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**Research Article**

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# Intracellular Acidification Associated with Changes in Free Cytosolic Calcium

## Evidence for $Ca^{2+}/H^+$ Exchange via a Plasma Membrane $Ca^{2+}$-ATPase in Vascular Smooth Muscle Cells

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## Abstract

**The purpose of this study was to define the mechanism whereby agonists that increase free cytosolic calcium ($Ca_i^{2+}$) affect intracellular pH ($pH_i$) in smooth muscle. Rat aortic vascular smooth muscle cells grown on coverslips were loaded with BCECF/AM or fura-2/AM for continuous monitoring of $pH_i$ or $Ca_i^{2+}$, respectively, in a $HCO_3^-/CO_2$-containing medium. Recovery from rapid increases in $Ca_i^{2+}$ produced by 1 $\mu$M angiotensin (Ang) II ($\Delta$ $Ca_i^{2+}$ $-229\pm43$ nM) or 1 $\mu$M ionomycin ($\Delta$ $Ca_i^{2+}$ $-148\pm19$ nM) was accompanied by a fall in $pH_i$ ($\Delta$ $pH_i$, $-0.064\pm0.0085$ $P < 0.01$, and $-0.05\pm0.012$ pH units, $P < 0.01$, respectively). Neither the fall in $pH_i$ nor the rise in $Ca_i^{2+}$ elicited by Ang II was prevented by pretreatment with agents which block the action of this agonist on $pH_i$ via the stimulation of the Cl/$HCO_3$ exchangers (DIDS, 50 $\mu$M) or the $Na^+/H^+$ antiporter (EIPA, 50 $\mu$M). In the presence of DIDS and EIPA, Ang II produced a fall in $pH_i$ ($\Delta$ $pH_i$, $-0.050\pm0.014$, $P < 0.01$) and a rise in $Ca_i^{2+}$ ($\Delta$ $Ca^{2+}$ $252\pm157$ nM, $P < 0.01$). That the change in $pH_i$ was secondary to changes in $Ca_i^{2+}$ was inferred from the finding that, when the rise in $Ca_i^{2+}$ elicited by Ang II was prevented by preincubation with a $Ca^{2+}$ buffer, BAPTA (60 $\mu$M), the fall in $pH_i$ was abolished as well ($\Delta$ $pH_i$, $0.0014\pm0.0046$). The $pH_i$ fall produced by Ang II and ionomycin was prevented by cadmium at a very low concentration (20 nM) which is known to inhibit plasma membrane $Ca^{2+}$-ATPase activity ($\Delta$ $pH_i$ $-0.002\pm0.0006$ and $-0.0016$ pH units, respectively). Cadmium also blunted $Ca_i^{2+}$ recovery after Ang II and ionomycin. These findings suggest that the fall in $pH_i$ produced by these agents is due to $H^+$ entry coupled to $Ca^{2+}$ extrusion via the plasma membrane $Ca^{2+}$-ATPase. Our results indicate that agonists that increase $Ca_i^{2+}$ cause intracellular acidification as a result of $Ca^{2+}/H^+$ exchange across the plasma membrane. This process appears to be mediated by a plasma membrane $Ca^{2+}$-ATPase which, in the process of extruding $Ca^{2+}$ from the cell, brings in $[H^+]$ and thus acidifies the cell. (*J. Clin. Invest.* 1995. 95:1480–1489.)**

**Key words: muscle • smooth • vascular • $Ca^{2+}$-transporting-ATPase • intracellular pH • $Ca^{2+}/H^+$ exchanger • angiotensin II • $Cl^-/HCO_3^-$ exchanger**

## Introduction

In vascular smooth muscle both free cytosolic calcium ($Ca_i^{2+}$)[1] and intracellular pH ($pH_i$) are involved in contraction and proliferation (1). For this reason the study of interactions between $Ca_i^{2+}$ and $pH_i$ is of particular interest in vascular tissue. In a recent study, we found that both increases and decreases in the $pH_i$ of cultured rat aortic vascular smooth muscle cells (VSMC) result in an increase in $Ca_i^{2+}$ by mobilizing internal calcium stores (2). In this report we examine the $pH_i$–$Ca_i^{2+}$ interaction from a different perspective, namely, to what extent and by what mechanism elevations in $Ca_i^{2+}$ alter $pH_i$.

When smooth muscle type cells are exposed to agonists which increase $Ca_i^{2+}$ such as angiotensin II (Ang II) or vasopressin, a brief acidification followed by a marked alkalinization occurs under conditions when $HCO_3^-/CO_2^-$ is absent from the medium (3–5). Initial cell acidification occurs whether or not $HCO_3^-/CO_2^-$ is present in the medium (4–7). When the medium contains $HCO_3^-/CO_2^-$, however, the fall in $pH_i$ is not followed by a subsequent cell alkalinization (6, 7). To explain these discordant results, Ganz et al. (6) proposed that agonists simultaneously activate three ion transporters involved in $pH_i$ regulation, namely, $Na^+/H^+$ exchange, $Na^+$-dependent $Cl^-/HCO_3^-$ exchange, and $Na^+$-independent $Cl^-/HCO_3^-$ exchange. In the absence of $HCO_3^-/CO_2^-$, net alkalinization occurs due to stimulation of $H^+$ exit via stimulation of $Na^+/H^+$ exchange, the only exchanger operative under this unphysiological condition. By contrast, when $HCO_3^-/CO_2^-$ is present in the media, cell alkalinization is opposed and compensated by the concurrent activation of the $Na^+$-independent $Cl^-/HCO_3^-$ exchanger, a process that leads to cell $HCO_3^-$ exit and thus a fall in $pH_i$. Accordingly, Ganz et al. (6) proposed that activation of this exchanger is responsible for the cell acidification seen with exposure to agonists such as vasopressin. Other authors have suggested that the initial $pH_i$ fall may be somewhat related to the initial $Ca^{2+}$ surge produced by agonist-induced stimulation although the mechanism involved was not defined (4, 5, 8).

The purpose of this study was to explore the mechanism of

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1. *Abbreviations used in this paper:* Ang II, angiotensin II; AVP, arginine vasopressin; $Ca_i^{2+}$, free cytosolic calcium; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic; EIPA, ethylisopropylamiloride; $pH_i$, intracellular pH; $pH_o$, external pH; SR, sarcoplasmic reticulum; VSMC, vascular smooth muscle cells.

the cell acidification elicited by Ang II and ionomycin-induced $Ca^{2+}$ increases. We reasoned that the $pH_i$ fall could be directly related to the $Ca_i^{2+}$ increase which would activate $Ca^{2+}/H^+$ exchange across the plasma membrane. There is now evidence for multiple subtypes of $Ca^{2+}$-ATPases present in intracellular organelles and in the plasma membrane (9–12). It is well known that the sarcoplasmic reticulum (SR) $Ca_i^{2+}$-ATPase acts as a $Ca^{2+}/H^+$ exchanger (13–15). A plasma membrane $Ca_i^{2+}$-ATPase, originally characterized in bovine aorta by Furukawa and Nakamura (16), is the main cell calcium extrusion mechanism in vascular smooth muscle. An effect of this pump on $pH_i$, however, has not been previously demonstrated. We theorized that stimulation of a plasma membrane $Ca^{2+}$-ATPase by agonists that increase $Ca_i^{2+}$ could result in activation of $Ca^{2+}/H^+$ exchange, a process that would lead to intracellular acidification. We focused on this possible mechanism in light of evidence that the plasma membrane $Ca^{2+}$-ATPase acts as a $Ca^{2+}/H^+$ exchanger in red blood cells (17–20).

## Methods

### *Cell culture*

VSMC were obtained from the thoracic aortae of male rats weighing 300–500 g and isolated by collagenase and elastase digestion as previously described (2, 21). Cells were seeded onto 9 X 35–mm coverslips (Wheaton Industries, Mays Landing, NJ) resting in a 60-mm tissue culture dish. One aorta was used per culture. The cells were grown in Dulbecco's modified Eagle's medium supplemented with Ham's F12 nutrient (Sigma Immunochemicals, St. Louis, MO), 10% fetal calf serum (Hazleton Biologics, Inc., Lenexa, KS), penicillin (100 U/ml), streptomycin (100 μg/ml), and Fungizone (250 μg/ml) at 37°C in a humidified atmosphere of 5% $CO_2$. Cultures were fed or passaged twice weekly.

Experiments were performed on confluent subcultures between the 2nd and 10th passages obtained from primary cultures. 24–48 h before the study, the cells on coverslips were fed with serum-free media. After the coverslips were removed from the culture dish for experiments, cells remaining adherent to the culture dish were passaged onto fresh coverslips. The subcultures grew to confluency in 5–7 d.

