Divergent effects of extracellular and intracellular alkalosis on Ca^{2+} entry pathways in vascular endothelial cells

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Modulation by alkalosis of basal leak Ca^{2+} entry and store-depletion-induced Ca^{2+} entry was investigated in the vascular endothelial cell line ECV 304. Ca^{2+} entry was monitored as the increase in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) induced by elevation of the extracellular Ca^{2+} concentration. When ECV 304 cells were challenged with 100 nM thapsigargin in nominally Ca^{2+}-free solution, $[Ca^{2+}]_i$ increased transiently, and the increase in $[Ca^{2+}]_i$ during a subsequent cumulative elevation of extracellular Ca^{2+} (from nominally Ca^{2+}-free up to 5 mM) was markedly enhanced compared with non-stimulated cells (i.e. basal Ca^{2+} leak). Prolonged elevation of the extracellular pH (pH_o) from 7.4 to 7.9 did not affect resting $[Ca^{2+}]_i$ or the thapsigargin-induced $[Ca^{2+}]_i$ transient evoked in nominally Ca^{2+}-free solution, but increased leak Ca^{2+} entry as well as store-depletion-activated Ca^{2+} entry significantly. Basal Ca^{2+} leak and store-depletion-activated Ca^{2+} entry were enhanced either by acute elevation of pH_o from 7.4 to 7.9 or by chronic alkalosis ($pH_o = 7.9$). Stimulation of Ca^{2+} entry by extracellular alkalosis was observed both in normal and in high extracellular K^+ (110 mM) solution, suggesting that the effects of alkalosis are independent of membrane potential. The intracellular pH (pH_i) increased slightly during both acute and chronic extracellular alkalosis (from 7.22 ± 0.01 to 7.37 ± 0.04 and 7.45 ± 0.05 respectively). Elevation of pH_i to 7.60 ± 0.06 at constant pH_o by administration of 20 mM NH_4Cl failed to stimulate, and in fact inhibited, store-depletion-activated Ca^{2+} entry. Our results demonstrate that a decrease in the extracellular but not the intracellular proton concentration promotes both basal and stimulated Ca^{2+} entry into endothelial cells.

INTRODUCTION

The vascular endothelium plays an obligatory role in the regulation of vasotone via mediators such as prostacyclin, endothelium-derived relaxing factor (EDRF; nitric oxide) and endothelin [1–4]. The production and release of vasoactive mediators are to a large extent controlled by the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$), and are thus in principle enhanced by any mechanism that allows Ca^{2+} entry into endothelial cells. In resting non-stimulated cells, Ca^{2+} influx is thought to occur via a so-called 'leak' pathway [5,6]. Activation of endothelial cells by hormones and neurotransmitters involves a discharge of intracellular Ca^{2+} stores which is accompanied by a dramatic increase in the plasmalemmal Ca^{2+} conductance, and consequently in Ca^{2+} entry [7,8]. This agonist-induced activation of endothelial cells is characterized by a biphasic increase in $[Ca^{2+}]_i$, which is composed of an initial Ca^{2+} transient due to Ca^{2+} mobilization from internal stores triggered by inositol 1,4,5-trisphosphate, and a subsequent sustained elevation due to agonist-stimulated Ca^{2+} influx from the extracellular space [9,10]. The mechanisms that link the stimulation of membrane receptors to the activation of plasmalemmal Ca^{2+} channels have not yet been clarified. The depletion of intracelluar Ca^{2+} stores has been proposed as a key event in the signal transduction cascade leading to enhanced Ca^{2+} conductance of the plasma membrane [11,12], and this Ca^{2+} entry pathway has been termed store-depletion-activated Ca^{2+} entry or capacitive Ca^{2+} entry. This pathway has pharmacological properties distinct from those of the leak Ca^{2+} entry pathway [6].

The Ca^{2+}-dependent production of vasoactive factors by endothelial cells is known to be remarkably sensitive to changes in the extracellular pH (pH_o). This pH-dependence of endothelial function is thought to be of physiological and pathological significance. In rat aortic rings, extracellular-Ca^{2+}-dependent prostacyclin production increases as the pH_o is increased from 6.5 to 8.0 [13]. In cultured vascular endothelial cells, extracellular alkalosis was reported to induce the release of EDRF, which was dependent on the presence of extracellular Ca^{2+} [14]. Thus the Ca^{2+}-dependent formation of vasoactive factors in endothelial cells is clearly stimulated by extracellular alkalosis. *In vivo*, alkalosis is often associated with vasospasms [15,16], which are the result of an effect of pH_o on smooth muscle cells [17,18]. Alkalosis-induced stimulation of Ca^{2+} entry and formation of vasodilator substances in endothelial cells may thus serve as a mechanism that protects blood vessels from alkalosis-induced vasospasms.

