aortic smooth muscle with increasing concentrations of NH$_4$Cl on EIPA-sensitive ^{22}Na$^+$ uptake in control and 2-deoxy-D-glucose-exposed cells.** Cells were preincubated at 37 °C for 15 min with increasing concentrations (0–40 mM) of NH$_4$Cl in solution B (●) or solution B in which the glucose had been replaced with 5.5 mM 2-deoxy-D-glucose (O). Upon rapidly rinsing the cells with identical solutions free of NH$_4$Cl, ^{22}Na$^+$ (10^6 cpm) was rapidly added and its uptake determined in the presence and absence of EIPA over a 2.5-min interval. The data are typical of three similar experiments.

glucose-incubated cells was reduced by approximately 80% compared to control cells (Fig. 7).

DISCUSSION

Our results indicate that regulation of Na$^+$/H$^+$ antiport activity in vascular smooth muscle is dependent upon processes, presumably phosphorylation, involving calcium, calmodulin, and ATP. Phosphorylation processes dependent on protein kinase C have been suggested to be the mechanism by which serum and growth factors such as, for example, platelet-derived growth factor activate the antiport in fibroblasts (28), and lymphocytes (25). However, in other cells, mechanisms independent of protein kinase C activation have also been reported to increase Na$^+$/H$^+$ antiport activity. Thus, although phorbol ester-mediated activation of protein kinase C increases Na$^+$/H$^+$ antiport activity in lymphocytes, its activation by osmotic shrinking occurs by mechanisms independent of protein kinase C (29). In human fibroblasts (10–12) and in A$_{10}$ smooth muscle cell line (9), activation of the Na$^+$/H$^+$ antiport by serum and platelet-derived growth factor appears dependent on Ca^{2+}/calmodulin-dependent processes. Our results on the ability of calmodulin antagonists to inhibit Na$^+$/H$^+$ antiport activity in cultured aortic smooth muscle exposed to serum and ionomycin are also consistent with a calmodulin-dependent process being involved in its activation. The naphthalenesulfonamide calmodulin antagonist W-12 was found to be at least 3 times less potent than its 5-chloro analogue W-13 (20). Furthermore, indirect assessment of the ability of W-13 to inhibit smooth muscle calmodulin-dependent kinases, based on the equal activity of W-7 and W-13 to inhibit myosin light chain kinase activity (19, 20) and the reported potency of W-7 to inhibit phosphorylation in smooth muscle (23), indicates that the IC$_{50}$ value of 150 μM for the inhibition of Na$^+$/H$^+$ antiport activity by W-13 is consistent with inhibition of calmodulin-dependent phosphorylation processes in smooth muscle. Similarly, the inhibitory potencies of two other structurally unrelated compounds, haloperidol and diazepam, are also consistent with activation of Na$^+$/H$^+$ antiport activity in vascular smooth muscle being

mediated by calmodulin-dependent processes (see "Results"). Exposure of aortic smooth muscle to angiotensin II also increases Na$^+$/H$^+$ antiport activity by mechanisms independent of protein kinase C (5). Since angiotensin II is known to activate Ca^{2+}/calmodulin-dependent processes in vascular smooth muscle (30), it is also probable that the mechanism by which angiotensin II activates the antiport also involves Ca^{2+}/calmodulin. Our observations on the ability of a variety of calmodulin antagonists to attenuate activation of the Na$^+$/H$^+$ antiport by intracellular acidosis and by increased intracellular calcium suggest that calmodulin-dependent activation in aortic smooth muscle is not restricted to agents which activate the antiport via membrane receptor systems.

The role of cytoplasmic Ca^{2+} in activating the Na$^+$/H$^+$ antiport is controversial. In human fibroblasts (10–12) and the A$_{10}$ smooth muscle cell line (9), elevation of the cytoplasmic free Ca^{2+} concentration with the ionophore A23187 markedly stimulated Na$^+$/H$^+$ antiport activity. However, in other cell lines, calcium appears to play little role in antiport activation (13, 14). Since, as in the human fibroblasts and the A$_{10}$ cell line calmodulin appeared to be involved in activating the Na$^+$/H$^+$ antiport of vascular smooth muscle, the role of calcium was investigated. Increasing intracellular Ca^{2+} by exposing the cells to the calcium ionophore ionomycin induced a rapid but transient alkalinization which could be inhibited by EIPA. Consistent with this finding of Ca^{2+}-dependent activation of the antiport, EIPA-sensitive ^{22}Na$^+$ uptake was also increased during the alkalinization phase. This effect of ionomycin on antiport activity was also dependent on calmodulin, making it unlikely that it is due to cell shrinkage, as has been suggested for thymic lymphocytes (31). Taken together these effects of ionomycin are consistent with Ca^{2+}/calmodulin processes being involved in the activation of the Na$^+$/H$^+$ antiport of vascular smooth muscle.

Previous studies, including those on vascular smooth muscle (3, 4, 24), indicate that the Na$^+$/H$^+$ antiport is allosterically activated by intracellular protons. Such activation enables the antiport to function in pH$_i$ homeostasis because, whenever pH$_i$ decreases, the antiport becomes more active to restore pH$_i$. Our finding that the ability of the antiport to restore pH$_i$ following an acid load is attenuated by preincubating vascular smooth muscle with concentrations of W-7 or trifluoroperazine, which inhibit Ca^{2+}/calmodulin-dependent phosphorylation of myosin light chain in carotid smooth muscle (32), suggests that calmodulin processes also influence the degree to which allosteric mechanisms affect antiport activity.