### *Dye loading and superfusion*

On the day of the study, cells were loaded with either 4 μM fura-2/AM or 1.5 μM BCECF/AM for 30 min at 37°C. The coverslips were then washed 3 times with the assay buffer and allowed to sit for at least 15 min before beginning the experiment. Neither fura-2 nor BCECF loading altered VSMC morphology as assessed by light microscopy. At the start of the experiment, a coverslip was placed in a customized holder and inserted into a suction cuvette (Hellma, Jamaica, NY) resting in a water-jacketed cuvette holder. The temperature was maintained constant at 37°C. The coverslips were then superfused at a rate of 2 ml/min with the prewarmed assay buffer using a syringe pump, and the effluent was constantly removed with a peristaltic pump. When experimental solutions were switched during the course of an experimental maneuver, the superfusion rate was increased to 8 ml/min for 30 s to enhance the rate of exchange. Preliminary experiments demonstrated that at this rate there was a > 95% exchange of solutions within 20 s. The contribution of external fura-2 or BCECF to the total fluorescent signal due to dye leakage was found to be negligible at the rate of superfusion used as demonstrated by the lack of fluorescence of either dye in the effluent. All solutions and experimental agents were measured for intrinsic fluorescence, and these values were subtracted from total fluorescence before calculating $Ca_i^{2+}$. None of the solutions affected the calculation of $pH_i$ at the wavelengths used for BCECF.

### *Method of calculating $Ca_i^{2+}$*

Fura-2 fluorescence was measured using a spectrofluorometer (LS-5; Perkin Elmer Corp., Norwalk, CT) connected to an IBM PC-XT computer programmed to rapidly alternate between the desired excitation wavelengths (340 and 380 nm) for fura-2–loaded cells. The emission wavelength (510 nm) was constant. The excitation and emission slits were set at 5 and 10 nm, respectively. Free cytosolic $Ca^{2+}$ was calculated using the formula described by Grynkiewicz et al. (22),

$$Ca_i^{2+} = (R - R_{min}/R_{max} - R) \times (Sf/Sb) \times K_d,$$

where $R$ was the 340/380 nm ratio of the fluorescence signal. $R_{max}$ was the 340/380 ratio in the presence of saturating $Ca^{2+}$, $R_{min}$ was the 340/380 ratio in $Ca^{2+}$-free media with 10 mM EGTA added, and $Sf/Sb$ was the ratio of the 380 nm fluorescence measured in a $Ca^{2+}$-free solution to that measured in a $Ca^{2+}$-replete solution. The calibration parameters were obtained in a separate set of experiments using fura-2 acid in a solution designed to simulate the cytosol. $R_{min}$ was determined in a solution of 10% glycerol containing (mM): KCl 115, NaCl 10, $MgSO_4$ 2.0, $K_2H_2EGTA$ 10, and MOPS ($K_2$-[*N*-morpholino]-propane sulfonic acid) 10, at a pH of 7.2. For the determination of $R_{max}$, 2.0 mM $CaCl_2$ was added and CaEGTA was substituted for $K_2H_2EGTA$. The calibration values so determined were 6.98, 0.79, and 4.19 for $R_{max}$, $R_{min}$, and $Sf/Sb$, respectively (21).

The value for the fura-2 $K_d$ for calcium used in the $Ca_i^{2+}$ equation above was 224 nm, which is the $K_d$ determined by Grynkiewicz et al. (22). The value for the $K_d$ of fura-2 is pH dependent and is markedly altered as pH falls to < 6.5. Unless the $K_d$ is corrected, under very acidic conditions the $Ca_i^{2+}$ will be markedly underestimated. Alkaline excursions of pH have a much smaller effect on the fura-2 $K_d$, leading to a 10–20% overestimation of $Ca_i^{2+}$ if the $K_d$ at neutral pH is used (see Negulescu and Machen [23] and Batlle et al. [2]). In this study we used a constant value for the fura-2 $K_d$, as $pH_i$ in our studies was always well above 6.5, and, under these conditions, $K_d$ corrections had very little impact on the final estimate of $Ca_i^{2+}$.

### *Method of calculating $pH_i$*

BCECF fluorescence was measured by alternating between the desired excitation wavelengths (500 and 440 nm) with an emission wavelength of 510 nm. The excitation and emission slits were set at 5 and 10 nm, respectively. To calculate the $pH_i$, the 500/440 BCECF ratio was calibrated using nigericin (6 μg/ml) in 120 mM K buffer as previously described (2, 24, 25). The pH of the superfusate was adjusted in a stepwise fashion between 6.4 and 7.8 by progressive addition of NaOH.

### *Solutions*

All experiments were performed in $CO_2/HCO_3^-$ buffered solutions. The standard $HCO_3^-$ solution contained (mM): $Na^+$ 136, $K^+$ 4.7, $Cl^-$ 122, $HCO_3^-$ 22, $Ca^{2+}$ 1.25, $Mg^{2+}$ 1.25, $HPO_4$ 0.97, $H_2PO_4$ 0.23, glucose 3.0, and Hepes 5.0; and was equilibrated with 5% $CO_2$/95% $O_2$. The pH of this solution was 7.40. When $K^+$ was reduced to 0.25 mM, Cl was reduced to 117 mM.

*Solutions of different pH.* The pH of the bicarbonate-containing perfusate was varied from 6.8 to 8.0 by changing its bicarbonate concentration. The $PCO_2$ was kept constant at 40 mmHg. When the bicarbonate concentration was varied, the anion concentration was kept constant by appropriately varying the amount of chloride.

*Zero chloride solution.* This solution had the following composition: Hepes 5 mM, calcium acetate 1.8 mM, $MgSO_4$ 0.8 mM, glucose 5.6 mM, Na isethionate 101.3 mM, $NaH_2PO_4$ 1.0 mM, mannitol 50 mM, $NaHCO_3$ 16.7 mM, and $KHCO_3$ 5.3 mM.

### *Cadmium and vanadate*

The recovery of $Ca_i^{2+}$ after an increase induced by ionomycin or Ang II is largely due to plasma membrane $Ca^{2+}$-ATPase activation (26, 27). Although vanadate has a variety of nonspecific effects, cadmium at very low concentrations (nanomolar range) has been shown to inhibit $Ca^{2+}$-ATPase in a specific manner (28, 29).

### *Blockade of calcium release from the SR*

Two methods were used. The first involved blockade of calcium release from the SR with TMB-8, (8-diethylamino)-octyl-3,4,5, trimethoxybenzoate hydrochloride (Aldrich Chemical Co., Milwaukee, WI) (30). The effectiveness of TMB-8 blockade was tested by measuring the $Ca_i^{2+}$

response to Ang II. TMB-8 (100 $\mu$m) was added to the superfusate 5 min before exposure to the experimental solution as previously described by us (2). The second method used was to deplete SR of calcium by exposing the cells to angiotensin II ($10^{-6}$ M) in calcium-free solution for 15 min as previously described by us (2) and others (31). The perfusate was switched to a calcium-containing solution immediately before the actual experiment. Depletion of SR stores was verified in studies which showed no $Ca_i^{2+}$ rise after challenge with arginine vasopressin (AVP) ($10^{-6}$ M) (2).

### *Compounds used*

Nigericin, Hepes, choline chloride, AVP, and $NH_4Cl$ were obtained from Sigma Immunochemicals. BCECF/AM and fura-2/AM were purchased from Molecular Probes, Inc. (Eugene, OR). TMB-8 was obtained from Aldrich Chemical Co.

### *Statistical analysis*

Initial analyses of the time course of $Ca_i^{2+}$ or $pH_i$ changes factored by intervention were by repeated measures ANOVA with covariance, assessing the probability of a time effect and of a time by group interaction. Where a time by group interaction was found to exist, further analysis was by the Student $t$ test (i.e., to compare baseline values or peak change values between different groups) or by one-way ANOVA. Changes within the same experimental group were analyzed using paired $t$ testing. Differences were considered significant if $P < 0.05$. All data are reported as mean±SD.

## Results

*Effect of Ang II and ionomycin on $pH_i$ and $Ca_i^{2+}$.* Ang II (1 $\mu$M) caused a prompt rise in $Ca_i^{2+}$ ($\Delta$ $Ca_i^{2+}$, 290±46 nM, $P < 0.001$) with a peak response at ~ 30 s. This peak increase in $Ca_i^{2+}$, which is well known to result from release of $Ca^{2+}$ from the SR, was followed by rapid recovery, with $Ca_i^{2+}$ falling from 449±52 to 220±17 nM ($P < 0.001$) at 3 min ($\Delta$ $Ca_i^{2+}$ −229±43 nM). In the continued presence of Ang II, $Ca_i^{2+}$ remained ~ 30–40 nM above baseline for up to 15 min after initial exposure (Fig. 1, *bottom*).

Changes in $pH_i$ measured in parallel experiments are shown in the top panel of Fig. 1. Concurrent with or soon after the transient rise in $Ca_i^{2+}$, $pH_i$ began to decrease. The fall in $pH_i$ was maximal at 3 min ($\Delta$ $pH_i$, −0.064±0.0085 units, $P <$ 0.01) and coincided with the maximal fall in $Ca_i^{2+}$. The $pH_i$ then returned very slowly to baseline, such that by 12–15 min there was no longer a significant difference between the Ang II and the time control groups.