Up to now, only a few studies have addressed the question of how Ca^{2+} entry into vascular endothelial cells is promoted during extracellular alkalosis. Alkalosis (pH_o 8.5) was reported to augment Ca^{2+} influx into store-depleted endothelial cells [19,20]. Since Ca^{2+} influx into activated cells generally comprises the background leak Ca^{2+} influx and the Ca^{2+} influx occurring via activated Ca^{2+} channels, the relative contribution of pH modulation of either component to the overall effect is still unclear. Moreover, extracellular alkalosis necessarily results in intracellular alkalosis, the effect of which on Ca^{2+} influx is not yet known. We demonstrated recently that protons are able to block the store-depletion-activated Ca^{2+} entry pathway by interaction with an extracellular site [21]. It was thus of interest to test the hypothesis that the stimulatory effect of alkalosis is simply due to the removal of protons from this extracellular inhibitory site which controls the store-operated Ca^{2+} entry pathway. In the

Abbreviations used: $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; pH_o, extracellular pH; pH_i, intracellular pH; EDRF, endothelium-derived relaxing factor; BCECF, 2′,7′-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein.

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present study, we report on stimulation of both leak Ca^{2+} entry and store-activated Ca^{2+} entry by alkalosis, and present evidence for divergent effects of pH_o and intracellular pH (pH_i) on these Ca^{2+} entry pathways.

MATERIALS AND METHODS

Cell culture

ECV 304 cells were kindly provided by Dr. K. Takahashi (Fifth Department of Internal Medicine, Tokyo Medical College, Ibaraki, Japan) [22], and were cultured in 65 cm^2 dishes with Dulbecco's modified Eagle's medium containing antibiotics and 3% foetal bovine serum in a humidified atmosphere at 37 °C under 5% CO_2/95% O_2. Cells were subcultured at a ratio of 1:6, and the confluent cells were used for experiments measuring $[Ca^{2+}]_i$ and pH_i.

Measurement of $[Ca^{2+}]_i$ and pH_i

$[Ca^{2+}]_i$ and pH_i were ascertained using the fluorescent Ca^{2+} and pH indicators fura-2 and 2′,7′-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) respectively. Fura-2/AM and BCECF/AM were initially dissolved in DMSO at 5 mM and 2.5 mM respectively, and were used at final concentrations of 10 μM and 5 μM respectively. Confluent cells from one dish were collected and suspended in 5 ml of physiological solution [10 mM Hepes/Tris, pH 7.4, containing (in mM) 135 NaCl, 5 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 1 KH_2PO_4 and 10 glucose at 37 °C]. The suspended cells were loaded with fura-2 or BCECF for 60 min at 37 °C. After loading, the cells were washed once with Ca^{2+}-free solution containing 0.01 mM EGTA (nominally Ca^{2+}-free), and then resuspended in nominally Ca^{2+}-free solutions of different pH values. Experiments at elevated pH_o were started 20 min after resuspension of cells in a solution of pH 7.9 (chronic alkalosis).

Fluorescence measurements were carried out with a dual-wavelength spectrophotofluorimeter (Hitachi F2000) using a 2 ml cuvette maintained at 37 °C. For $[Ca^{2+}]_i$ measurement, excitation was at 340 nm and 380 nm, and emission was measured at 510 nm. For pH_i measurement, excitation wavelengths were 506 nm and 455 nm, and emission was collected at 530 nm. Using ratios (R) of fluorescence intensity (F) of F_{340}/F_{380} and F_{506}/F_{455}, the fractional changes in $[Ca^{2+}]_i$ and pH_i respectively were determined. The fluorescence after sequential addition of 0.1% Triton X-100 and EGTA to the cell suspension provided the maximum fluorescence ratio (R_{max}) and minimum fluorescence ratio (R_{min}) respectively. $[Ca^{2+}]_i$ was calculated using the formula described by Grynkiewicz et al. [23]:

$$[Ca^{2+}]_i = (R - R_{min})/(R_{max} - R) \times \beta \times K_d$$

where β is the ratio of the emission fluorescence values at 380 nm excitation in the presence of Triton X-100 and EGTA. Since the dissociation constant for Ca^{2+} (K_d) of fura-2 is affected by changes in pH, the K_d was corrected using the following equation [24]:

$$K_d = 224 \times \{1/[3.1 \times (pH_i - 5.77)] + 0.73\}.$$

To test for a possible pH-induced leakage of fluorescence dyes, we compared the ability of extracellular Mn^{2+} (100 μM) to quench fura-2 fluorescence when cells were kept at normal (pH 7.4) and elevated (pH 7.9; 30 min) pH. The Mn^{2+}-induced quenching of fura-2 fluorescence was 14±2% at pH 7.4 and 15±1% at pH 7.9 ($n = 7$), indicating that an elevation in pH did not promote dye leakage from endothelial cells. Calibration of pH_i measurements was performed in a nigericin (7 μM)-containing high-K^+ solution at various pH_o values. Fluorescence ratios obtained were analysed by linear regression analysis and the derived equation was used to calculate pH_i values.