Activation of the antiport in aortic smooth muscle by intracellular acidosis is also dependent upon the presence of adequate cellular levels of ATP. When cellular ATP is depleted by preincubation with 2-deoxy-D-glucose (26), aortic smooth muscle cells were unable to recover from the 15 mM NH$_4$Cl induced acid load. This effect of ATP depletion by 2-deoxy-D-glucose on antiport activation is not a consequence of any reduction in the transmembrane sodium gradient due to a fall in sodium extrusion by the Na$^+$/K$^+$-pump. Activation of the Na$^+$/H$^+$ antiport of aortic smooth muscle by intracellular acidosis has been shown to be unaffected by inhibition of the Na$^+$/K$^+$-pump for periods up to 1 h (4). The finding that pH$_i$ of the cells fell by ~0.4 pH units after 30 min incubation with 2-deoxy-D-glucose is also consistent with basal Na$^+$/H$^+$ antiport activity contributing to the maintenance of normal pH$_i$ in these cells (4, 7). Our observations that ATP depletion of aortic smooth muscle attenuates Na$^+$/H$^+$ antiport activity by shifting its dependence on the intracellular proton concentration to more acidic pH levels are

consistent with recent observations in cultured human A431 cells (26). However, in addition to the change in pH_i dependence, ATP depletion in aortic smooth muscle also reduces the maximal activity of the antiport. This effect of ATP depletion on the maximal activity of the Na^+/H^+ antiport is unlikely to be due to any alteration in the number of antiport units present in the cell membrane since a rapid insertion of antiport units from internal membrane sources does not appear to be an important mechanism by which antiport activity is increased (32). Furthermore, activation of the Na^+/H^+ antiport by intracellular acidosis, at least in isolated membrane vesicles, is not directly dependent upon the presence of ATP (1). Taken together, the effects of ATP depletion on both the pH_i dependence and maximal activity of the Na^+/H^+ antiport suggests the presence of at least two ATP-dependent regulatory sites for the antiport. The most likely mechanism by which ATP would be involved in this regulation would be via multiple sites of protein phosphorylation. Certainly phosphorylation has been proposed as a mechanism by which Na^+/H^+ antiport activity could be regulated under some circumstances (2, 25, 26, 29). Moreover, phosphorylation appears to be an important mechanism by which a number of transmembrane ion transport systems are regulated. Recently it has been shown that the dihydropyridine-sensitive class of voltage-activated calcium channel must be phosphorylated in order to open when the membrane is depolarized (33). Multiple phosphorylation processes have also been shown to regulate the activity of the Ca^{2+}-pump ATPase in the sarcoplasmic reticulum of muscle (34, 35). Our observation suggesting that the Na^+/H^+ antiport is regulated by multiple phosphorylation mechanisms is consistent with recent observations in myoblasts where activation of the antiport by serum was associated with a shift of its pH_i dependence toward more alkaline pH_i values and an increase in its maximal activity at acidic pH (36).

Much evidence has now accumulated which implicates the Na^+/H^+ antiport in regulating cell volume, intracellular pH, and cell proliferation (1, 2). In aortic smooth muscle this antiport is the predominant mechanism by which the cells regulate pH_i (4, 5, 15) and is also responsible for a large component of the sodium which enters via electroneural processes (3, 5). Alterations in activity of the Na^+/H^+ antiport could, by its effects on sodium influx and proton efflux, both increase and reduce vascular smooth muscle tone. Activation of the antiport, by increasing the rate of proton efflux from cells, would elevate pH_i, thereby increasing the affinity of Ca^{2+} for myofibrils (37). Similarly, any reduction in antiport activity is ensued by a lowering of pH_i (4, 32, 38), thereby lowering the affinity of Ca^{2+} for myofibrils and vascular smooth muscle tone. Both increases and decreases in constrictor tone have been reported when rat aortic strips, under conditions which induce intracellular alkalosis and acidosis, are exposed to the agonist, noradrenaline (39).

Our observations indicating that the affinity of the Na^+/H^+ antiport for intracellular protons as well as its maximum activity in cultured aortic smooth muscle are dependent on calmodulin, Ca^{2+}, and ATP are consistent with the involvement of calmodulin-dependent protein kinase systems in its regulation. This, taken, together with the recent finding that protein kinase C-dependent processes also activate this antiport (5), indicates that antiport activity in vascular smooth muscle may be subject to regulation via a number of kinase systems, as has been reported for lymphocytes (2, 25, 29). Precisely how these kinases might be involved in regulating activity clearly requires further investigation. However, the recent finding that platelet-derived growth factor, a potent

constrictor of coronary smooth muscle (40), also activates the Na^+/H^+ antiport in cultured aortic smooth muscle by calmodulin-dependent processes (6) supports previous suggestions that the Na^+/H^+ antiport facilitates or contributes to the maintenance of contracture (4, 5). Whether calmodulin-dependent activation represents a general mechanism by which the antiport is activated when vascular smooth muscle is exposed to different constrictor agents requires further investigation.

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