Very similar results were obtained using a 1 $\mu$M concentration of ionomycin (Fig. 2). The peak increment in calcium was 199±23 nM, $P < 0.001$. By 3 min, $Ca_i^{2+}$ had fallen from 356±30 to 209±30 nM $P < 0.001$ ($\Delta$ $Ca_i^{2+}$ −148±19 nM). The $pH_i$ decreased by 0.050±0.012 units, $P < 0.01$ at 3 min and then slowly returned to baseline (Fig. 2, *top*).

In additional control experiments, $Ca^{2+}$ and $pH_i$ were measured by switching to solutions not containing either Ang II or ionomycin. Under these conditions, the increase in superfusion rate inherent to changing solutions did not affect either $Ca_i^{2+}$ ($\Delta$ $Ca_i^{2+}$ 0.19 ±1.75 nM at 1 min) or $pH_i$ ($\Delta$ $pH_i$ 0.008±0.0005 at 1 min).

*Effect of Ang II on $pH_i$ and $Ca_i^{2+}$ after pretreatment with BAPTA.* The baseline $Ca_i^{2+}$ values in the control and BAPTA-pretreated cells were similar (Fig. 3, *bottom*). Pretreatment of the cells with 60 $\mu$M BAPTA completely abolished the $Ca_i^{2+}$ response to Ang II (Fig. 3). The peak increase in $Ca_i^{2+}$ was reduced from 385±84 in the control studies to 3.6±4.2 nM in the BAPTA group ($P < 0.001$). As shown in the top panel of Fig. 3, BAPTA pretreatment also abolished completely the $pH_i$ decrease associated with Ang II ($\Delta$ $pH_i$ change −0.055±0.008 in control cells vs -0.002±0.0008 pH units in BAPTA-pretreated cells [$P < 0.001$]).

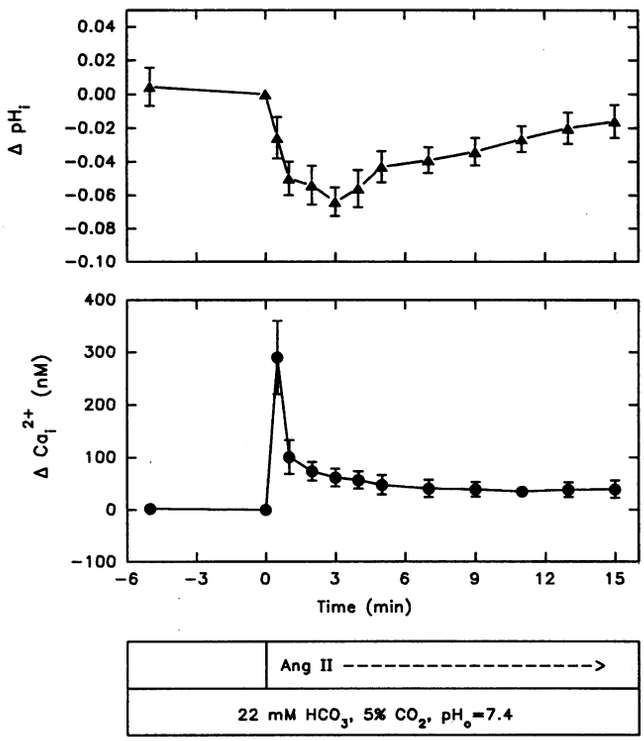


*Figure 1.* Effect of Ang II on $pH_i$ (*top*) and $Ca_i^{2+}$ (*bottom*). Ang II caused a prompt fall in $pH_i$, with a maximum drop observed at ~ 3 min. Subsequently, $pH_i$ recovered slowly toward baseline. In parallel experiments summarized in the lower panel it was found that Ang II exposure was associated by a rapid $Ca_i^{2+}$ increase (peak response ~ 30 s), followed by a prompt, partial recovery toward baseline, to a plateau $Ca_i^{2+}$ increase of ~ 30 nM. As is evident from the traces, the decrease in $pH_i$ and the $Ca_i^{2+}$ recovery were temporally correlated ($n = 9$).

*Effect of Ang II on $pH_i$ and $Ca_i^{2+}$ after DIDS and EIPA.* The $pH_i$ effect of Ang II was studied in cells sequentially pretreated with 50 $\mu$M ethylisopropylamiloride (EIPA) to block Na/H exchange, and 50 $\mu$M 4,4′-diisothiocyanatostilbene-2,2′-disulfonic (DIDS) to block both Na-dependent and Na-independent $Cl/HCO_3$ exchange. EIPA addition resulted in cell acidification, and, with both blockers added, the baseline $pH_i$ was further reduced (from 7.36±0.035 to 7.24±0.027, $P < 0.001$) (Fig. 4, *top*). In the continued presence of both EIPA and DIDS, addition of Ang II to the perfusate resulted in a further fall in $pH_i$ (from 7.24±0.27 to 7.17±0.032 at 3 min, $P < 0.01$), whereas in controls perfused with only EIPA and DIDS $pH_i$ had stabilized by this time (Fig. 4).

The decrease in $pH_i$ associated with perfusion with Ang II was similar or even greater than the fall in $pH_i$ observed in control cells exposed to Ang II but not pretreated with EIPA/DIDS (compare Fig. 4 with Fig. 1). In the presence of EIPA/DIDS, however, there was no recovery of $pH_i$ from the fall associated with Ang II; in fact, in the continued presence of Ang II, EIPA, and DIDS, $pH_i$ continued to fall over the 15-min monitoring period (Fig. 4). This is consistent with the notion that recovery from cell acidification requires activation of $Na^+/H^+$ exchange and Na-dependent $Cl/HCO_3$ exchange (6).

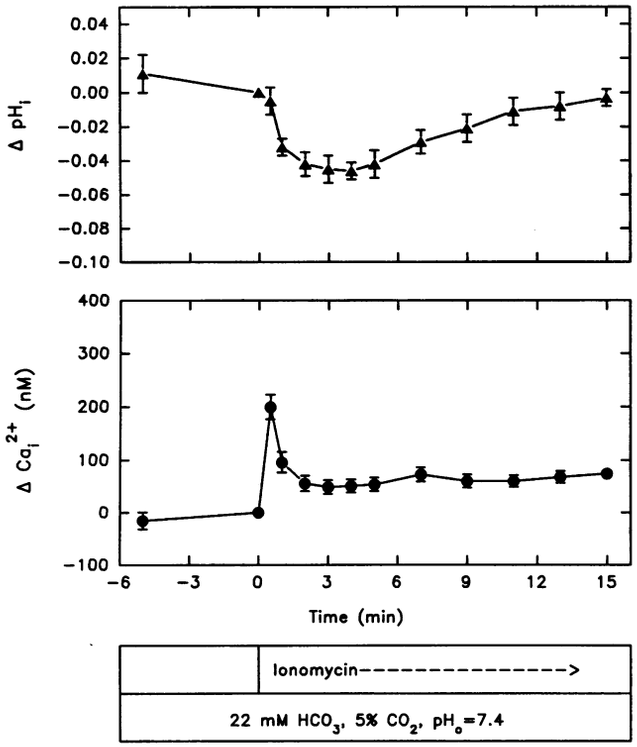


*Figure 2.* Effect of ionomycin on $pH_i$ (*top*) and $Ca_i^{2+}$ (*bottom*). The same experiment as in Fig. 1, except that ionomycin (1 $\mu$M) was used. Changes observed in $pH_i$ ($n$ = 7) and in $Ca_i^{2+}$ ($n$ = 11) were similar to those found after exposure to Ang II (compare with Fig. 1).

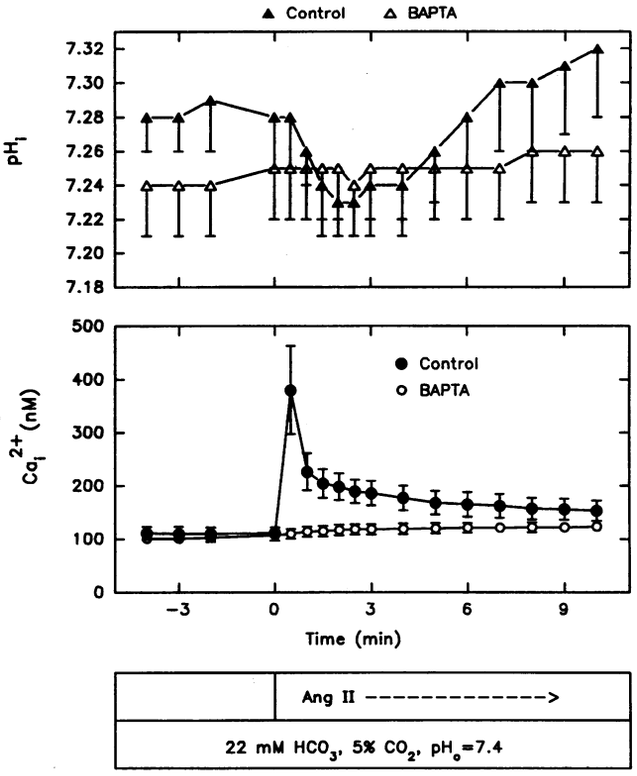


*Figure 3.* Ang II–associated cell acidification: effect of BAPTA (60 $\mu$M). In control studies ($n$ = 4), the usual profile of $pH_i$ and $Ca_i^{2+}$ changes was observed. However, BAPTA pretreatment ($n$ = 6) completely abolished both the decrease in $pH_i$ (*top*) and the increase in $Ca_i^{2+}$ (*bottom*), suggesting that the two are linked.