Results are expressed as means±S.E.M. The significance of differences was assessed by analysis of variance and subsequent Scheffé F-test. P values of < 0.05 were considered significant.

RESULTS

Extracellular alkalosis (pH_o 7.9) promotes Ca^{2+} entry into store-depleted endothelial cells

Figure 1 illustrates a representative experiment in which $[Ca^{2+}]_i$ was measured at pH_o values of 7.4 and 7.9. Intracellular Ca^{2+} stores were discharged by the administration of 100 nM thapsigargin in nominally Ca^{2+}-free solution, which resulted in a transient increase in $[Ca^{2+}]_i$. At 200 s after the addition of thapsigargin, the extracellular Ca^{2+} concentration was elevated in a cumulative manner, resulting in a concentration-dependent increase in $[Ca^{2+}]_i$. Whereas the resting $[Ca^{2+}]_i$ and the thapsigargin-induced Ca^{2+} transient remained unaffected, the extracellular-Ca^{2+}-dependent rise in $[Ca^{2+}]_i$ was clearly enhanced at a pH_o of 7.9. This enhancement was obvious at extracellular Ca^{2+} concentrations as low as 0.5 mM.

Table 1 compares the mean values of $[Ca^{2+}]_i$ measured at pH_o values of 7.4 and 7.9. The basal $[Ca^{2+}]_i$ was not significantly

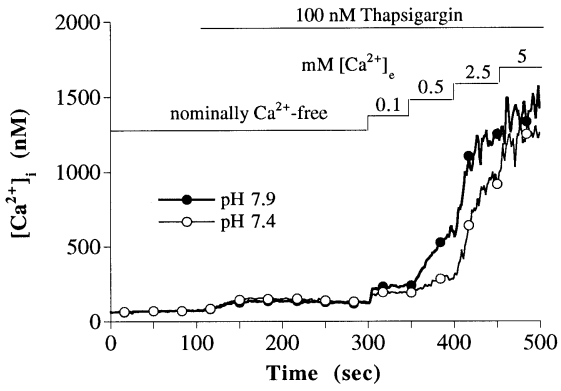

Figure 1 Representative changes in $[Ca^{2+}]_i$ measured by fura-2 fluorescence in solutions of pH 7.4 and 7.9

In nominally Ca^{2+}-free solution, thapsigargin (100 nM) was added at 100 s, and $CaCl_2$ ($[Ca^{2+}]_e$; 0.5–5 mM) was increased cumulatively as indicated.

Table 1 Effects of extracellular alkalosis (pH_o 7.9) on $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured in non-stimulated cells (basal), during thapsigargin (TG; 100 nM)-induced Ca^{2+} release in nominally Ca^{2+}-free solution (peak value), and 2 min and 20 min after the addition of Ca^{2+} in the presence of thapsigargin. *Significant difference ($P < 0.05$) compared with values at pH 7.4.

Conditions	$[Ca^{2+}]_i$ (nM)	
	pH_o 7.4	pH_o 7.9
Basal	65±8	55±12
+TG (Ca^{2+}-free)	146±16	113±13
+TG+2.5 mM Ca^{2+} (2 min)	865±60	1096±61*
+TG+2.5 mM Ca^{2+} (20 min)	636±80	1011±72*

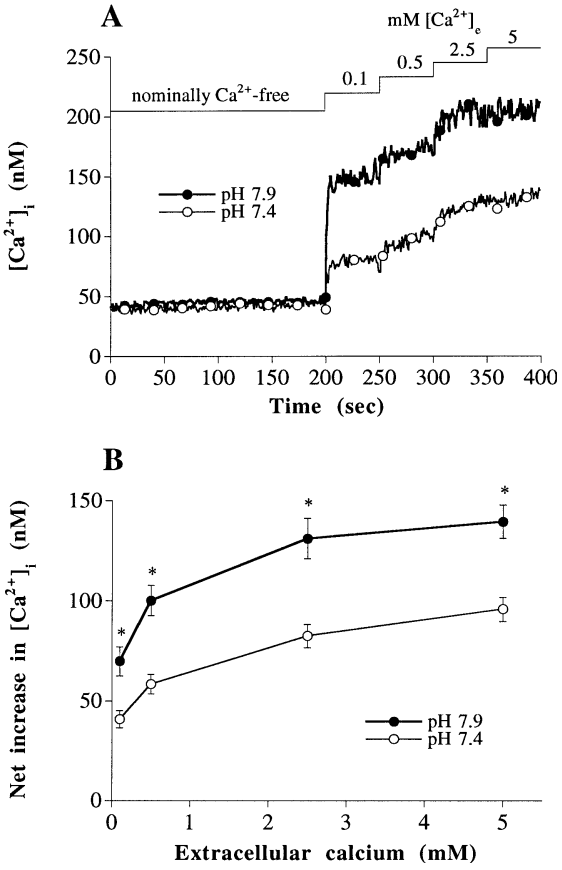

Figure 2 Changes in $[Ca^{2+}]_i$ due to leak Ca^{2+} influx in solutions of pH 7.4 and 7.9

(**A**) $CaCl_2$ ($[Ca^{2+}]_e$; 0.5–5 mM) was elevated cumulatively as indicated. (**B**) Net $[Ca^{2+}]_i$ increase due to leak Ca^{2+} influx. Net leak Ca^{2+} influx was calculated by subtraction of basal $[Ca^{2+}]_i$ from $[Ca^{2+}]_i$ after the addition of Ca^{2+}. Asterisks denote significant differences ($P < 0.05$) compared with the values at pH 7.4.

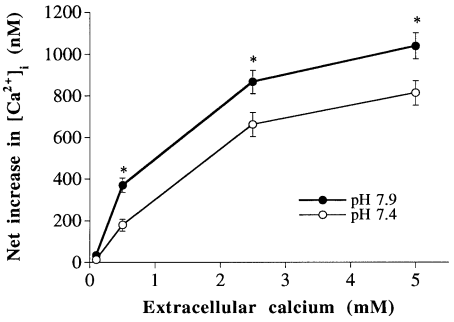

Figure 3 Effects of extracellular alkalosis on the net $[Ca^{2+}]_i$ increase elicited by store depletion

Thapsigargin (100 nM) was added for 200 s to deplete Ca^{2+} stores. Net activated Ca^{2+} entry was quantified as the difference between $[Ca^{2+}]_i$ in stimulated and non-stimulated cells (leak entry). Asterisks denote significant differences ($P < 0.05$) compared with the values at pH 7.4.

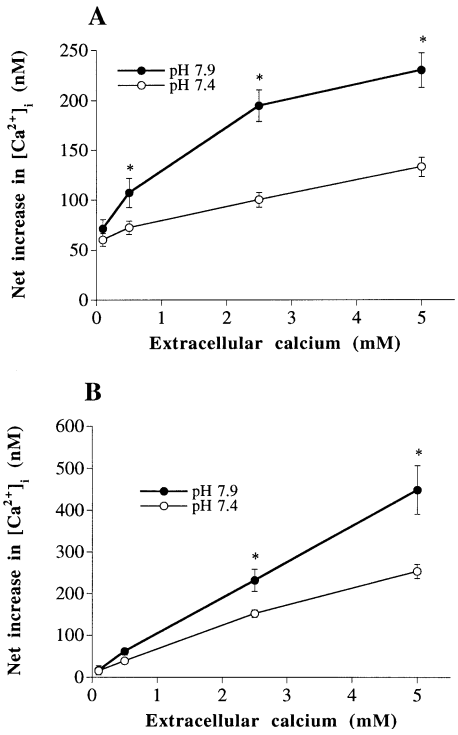

Figure 4 Net $[Ca^{2+}]_i$ increases in high-K^+ (110 mM) solutions due to (A) leak Ca^{2+} entry and (B) store-depletion-activated Ca^{2+} entry at pH 7.4 and 7.9

The high K^+ concentration was osmotically compensated for by an equimolar decrease in the NaCl concentration. Net increases in leak and stimulated Ca^{2+} entry were quantified as described for Figures 2(B) and 3. Asterisks denote significant differences ($P < 0.05$) compared with values at pH 7.4.

different, and the transient peak level of $[Ca^{2+}]_i$ evoked by thapsigargin in nominally Ca^{2+}-free solution was slightly but not significantly lower, in the solution of pH 7.9 compared with that of pH 7.4. The thapsigargin-induced net increase in $[Ca^{2+}]_i$ induced by thapsigargin addition in nominally Ca^{2+}-free solution was slightly but not significantly decreased by extracellular alkalosis [81.2 ± 9.0 nM (pH 7.4) compared with 58.2 ± 6.4 nM (pH 7.9)]. In contrast, the $[Ca^{2+}]_i$ values obtained in the presence of cumulative increases in the extracellular Ca^{2+} concentration were significantly higher at pH 7.9 than at pH 7.4. As shown in Table 1, $[Ca^{2+}]_i$ was higher when measured immediately (2 min) after initiation of Ca^{2+} entry as well as after a prolonged period (20 min) of Ca^{2+} entry at physiological extracellular Ca^{2+} concentrations (2.5 mM). Since both leak Ca^{2+} influx and store-depletion-activated Ca^{2+} influx contribute to the extracellular-Ca^{2+}-induced increase in $[Ca^{2+}]_i$ in store-depleted cells, the two components were analysed separately.