We also studied the effects of Ang II on $Ca_i^{2+}$ after EIPA and DIDS pretreatment (50 $\mu$M EIPA and 50 $\mu$M DIDS). Because EIPA interferes with the fluorescence evaluation of fura-2 (but not BCECF), EIPA and DIDS were washed out 30 s before exposure of the cells to Ang II (Fig. 4, *bottom*). Under these conditions, with blocking of both the $Na^+/H^+$ antiporter and the $Cl/HCO_3$ exchangers, Ang II also produced a typical increase in $Ca_i^{2+}$ followed by rapid recovery (Fig. 4, *bottom*).

*Effect of Ang II and ionomycin on $pH_i$ and $Ca_i^{2+}$ in the presence of cadmium.* Effects of cadmium (20 nM) on Ang II or ionomycin-associated cell acidification are presented in the upper panels of Figs. 5 and 6, respectively. Cadmium abolished the acidification induced by either of these agents. The $pH_i$ change after Ang II was $-0.058\pm0.006$ in the control studies and was reduced to $-0.0022\pm0.0006$, $P < 0.001$ in the presence of cadmium (Fig. 5). The $pH_i$ fall elicited by ionomycin in control studies ($-0.042\pm0.0038$) also was obliterated in the presence of cadmium ($\Delta$ $pH_i$, $-0.0016\pm0.0004$, $P < 0.001$) (Fig. 6, *top*).

The effect of Ang II or ionomycin on $Ca_i^{2+}$ in the presence of cadmium is shown in the lower panels of Figs. 5 and 6, respectively. Cadmium (20 nM) had no effect on the peak $Ca_i^{2+}$ levels achieved with either Ang II or ionomycin. However, in cadmium-treated cells, $Ca_i^{2+}$ recovery was reduced as compared with cells perfused with either Ang II or ionomycin not treated with cadmium. The inhibitory effect of cadmium on $Ca_i^{2+}$ recovery was more pronounced with ionomycin than with Ang II, but a significant inhibitory effect on the $Ca_i^{2+}$ recovery curve was present with both agents. These results support the notion that cadmium inhibits cell $Ca^{2+}$ exit as a result of inhibition of a plasma membrane $Ca^{2+}$-ATPase (28, 29).

*Effects of cadmium on baseline $Ca_i^{2+}$ and $pH_i$.* It has been shown previously in erythrocyte ghosts that very low doses of cadmium ($K_i$ = 2.0 nM) cause inhibition of $Ca^{2+}$-ATPase activity (32). In our experimental preparation, introduction of 20 nM cadmium into the perfusate caused a slight but significant increase in the $Ca_i^{2+}$ level, ($\Delta$ $Ca_i^{2+}$ 15±6.8 nM at 3 min vs $-2.69\pm4.11$ nM in time controls, $P < 0.05$) (Fig. 7, *bottom*). $pH_i$ also increased slightly ($\Delta$ $pH_i$ $0.021\pm0.006$ units vs a time control value of $0.003\pm0.01$). The $pH_i$ differences between cadmium-treated and control cells were statistically significant (Fig. 7, *top*). These data are consistent with the hypothesis that cadmium at low doses inhibits a plasma membrane $Ca^{2+}/H^+$ exchanger.

*Effects of DIDS and cadmium on $pH_i$ recovery from cell alkalinization.* In the EIPA/DIDS experiments depicted in Fig. 4, it might be argued that the dose of DIDS used (50 $\mu$m) was not sufficient to fully inhibit Na-independent $Cl/HCO_3$ exchange. If this were the case, stimulation of this exchanger by Ang II could have been responsible for a fall in $pH_i$ as proposed by Ganz et al. (6). To verify that the dose of DIDS used by us inhibits the Na-independent $Cl/HCO_3$ exchanger, cells were acutely alkalinized using zero chloride solution. Readdition of chloride resulted in a prompt restoration of $pH_i$ to baseline (Fig. 8), an effect which is thought to be due to operation of the Na-independent $Cl/HCO_3$ exchanger (33, 34). In the presence of 50 $\mu$M DIDS, the $pH_i$ fall on readdition of chloride was completely blocked (Fig. 8). This attests to the efficacy of this concentration of DIDS in blocking Na-independent $Cl/HCO_3$ exchange.

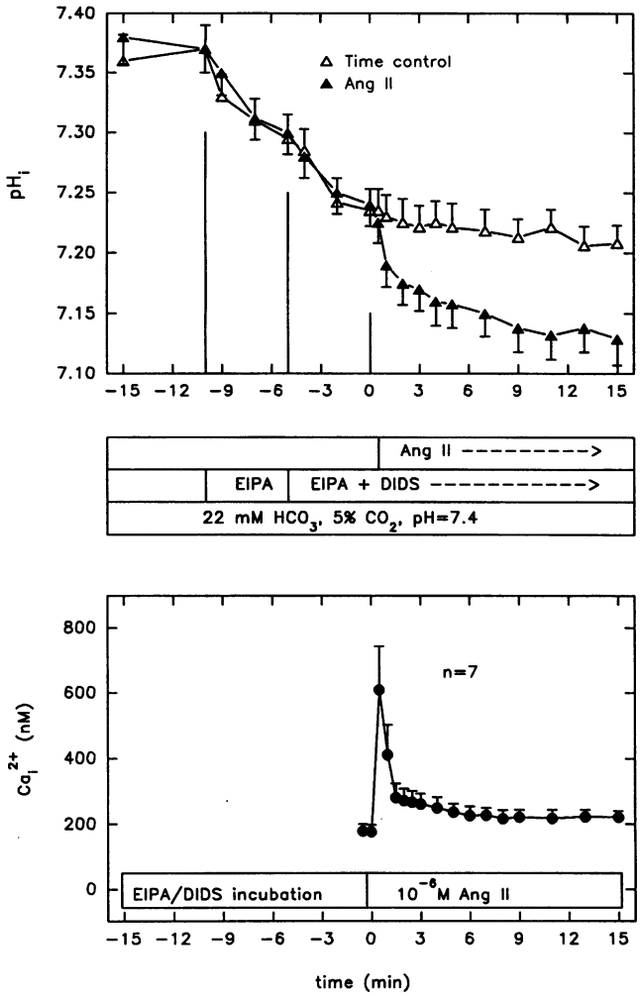


*Figure 4.* Ang II–associated cell acidification: effect of blocking Cl/$HCO_3$ exchange and Na/H exchange with DIDS (50 $\mu$M) and EIPA (50 $\mu$M), respectively. In control experiments (*open triangles*), in which Ang II was not added to the perfusate, sequential addition of EIPA and DIDS caused cell acidification. When Ang II was introduced in cells pretreated with EIPA/DIDS (*filled triangles*), a further rapid decline in $pH_i$ was seen. This Ang II–associated $pH_i$ response was similar in magnitude and time course to that seen when Ang II was introduced to untreated cells (compare with Fig. 1), except that with EIPA/DIDS pretreatment there was no recovery of $pH_i$ toward baseline ($n = 15$). In the bottom panel, the $Ca_i^{2+}$ response after Ang II is shown in cells pretreated with the same dose of EIPA and DIDS. EIPA and DIDS were washed out 30 s before introduction of Ang II for reasons explained in the text. EIPA/DIDS pretreatment had no effect on either the $Ca_i^{2+}$ peak or recovery observed during Ang II superfusion.

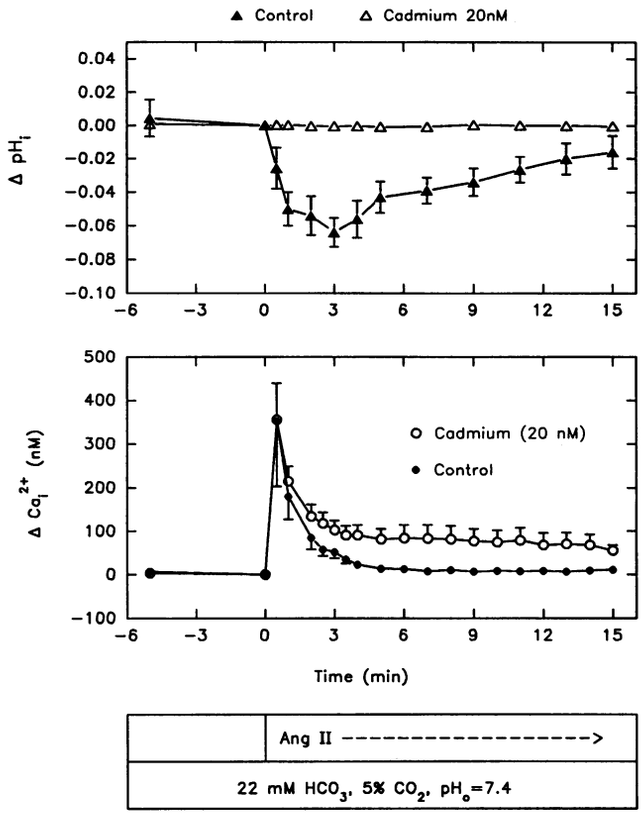


*Figure 5.* Ang II–associated cell acidification: effect of cadmium (20 $\mu$M). Cadmium ($n = 7$) (*open triangles*) blocked the fall in $pH_i$ caused by Ang II (1 $\mu$M) in control preparations (*filled triangles*). The cadmium effect was highly significant ($P < 0.001$) compared with time controls ($n = 9$). In the bottom panel, the effects of cadmium on the $Ca_i^{2+}$ response to Ang II are shown. Cadmium did not affect the early rise in $Ca_i^{2+}$ but substantially delayed $Ca_i^{2+}$ recovery ($P < 0.05$ by two-way ANOVA).