Extracellular alkalosis promotes leak Ca^{2+} influx

Figure 2(A) shows representative changes in $[Ca^{2+}]_i$ in a non-stimulated (resting) cell induced by elevation of the extracellular Ca^{2+} concentration. An extracellular-Ca^{2+}-dependent rise in $[Ca^{2+}]_i$ was observed when the extracellular Ca^{2+} concentration was raised above nominally Ca^{2+}-free to concentrations of 0.1–5 mM. The observed extracellular-Ca^{2+}-induced rise in $[Ca^{2+}]_i$ was taken to represent measured Ca^{2+} entry via the leak conductance of the cell membrane. Net leak Ca^{2+} entry, which was calculated by subtraction of basal $[Ca^{2+}]_i$ from $[Ca^{2+}]_i$ after elevation of the extracellular Ca^{2+} concentration, was significantly higher at pH_o 7.9 than at pH_o 7.4 (Figure 2B). The alkalosis-induced elevation of $[Ca^{2+}]_i$ in non-stimulated cells

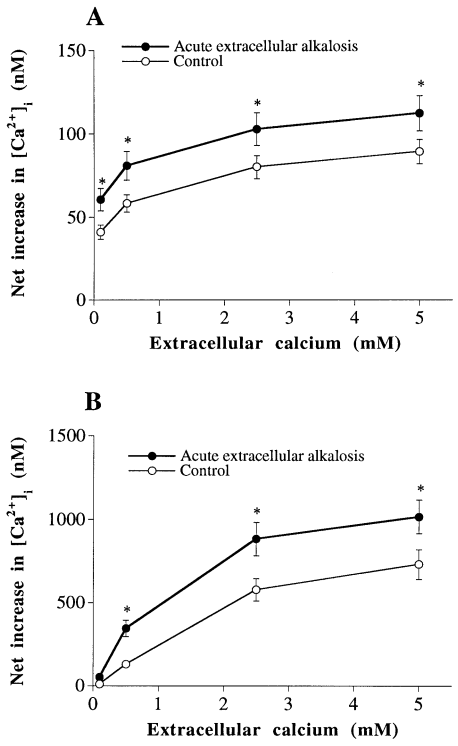

Figure 5 Effects of acute extracellular alkalosis on net $[Ca^{2+}]_i$ increases due to leak Ca^{2+} entry (A) and store-depletion-activated Ca^{2+} entry (B)

Acute extracellular alkalosis was induced by the addition of an adequate volume of Tris to elevate pH_o from 7.4 to 7.9 at 20 s before Ca^{2+} addition. Net increases in leak and stimulated Ca^{2+} entry were quantified as described for Figures 2(B) and 3. Asterisks denote significant differences ($P < 0.05$) compared with values at pH 7.4.

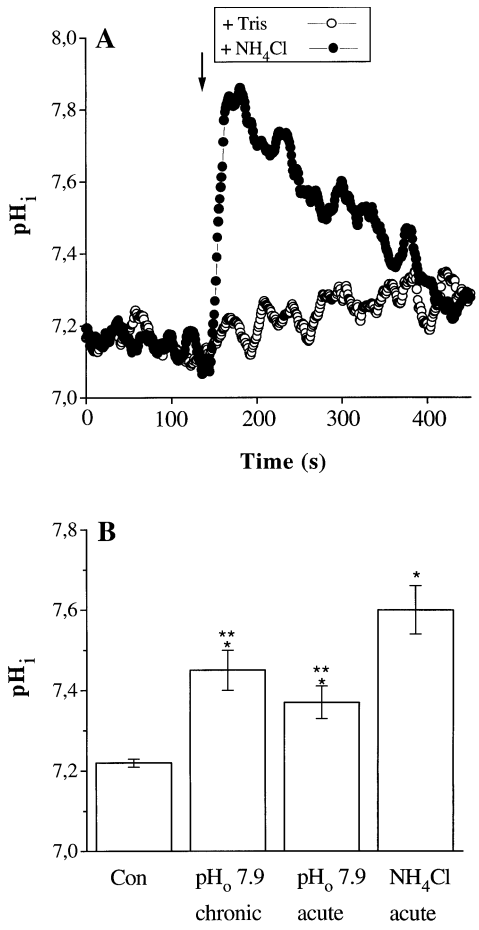

Figure 6 Intracellular alkalosis is induced by extracellular alkalosis and NH_4Cl

(**A**) Representative changes in pH_i measured by BCECF fluorescence during acute extracellular and intracellular alkalosis induced by the addition of an adequate amount of Tris or 20 mM NH_4Cl respectively. Note that, in fura-2 experiments, Ca^{2+} entry was initiated 20 s after the addition of Tris or NH_4Cl. (**B**) Comparison of pH_i under various experimental conditions. The stable pH_i values in solutions of pH 7.4 (Con) and pH 7.9 (chronic), and the peak pH_i values at the time corresponding to the addition of $CaCl_2$ in the protocol of $[Ca^{2+}]_i$ measurements in acute extracellular (acute) and intracellular alkalosis induced by 20 mM NH_4Cl (NH_4Cl) are compared. *Significantly different ($P < 0.05$) from control; **significantly different from NH_4Cl.

persisted for more than 20 min at physiological levels of extracellular Ca^{2+} ($n = 3$; results not shown).