In contrast, the fall in $pH_i$ on readdition of chloride was identical in the presence and absence of 20 nM cadmium (Fig. 8). This shows that cadmium has no inhibitory effect on Na-independent Cl/$HCO_3$ exchange. Thus, the blockade of Ang II–induced cell acidification by cadmium that we observed (Figs. 5 and 6) must have been unrelated to blockade by cadmium of cell acidification caused by cell $HCO_3$ exit via stimulation of the Na-independent Cl/$HCO_3$ exchanger.

*Effects of cadmium on $pH_i$ changes produced by cell hyperpolarization.* Cadmium has been shown to inhibit proton currents in cell membranes (albeit at high concentration, 40 $\mu$M) (35). It remained thus possible that the inhibitory effect of cadmium on agonist-induced acidification that we observed was not due to inhibition of $Ca^{2+}/H^+$ exchange via the plasma membrane Ca-ATPase but rather to an effect on a proton conductance pathway. Hyperpolarization should induce a decrease in $pH_i$ by increasing $H^+$ entry through this pathway (36). To explore this question, membrane hyperpolarization was induced by lowering the perfusate potassium concentration from 4.7 to 0.25 mM. Lowering perfusate potassium did indeed effect a significant fall in $pH_i$, averaging ~ 0.7 pH units 3 min after solution change (Fig. 9). When the experiments were performed in the presence of 20 nM cadmium, the fall in $pH_i$ associated with lowering perfusate $K^+$ was not significantly different than that without cadmium (Fig. 9). These results indicate that the low concentration of cadmium used in our study does not affect $pH_i$ by inhibiting proton conductance through the plasma membrane.

*Effect of external pH on $Ca_i^{2+}$ and $pH_i$.* If a plasma membrane Ca/H exchanger were present and tonically active, one would expect that lowering external pH ($pH_o$) (increasing the perfusate $[H^+]$) would accelerate calcium extrusion and thereby reduce $Ca_i^{2+}$, whereas increasing $pH_o$ (lowering perfusate $[H^+]$) should slow down the operation of such an exchanger causing an increase in $Ca_i^{2+}$. $pH_o$ was thus altered by changing the $HCO_3$ level in the perfusate, keeping the $pCO_2$ level at

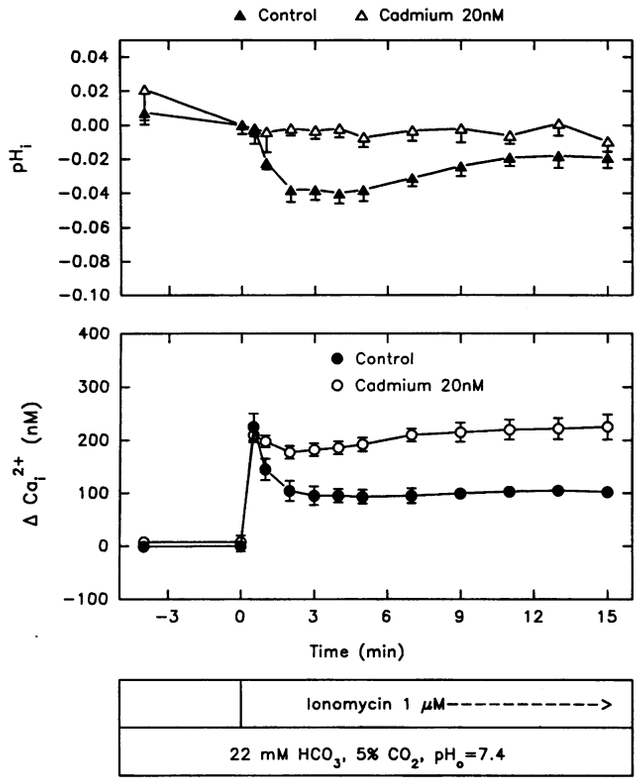


*Figure 6.* Ionomycin-associated cell acidification: effect of cadmium (20 nM). The $pH_i$ results are presented in the top panel ($n$ = 7 each for cadmium and controls), and $Ca_i^{2+}$ data are shown in the lower panel ($n$ = 8 each for cadmium and controls). Cadmium completely blocked the $pH_i$ fall associated with ionomycin (1 $\mu$m). Cadmium did not inhibit the early rise in $Ca_i^{2+}$ associated with ionomycin, but prevented the subsequent $Ca_i^{2+}$ recovery ($P < 0.05$ by two-way ANOVA).

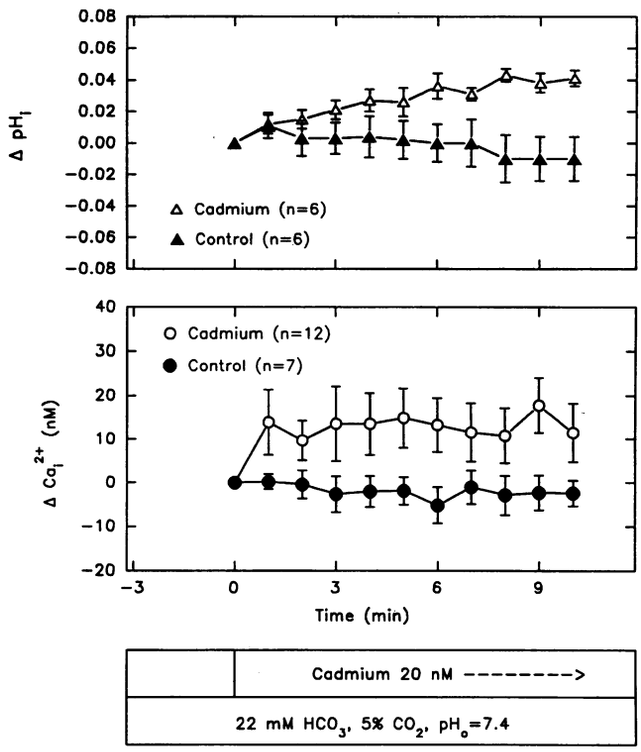


*Figure 7.* The effects of low concentration cadmium (20 nM) on resting (steady state) $pH_i$ (*top*) and $Ca_i^{2+}$ (*bottom*). This concentration of cadmium caused a slight but statistically significant increase in both $pH_i$ and $Ca_i^{2+}$ as compared with time controls ($P < 0.05$ by two-way ANOVA).

40 Torr as previously described by us (2). $HCO_3$-containing solutions with pH values ranging from 6.8 to 8.0 were used (Fig. 10).

Alkalinizing the perfusate to 7.8 or 8.0 caused $Ca_i^{2+}$ to increase, whereas acidifying the perfusate caused a small decrease in $Ca_i^{2+}$. The $Ca_i^{2+}$ changes were rather prompt and were well established by 3 min after perfusate change. Changing to $pH_o$ of 8.0, 7.8, 7.0, and 6.8, respectively, produced $Ca_i^{2+}$ increments of +40±7.8, +11±3.8, −3.7±3.1, and −4.4±3.3 nM, at 3 min, respectively. By ANOVA, the $Ca_i^{2+}$ changes associated with increasing perfusate pH to 8.0 or 7.8 were significantly different from baseline and from each other ($P < 0.01$) (Fig. 10).

$pH_i$ was measured in control experiments conducted in parallel. Results are presented in the top panel of Fig. 10. Changes in $pH_o$ to 8.0 and 6.8 were reflected by corresponding changes in $pH_i$ in the same direction. However, $pH_i$ changes were delayed with respect to the imposed $pH_o$ change. The increase in $pH_i$ elicited by perfusate alkalinization to pH 8.0 was gradual, requiring several minutes to manifest ($\Delta$ $pH_i$ +0.31±0.05 units at 12 min), whereas the $Ca_i^{2+}$ increase was more rapid and already maximal at 3 min (see Fig. 10, *bottom*). Increasing $pH_i$ (that is, lowering cytosolic $H^+$) could only facilitate $Ca^{2+}$ reuptake by the SR $Ca^{2+}$-ATPase, a process that would lower $Ca_i^{2+}$. The increase in $pH_i$ secondary to external alkalinization, however, could have resulted in an increased $Ca_i^{2+}$ by stimulating $Ca^{2+}$ release from internal stores (2). To rule out this possi-

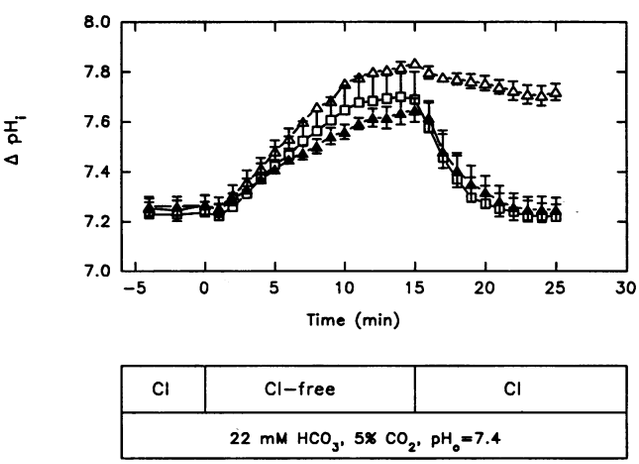


*Figure 8.* Effects of cadmium (20 nM) or DIDS (50 $\mu$M) on Na-independent $Cl/HCO_3$ exchange. Cells were alkalinized by exposing them to a zero chloride perfusate. In control cells (*filled triangles*), readdition of chloride to the perfusate caused a prompt resolution of cell alkalinization and restoration of baseline $pH_i$. This restoration of $pH_i$ is presumably due to expulsion of $HCO_3$ from the cell in exchange for chloride via Na-independent $Cl/HCO_3$ exchange. DIDS (*open triangles*) completely abolished the restoration of $pH_i$ associated with readdition of chloride to the perfusate, demonstrating that the dose of DIDS used in this study completely blocked Na-independent $Cl/HCO_3$ exchange. In contrast, cadmium pretreatment (*open squares*) failed to affect the restoration of $pH_i$ associated with readdition of chloride to the perfusate, demonstrating that cadmium has no effect on Na-independent $Cl/HCO_3$ exchange.

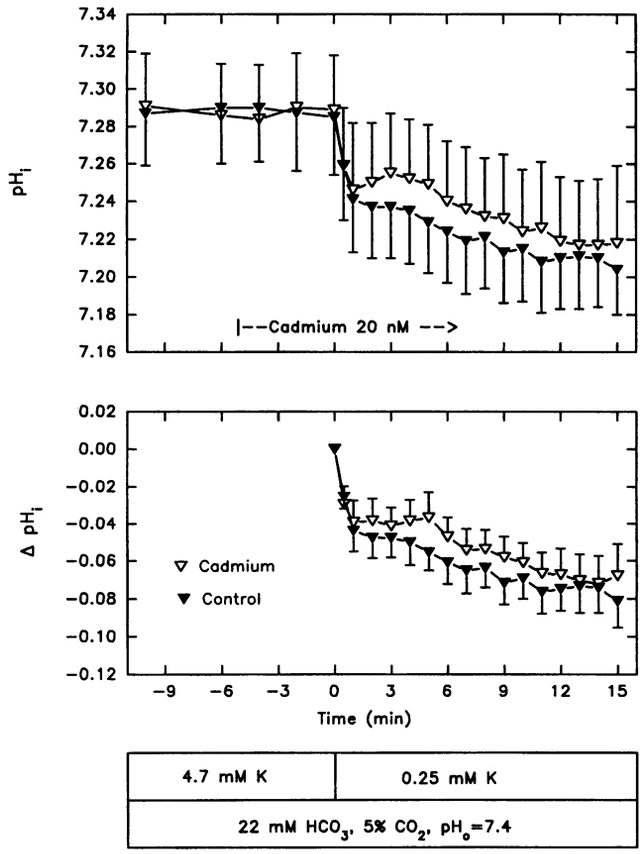


*Figure 9.* The effects of membrane hyperpolarization induced by reducing perfusate K from 4.7 to 0.25 mM on $pH_i$ are shown in both the presence and absence of 20 nM cadmium. Absolute $pH_i$ changes are shown in the top panel and $\Delta$ $pH_i$ changes are shown in the lower panel. If a proton conductance were present, membrane hyperpolarization would increase $H^+$ entry, resulting in cell acidification. The data presented show that cell acidification does indeed occur under these conditions and are consistent with the existence of a proton conductance in our cells. However, 20 nM cadmium has no apparent effect on this proton conductance, as the $pH_i$ changes subsequent to membrane hyperpolarization were not significantly different in the presence and absence of cadmium.

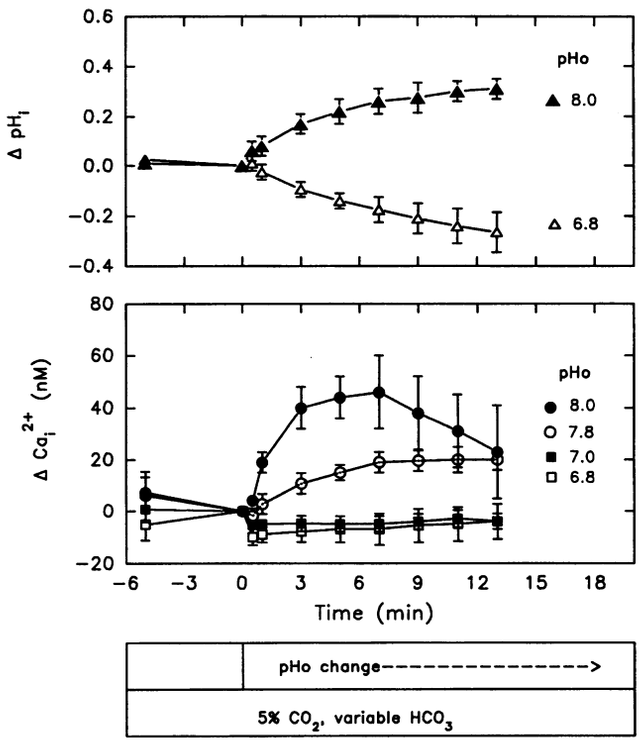


*Figure 10.* Effects of changing perfusate pH ($pH_o$) on $pH_i$ (*top*) and $Ca_i^{2+}$ (*bottom*). Baseline perfusate pH was 7.4. As shown, increasing $pH_o$ from 7.4 to 7.8 or 8.0 caused a rather prompt rise in $Ca_i^{2+}$, with the response being more rapid and pronounced at pH 8.0 than at 7.8. Reducing perfusate pH from 7.4 to 7.0 or 6.8 caused a slight decrease in $Ca_i^{2+}$. A temporal comparison of the $pH_i$ and $Ca_i^{2+}$ traces suggests that changes in $Ca_i^{2+}$ associated with perfusate alkalinization were not secondary to changes in $pH_i$.

bility, we examined the effect of $pH_o$ on $Ca_i^{2+}$ under conditions where internal mobilization of $Ca^{2+}$ was blocked (see below).

*Role of the SR in the $Ca_i^{2+}$ increase elicited by perfusate alkalinization.* SR blockade of $Ca_i^{2+}$ release was affected using TMB-8 or SR depletion by prior exposure to Ang II as previously described by us (2). Both these maneuvers completely block the rise in $Ca^{2+}$ produced by rapid cell alkalinization (2). In contrast, in the present study neither one of these maneuvers affected the initial rise in $Ca_i^{2+}$ secondary to perfusate alkalinization to pH 8.0 (Fig. 11). The $Ca_i^{2+}$ increases at 3 min in the control cells (40±7.8 nM), TMB-8–treated cells (66.3±12.4 nM), and SR-depleted cells (44.9±5.6 nM) were not significantly different by ANOVA ($P = 0.112$). These results indicate that internal $Ca^{2+}$ mobilization was not the source for the $Ca_i^{2+}$ increase associated with external alkalinization and the associated increase in internal pH.

The $Ca_i^{2+}$ recovery profile in the SR-depleted cells was different from both the control curve and TMB-8 curve by repeated measures by ANOVA with covariance ($P < 0.01$). The difference was due to a more prompt resolution of the $Ca_i^{2+}$ surge in the SR-depleted cells, probably reflecting enhanced $Ca^{2+}$ reuptake by the SR (Fig. 11).