Extracellular alkalosis promotes net Ca^{2+} entry induced by Ca^{2+} store depletion

Figure 3 shows the net store-depletion-stimulated increase in $[Ca^{2+}]_i$, which was calculated by subtraction of the leak Ca^{2+} entry from total Ca^{2+} entry. At pH 7.9, the net store-depletion-induced $[Ca^{2+}]_i$ increment was significantly higher than at pH 7.4.

Alkalosis-induced stimulation of leak Ca^{2+} entry and store-depletion-induced Ca^{2+} entry is independent of the extracellular K^+ concentration

Figure 4(A) shows leak Ca^{2+} entry in a solution containing a high K^+ concentration (110 mM). In high-K^+ solution, which depolarizes endothelial cells, leak Ca^{2+} influx was significantly greater than that in normal K^+ solution [maximum leak Ca^{2+} influx in the presence of 5 mM Ca^{2+}: 95.7 ± 6.1 nM (normal K^+) compared with 139.4 ± 8.3 nM (high K^+) ($P < 0.05$)]. Alkalosis (i.e. elevation of pH_o to 7.9) significantly increased leak Ca^{2+} entry in the presence of high extracellular K^+ (Figure 4A). Figure 4(B) shows net Ca^{2+} entry stimulated by store depletion with thapsigargin in high-K^+ solution. The net store-depletion-induced Ca^{2+} influx was clearly lower than in the normal-K^+ solution [maximum stimulated Ca^{2+} influx in the presence of 5 mM Ca^{2+}: 845.4 ± 34.4 nM (normal K^+) compared with 253.4 ± 34.4 nM (high K^+) ($P < 0.05$)]. Elevation of pH_o to 7.9 significantly increased store-depletion-induced Ca^{2+} entry in high-K^+ solution (Figure 4B).

Acute extracellular alkalosis is sufficient to augment both leak Ca^{2+} entry and store-depletion-activated Ca^{2+} entry

In order to test whether the effect of alkalosis on Ca^{2+} entry is induced acutely or requires long-term elevation of pH_o, we studied the effects of acute extracellular alkalosis (pH_o 7.9) on leak and stimulated Ca^{2+} influx. Acute alkalosis was induced by the addition of an appropriate volume of Tris base 20 s prior to the initiation of Ca^{2+} entry. Similar to the effects observed after prolonged exposure of the cells to alkaline solution (chronic effects), both leak Ca^{2+} influx and net Ca^{2+} influx stimulated by Ca^{2+} store depletion were augmented by acute extracellular alkalosis (Figures 5A and 5B).

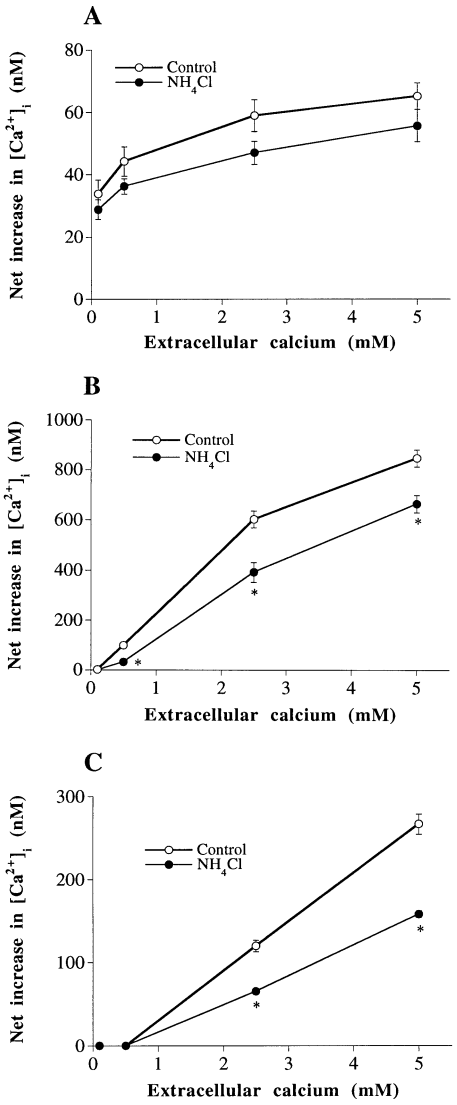

Figure 7 Effects of acute intracellular alkalosis on net $[Ca^{2+}]_i$ increases due to leak Ca^{2+} entry (A) and store-depletion-activated Ca^{2+} entry (B) in normal-K^+ solution, and to store-depletion-activated Ca^{2+} entry in high-K^+ solution (C)

The high K^+ concentration (110 mM) was osmotically compensated for by an equimolar decrease in the NaCl concentration. Acute intracellular alkalosis was induced by the addition of 20 mM NH_4Cl at 20 s before Ca^{2+} addition. Net increases in leak and stimulated Ca^{2+} entry were quantified as described for Figures 2(B) and 3. Asterisks denote significant differences ($P < 0.05$) compared with the control treated with vehicle (20 mM NaCl).