*Effect of external calcium on the $Ca_i^{2+}$ response to perfusate alkalinization.* The rise in $Ca^{2+}$ associated with perfusate alkalinization ($\Delta$ $Ca_i^{2+}$ 41±4.5 nM at 3 min) was markedly reduced when such alkalinization was affected in the nominal absence of calcium in the perfusate ($\Delta$ $Ca_i^{2+}$ 8.0±1.6 nM, $P < 0.001$) (Fig. 12). This finding is consistent with the finding of Muallem et al. (37) that external alkalinization enhances cell calcium entry. Removal of external $Ca^{2+}$, however, is a drastic maneuver which normally produces a fall in $Ca_i^{2+}$. The effect of perfusate alkalinization in a zero $Ca^{2+}$ perfusate on $Ca_i^{2+}$ was thus compared with a time control where $Ca_i^{2+}$ was measured after switching to a zero $Ca^{2+}$ perfusate. In the time control study ($pH_o$ 7.4) $Ca_i^{2+}$ decreased ($\Delta$ $Ca_i^{2+}$ −14±3.1 nM at 3 min) after perfusate change to zero calcium, compared with the $\Delta$ $Ca_i^{2+}$ increase of 8.0 nM noted when alkalinization (pH 8.0) was affected also in a zero calcium perfusate ($P < 0.05$). The lack of a fall, and in fact the slight initial rise in $Ca_i^{2+}$ associated with perfusate alkalinization despite zero $Ca^{2+}$ external conditions, is consistent with inhibition of a $Ca^{2+}$ extrusion mechanism by an alkaline $pH_o$. The slight initial rise in $Ca_i^{2+}$ associated with external alkalinization in a zero calcium perfusate was of similar magnitude to that seen with addition of 20 nM cadmium to a 1.25 mM $Ca^{2+}$ perfusate (compare Figs. 12 and 7). Taken together, the data suggest that both perfusate alkaliniza-

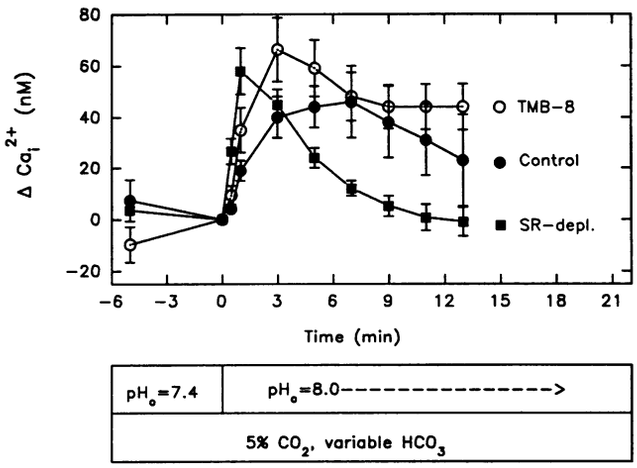


*Figure 11.* SR blockade using TMB-8 did not alter the initial increase in $Ca_i^{2+}$ induced by changing $pH_o$ from 7.4 to 8.0. SR depletion by prior exposure to Ang II also did not prevent the early rise in $Ca_i^{2+}$ associated with perfusate alkalinization. The results indicate that internal $Ca^{2+}$ mobilization was not the source of the $Ca_i^{2+}$ increase produced by perfusate alkalinization. In the SR-depleted cells, $Ca_i^{2+}$ recovery was enhanced ($n$ = 8 per group).

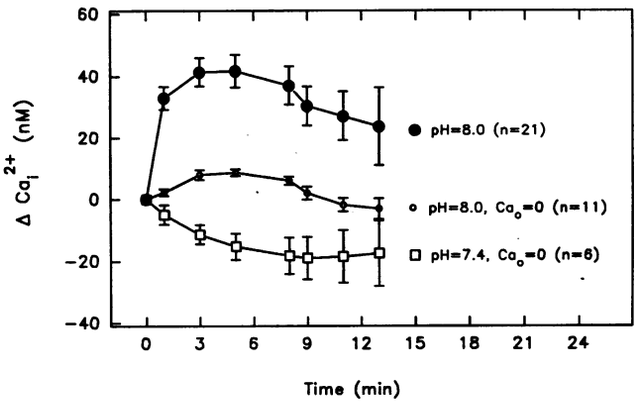


*Figure 12.* The effects of external $Ca^{2+}$ on the $Ca_i^{2+}$ response to perfusate alkalinization. The control trace (perfusate $Ca^{2+}$ = 1.25 mM) shows that there is an increase in $Ca_i^{2+}$ within minutes of increasing perfusate pH from 7.4 to 8.0. The increase in $Ca_i^{2+}$ associated with perfusate alkalinization is substantially diminished when perfusate $Ca^{2+}$ is lowered to zero before perfusate alkalinization. However, the $Ca_i^{2+}$ profile associated with an alkalinized ($pH_o$ 8.0) zero calcium perfusate is substantially higher ($P < 0.01$) than that of time controls exposed to zero calcium perfusate at $pH_o$ 7.4 (see text for details).

tion and cadmium inhibit a calcium extrusion mechanism that exchanges $Ca^{2+}$ for $H^+$ via the plasma membrane $Ca^{2+}$-ATPase. However, perfusate alkalinization also appears to increase $Ca_i^{2+}$ by an additional effect that is mediated by enhanced calcium entry.

## Discussion

This study shows that, in cultured rat aortic VSMC assayed in a $HCO_3$-containing medium, rapid increases in $Ca_i^{2+}$ are associated with cell acidification which occurs in parallel with recovery from the $Ca_i^{2+}$ surge. The data strongly suggest that this cell acidification is due to entry of acid equivalents into the cell via the plasma membrane $Ca^{2+}$-ATPase acting as a $Ca^{2+}/H^+$ exchanger. Rapidly raising $Ca_i^{2+}$ about two- to threefold by mobilization of $Ca^{2+}$ from SR stores produced by either a vasoactive hormone (Ang II) (38, 39) or by a non–receptor-mediated mechanism using ionomycin (40) resulted in rapid cell acidification which was temporally associated with an equally rapid $Ca_i^{2+}$ recovery (Figs. 1 and 2). Our finding that cell acidification did not occur when the increase in $Ca_i^{2+}$ was prevented using BAPTA (Fig. 3) indicates that changes in $Ca_i^{2+}$ (rather than other $Ca_i^{2+}$-independent actions of agonists such as Ang II) are responsible for the initial fall in $pH_i$ observed with such agonists. The $Ca^{2+}/pH_i$ interaction was inferred to involve $Ca^{2+}/H^+$ exchange across the plasma membrane because cadmium, an agent that blocks $Ca^{2+}$ exit via the plasma membrane $Ca^{2+}$-ATPase, blunted both the cell acidification and $Ca_i^{2+}$ recovery after the $Ca_i^{2+}$ surge induced by Ang II or ionomycin (Figs. 5 and 6).

Blockade of Ang II–induced cell acidification produced by cadmium (20 nM) was not related to inhibition of a proton conductance at the plasma membrane. Kapus et al. (35) have described an electrogenic $H^+$-conducting pathway in the membrane of neutrophils that was inhibited by high concentrations of cadmium (40 $\mu$M). The concentration of cadmium used in our studies (20 nM), however, was an order of magnitude lower than that used by Kapus et al. (35). To exclude the possibility that cadmium at such a low dose inhibits proton conductance, we performed experiments in which cell acidification was induced by hyperpolarizing the plasma membrane (by markedly reducing the external K concentration) (36). Cadmium (20 nM) had no inhibitory effect on the degree of cell acidification produced by cell hyperpolarization (Fig. 9). This indicates an absence of effect of low dose cadmium on proton conductance.

Previous evidence for Ca/H exchange via a plasma membrane $Ca^{2+}$-ATPase in smooth muscle, to our knowledge, is lacking. The concept that the plasma membrane $Ca^{2+}$-ATPase might act as a $Ca^{2+}/H^+$ antiporter, however, has been advanced by Smallwood et al. (18) based on studies in erythrocytes. Rossi and Schatzman (17) and others (18–20) suggested that the plasma membrane $Ca^{2+}$-ATPase pump of erythrocytes was electrogenic and that the cation most probably implicated in compensation for the voltage generated was the hydrogen ion. Our findings are consistent with the plasma membrane $Ca^{2+}$-ATPase acting as a $Ca^{2+}/H^+$ exchanger rather than being electrogenic. Because the affinity of the $Ca^{2+}$-ATPase for calcium is high (relative to that of the Na/Ca exchanger), it is believed that the plasma membrane $Ca^{2+}$-ATPase is responsible for maintenance of $Ca_i^{2+}$ at a low level (9, 10, 12, 21). Tonic operation of this pump to maintain steady state $Ca_i^{2+}$ would thereby result in entry of $H^+$ into the cell cytoplasm and thus impact on $pH_i$ regulation. If a plasma membrane Ca-ATPase were tonically acting as a Ca/H exchanger, its inhibition might be expected to cause an increase in $Ca_i^{2+}$. In fact, when we measured $Ca_i^{2+}$ in cells exposed to 20 nM cadmium, a small but significant rise in $Ca_i^{2+}$ was observed (Fig. 9, *bottom*). At the same time, the $pH_i$ rose slightly, consistent with inhibition by cadmium of proton entry via Ca/H exchange. These data support the concept that a plasma membrane Ca-ATPase present in VSMC functions as an obligatory Ca/H exchanger.

Cadmium at very low concentrations is a reasonably specific blocker of the plasma membrane $Ca^{2+}$-ATPase (28, 29, 32). Consistent with this was our finding that recovery of $Ca_i^{2+}$ from both ionomycin and Ang II stimulation was blunted, but not

completely prevented, by the concentration of cadmium used by us (20 nM). This low concentration of cadmium may not have provided complete inhibition owing to incomplete penetration in the cell interior, the site of the cadmium inhibitory effect on the plasma membrane $Ca^{2+}$-ATPase (32). We found that higher concentrations of cadmium (20 $\mu$M) markedly increase steady state $Ca_i^{2+}$ and block $Ca_i^{2+}$ recovery after Ang II (data not shown). These studies were performed using a low concentration of cadmium (20 nM) to avoid the effects of higher concentrations on proton conductance and other possible nonspecific actions. Vanadate, another agent which inhibits the plasma membrane $Ca^{2+}$-ATPase (but with many nonspecific effects) also obliterated $Ca_i^{2+}$ recovery after Ang II stimulation (data not shown). These observations are in accord with the known role of the plasma membrane $Ca^{2+}$-ATPase in mediating, in part, $Ca_i^{2+}$ recovery after agonist-induced increases in $Ca_i^{2+}$ within the physiologic range.