Extracellular alkalosis is associated with intracellular alkalosis

Figure 6(A) shows representative changes in pH_i measured with BCECF. Addition of Tris base, which resulted in elevation of pH_o from 7.4 to 7.9, induced a slow and gradual increase in pH_i. At the time of initiation of Ca^{2+} entry (20 s after addition of Tris), elevation of pH_i by acute extracellular alkalosis was clearly less than the increase in pH_i induced at the same time by 20 mM NH_4Cl. In cells kept for 20 min at pH_o 7.9 (chronic extracellular alkalosis), pH_i was significantly higher than in control cells (pH_o 7.4), but significantly lower than during acute intracellular alkalosis induced by 20 mM NH_4Cl (Figure 6B).

Acute intracellular alkalosis inhibits store-depletion-induced Ca^{2+} entry

Addition of NH_4Cl (20 mM), which evokes a substantial increase in pH_i (see above), suppressed leak Ca^{2+} entry slightly but not significantly (Figure 7A). The net Ca^{2+} influx stimulated by Ca^{2+} store depletion, however, was significantly inhibited by NH_4Cl (Figure 7B). In order to test whether this inhibitory effect of intracellular alkalosis is related to changes in membrane potential, we studied the effect of intracellular alkalosis on store-depletion-induced Ca^{2+} entry under conditions which clamp the cell potential close to 0 mV, i.e. high extracellular K^+. Cell depolarization failed to prevent the inhibitory effect of intracellular alkalosis (Figure 7C), indicating that this is independent of membrane potential.

DISCUSSION

The present study demonstrates that elevation of pH_o results in complex modulation of endothelial cell Ca^{2+} entry mechanisms. We provide evidence that the observed stimulation of Ca^{2+} entry is based on a marked enhancement of both leak Ca^{2+} entry and the store-depletion-activated Ca^{2+} entry pathway by an extracellular mechanism of action. This extracellular effect is blunted by the inhibition of store-depletion-activated Ca^{2+} entry due to the elevation of pH_i that accompanies extracellular alkalosis.

The results presented here are in keeping with a previous investigation in calf pulmonary arterial endothelial cells, which provided evidence that Ca^{2+} influx into store-depleted cells is increased by raising pH_o from 7.4 to above 8.5 [20]. In contrast, in pig aortic endothelial cells, ATP-stimulated $^{45}Ca^{2+}$ uptake was reported to be insensitive to severe extracellular alkalosis (pH_o 9.2) [6]. Several reasons for this discrepancy appear possible, e.g. differences in the methods of determination of Ca^{2+} influx; different types of cells; degree of alkalosis. In the present study we chose a rather moderate degree of alkalosis and found that this relatively mild change in pH_o was sufficient to affect Ca^{2+} entry into ECV cells. Interestingly, Demirel et al. [6] reported that leak $^{45}Ca^{2+}$ influx was higher under conditions of extracellular alkalosis (pH 9.2). In the present study, which was aimed at analysing the effects of alkalosis on both leak and store-depletion-activated Ca^{2+} entry, we also observed promotion of leak entry. In addition, our results demonstrate unequivocally that the net $[Ca^{2+}]_i$ increase corresponding to store-depletion-activated Ca^{2+} entry is also increased by moderate extracellular alkalosis (pH_o 7.9). Thus extracellular alkalosis augments both types of Ca^{2+} entry.

Since extracellular alkalosis did not affect the net increment in $[Ca^{2+}]_i$ induced by Ca^{2+} release from internal storage sites, a process distal to store depletion appears to be modulated by pH_o. These results are in line with a previous study which reported that basal $[Ca^{2+}]_i$ and Ca^{2+} release from the stores remained unaffected in alkalosis [20].

Membrane potential has been recognized as a major determinant of Ca^{2+} entry and consequently of EDRF production [25–27]. Ca^{2+} influx activated by store depletion after bradykinin stimulation was found to be suppressed in high extracellular K^+ conditions in bovine aortic endothelial cells [5,28]. In the present study, Ca^{2+} influx initiated by store depletion with thapsigargin was suppressed, whereas leak Ca^{2+} entry was increased, in high-K^+ solution. Thus these Ca^{2+} entry mechanisms exhibit different dependence on membrane potential. Since the stimulatory effects of extracellular alkalosis on leak and activated Ca^{2+} entry were not prevented in high-K^+ solution, the observed pH effects may not involve changes in membrane potential. We have recently

reported [21] that extracellular acidosis (pH_o 6.9) inhibits store-depletion-activated Ca^{2+} entry, but not leak Ca^{2+} entry, into ECV 304 cells. Again, this inhibitory effect of extracellular acidosis was not affected by membrane depolarization.