While the plasma membrane $Ca^{2+}$-ATPase subserves a primary role in the regulation of $Ca_i^{2+}$, our data suggest that it may modify $pH_i$ in the process of extruding $Ca^{2+}$, particularly when activated by agonists that increase $Ca_i^{2+}$. This assumes particular relevance because concurrent changes in both $Ca_i^{2+}$ and $pH_i$, such as those depicted in Fig. 1, occur with a wide array of vasoactive compounds and growth factors. $pH_i$ regulation is generally accomplished via well known plasma membrane transporters (Na/H exchange, Na-dependent $Cl/HCO_3$ exchange, and Na-independent $Cl/HCO_3$ exchange) which, acting in concert, prevent departures of $pH_i$ from normal (6, 7, 33, 34). Recovery from cell acidification can be easily affected by stimulation of Na/H exchange and Na-dependent $Cl/HCO_3$ exchange. Our data show that this is the case, since the $pH_i$ fall after Ang II was markedly prolonged in cells pretreated with EIPA and DIDS (compare the $pH_i$ traces in Figs. 1 and 4). Similarly, Kikeri et al. (7) found that AVP did not acidify A10 cells when $pH_i$ regulatory mechanisms were intact, but the AVP effect was unmasked when cells were pretreated with amiloride, which prevents compensatory proton extrusion.

As noted in the introduction, Ganz et al. (6) did show that exposure of cultured mesangial cells to vasopressin, an agonist with actions in smooth muscle similar to those of Ang II, resulted in concurrent stimulation of three $pH_i$-dependent transporters, Na/H exchange, Na-dependent $Cl/HCO_3$ exchange, and Na-independent $Cl/HCO_3$ exchange. Only one of these, Na-independent $Cl/HCO_3$ exchange, could, when stimulated, result in cell acidification. Consistent with this idea is the finding by Green et al. (41) in osteoblasts, showing that a transient increase in $Ca_i^{2+}$ may alter the sensitivity of the Na-independent $Cl/HCO_3$ exchanger to internal bicarbonate, causing it to operate at a $pH_i$ range (i.e., near steady state) where this exchanger is normally quiescent. This would result in increased efflux of $HCO_3$ from the cell and net acidification . Our findings, however, do not support a role for $Cl/HCO_3$ exchange as the primary mechanism of agonist-induced cell acidification in VSMC. This can be inferred from the finding that when Ang II was applied after pretreatment of the cells with DIDS (and EIPA) to block the Na-independent $Cl/HCO_3$ exchanger, the rapid fall in $pH_i$ was intact and similar to that seen in control cells not pretreated with DIDS and EIPA (compare Fig. 4 with Fig. 1). It should also be noted that cadmium did not affect the $pH_i$ recovery after cell alkalinization, which is mediated by the Na-independent $Cl/HCO_3$ exchanger (Fig. 8). Thus, cadmium blocks the $pH_i$ fall produced by Ang II and ionomycin (Figs. 5 and 6) but does not inhibit the Na-independent $Cl/HCO_3$ exchanger which is the only known $pH_i$ regulatory transporter which, when stimulated, is capable of lowering $pH_i$.

$Ca^{2+}/H^+$ exchange via the SR $Ca^{2+}$-ATPase, like that postulated above to take place via the plasma membrane $Ca^{2+}$-ATPase, could result in cytosolic acidification in association with rapid recovery from the $Ca^{2+}$ surge induced by agonists. In vascular smooth muscle the activity of the SR $Ca^{2+}$-ATPase is modulated by pH, to the point that proton countertransport causes this enzyme to function as a $Ca^{2+}/H^+$ exchanger (13–15). To distinguish $Ca^{2+}/H^+$ exchange across the plasma membrane from that occurring at the level of the SR, $Ca_i^{2+}$ was monitored while the concentration of external protons was altered acutely. We reasoned that increasing $pH_o$ should have opposite effects on plasma membrane and SR $Ca^{2+}$-ATPases. External alkalinization should impede proton entry into, and $Ca^{2+}$ exit from, the cell, leading to an increase in $Ca_i^{2+}$ if there is tonic operation of plasma membrane $Ca^{2+}/H^+$ exchange. By contrast, external alkalinization should theoretically lead to a fall in $Ca_i^{2+}$ via SR $Ca^{2+}$ exchange because the expected resultant cytosolic alkalinization should favor proton exit from, and $Ca^{2+}$ entry into, the SR. However, external alkalinization could increase $Ca^{2+}$ by increasing $pH_i$ since it is known that an increase in $pH_i$ causes an increase in $Ca_i^{2+}$ due to $Ca^{2+}$ mobilization from an SR-associated store (2). The mechanism for this is unknown, but seems related to modulation of the interaction between the SR and $IP_3$ (23, 42).

The experimental approach to distinguish these possibilities was to change perfusate pH ($pH_o$) and monitor $Ca_i^{2+}$. Under these conditions $pH_i$ changes follow alterations in $pH_o$ in a delayed fashion (Fig. 10, *top*). This experimental design allowed for a temporal separation of the effects of increased $pH_o$ on plasma membrane $Ca^{2+}$-ATPase versus the effect of $pH_i$ on increased $Ca^{2+}$ release from the SR. The observed increases in $Ca_i^{2+}$ in response to alkaline $pH_o$ occurred well before marked changes in $pH_i$ took place (Fig. 10, *bottom*). This delay argues against the rise in $Ca^{2+}$ being mediated via SR $Ca^{2+}$ release associated with increased $pH_i$. To further exclude the possibility that an alkaline $pH_o$ had caused $Ca_i^{2+}$ to increase via SR release of $Ca^{2+}$ triggered by the secondary rise in $pH_i$, we used maneuvers directed to deplete the SR of calcium (preexposure to Ang II) or to block calcium uptake by the SR (TMB-8) (2). After these maneuvers, perfusate alkalinization still caused a rapid rise in $Ca_i^{2+}$ (Fig. 11). These maneuvers, by contrast, are known to block completely the increase in $Ca_i^{2+}$ associated with abrupt increases in $pH_i$ (2). Thus, the data indicate that $Ca^{2+}$ release from the SR is not the mechanism whereby external alkalinization increases $Ca^{2+}$.

Our data are thus consistent with the proposal that perfusate alkalinization increases $Ca_i^{2+}$ by inhibition of a plasma membrane Ca/H exchanger. The possibility that perfusate alkalinization might also increase $Ca_i^{2+}$ by augmenting calcium entry, however, needed to be explored as well. To investigate the latter possibility we compared the effects of alkalinization with a 1.25 mM $Ca^{2+}$ perfusate versus a 0 mM $Ca^{2+}$ perfusate on $Ca_i^{2+}$. Alkalinization ($pH_o$ 8.0) with a zero calcium perfusate was accompanied by only a very slight rise in $Ca_i^{2+}$, an increase that was greatly attenuated compared with the effects of alkalinizing with a 1.25 mM $Ca^{2+}$ perfusate (Fig. 12). These data suggested that perfusate alkalinization was increasing $Ca_i^{2+}$ by augmenting calcium entry. However, a close comparison of the $Ca_i^{2+}$ profile in zero calcium perfusate with and without perfusate alkalinization shows that perfusate alkalinization greatly retards, and in fact reverses, the fall in $Ca_i^{2+}$ which normally

occurs when these cells are exposed to a zero calcium perfusate (Fig. 12). Taken together, our data suggest that the effects of perfusate alkalinization on $Ca_i^{2+}$ involve both augmented $Ca^{2+}$ entry (presumably via a $pH_o$ sensitive $Ca^{2+}$ channel) as suggested by Muallem et al. (37) and inhibition of calcium extrusion via $Ca^{2+}/H^+$ exchange across the plasma membrane.

In summary, agonist-induced cell acidification is due to changes in $Ca^{2+}$ transport rather than to the activation of $pH_i$ regulatory transporters that acidify the cell such as the Na-independent $Cl/HCO_3$ exchanger. $Ca_i^{2+}$-mediated activation of a plasma membrane $Ca^{2+}$-ATPase, acting as a $Ca^{2+}/H^+$ exchanger, is proposed as the mechanism involved in the rapid cytosolic acidification seen with a variety of agonists that increase $Ca_i^{2+}$. While this plasma membrane $Ca^{2+}$-ATPase is primarily involved in $Ca_i^{2+}$ regulation, it may play a secondary role in $pH_i$ regulation such that, in the process of extruding $Ca^{2+}$, particularly during stimulation by agonists that increase $Ca_i^{2+}$, it brings acid equivalents into the cell.

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