The degree of increase in pH_i induced by extracellular alkalosis varies among different types of cells [29–32]. In ECV 304 cells, acute extracellular alkalosis resulted in a gradual increase in pH_i, which was about 0.2 pH units higher under chronic extracellular alkaline conditions (pH_o 7.9) than in normal solution (pH 7.4). Intracellular alkalinization has been considered as a process of intracellular signal transduction initiated by agonist stimulation [33,34]. Activation of the receptor by ligands such as ATP and thrombin induces phosphoinositide breakdown, resulting in protein kinase C activation and consequent stimulation of Na^+–H^+ exchange, which then results in intracellular alkalinization [35,36]. A previous study has shown that bradykinin stimulation of bovine aortic endothelial cells caused increases in $[Ca^{2+}]_i$ and pH_i, both of which were suppressed by a Na^+–H^+ pump inhibitor [37]. Thus agonist-induced changes in $[Ca^{2+}]_i$ and pH_i appear to be closely related in vascular endothelial cells.

NH_4Cl is frequently used as a tool to induce intracellular alkalinization. When cells are exposed to NH_4Cl, a weak base, at constant pH_o, intracellular alkalinization occurs due to the entry of NH_3, which is re-protonated in the cytoplasm to form NH_4^+ [38]. In ECV 304 cells, NH_4Cl (20 mM) induced acute intracellular alkalosis, the degree of which was greater than that accompanying extracellular alkalosis. Acute intracellular alkalosis induced by NH_4Cl clearly inhibited store-depletion-activated Ca^{2+} entry, but did not significantly affect leak Ca^{2+} entry. It is thus obvious that the stimulatory effects of extracellular alkalosis on Ca^{2+} entry are not mediated by concomitant changes in pH_i, but rather are due to a direct action of extracellular protons. The observed inhibition of activated Ca^{2+} entry by intracellular alkalosis is in keeping with a previous study by Thuringer et al. [19] demonstrating that Ca^{2+}-activated K^+ channel activity induced by bradykinin, which reflects agonist-stimulated Ca^{2+} influx, is suppressed by application of NH_4Cl (20 mM).

The mechanism of suppression of Ca^{2+} entry by intracellular alkalosis which counteracts the effects of extracellular alkalosis is as yet unknown. Danthuluri et al. [39] suggested that intracellular alkalinization by ammonium ions impairs the refilling of intracellular Ca^{2+} stores in bovine aortic endothelial cells. Recently, NH_4Cl has been reported to induce membrane depolarization, followed by trans-plasmalemmal Ca^{2+} influx through the voltage-dependent Ca^{2+} channels in vascular smooth muscle cells [40,41]. However, inhibition of the activated Ca^{2+} entry by NH_4Cl in ECV 304 cells was not affected by membrane depolarization (Figure 7). Thus the mechanism of the inhibitory effect of NH_4Cl does not involve changes in membrane potential, and remains to be clarified in future investigations.

Our results demonstrate that decreases in the extracellular proton concentration augment, while increases in the extracellular proton concentration inhibit [21], store-depletion-activated Ca^{2+} entry. The changes in depletion-activated Ca^{2+} entry elicited by a decrease in pH_o from 7.4 to 6.9 on the one hand and by an elevation to 7.9 on the other hand were comparable. In contrast, leak Ca^{2+} entry appears to be highly sensitive to extracellular alkalosis but barely sensitive to acidosis. Elevation of pH_o to 7.9 produced a marked increase in Ca^{2+} influx, while acidification (pH_o 6.9) does not inhibit the influx pathway [21], indicating that the leak pathway is already maximally inhibited at a physiological pH_o of 7.4. The regulation of trans-plasmalemmal Ca^{2+} entry by pH_i is biphasic, in that both intracellular acidosis and alkalosis inhibit store-depletion-activated Ca^{2+} entry in vascular endothelial cells (but the latter action is stronger than the former), indicating the existence of a pH_i optimum for depletion-activated Ca^{2+} entry into ECV 304 cells. We have summarized these findings in Table 2, which combines the present results with our previous observations in ECV 304 cells [21].

Table 2 Effects of pH on transmembrane Ca^{2+} entry pathways in ECV 304 cells

Extracellular alkalosis and acidosis correspond to pH_o values of 7.9 (the present study) and 6.9 [21]. Intracelluar alkalosis and acidosis were induced by NH_4Cl (20 mM; the present study) and propionate (20 mM; [21]) respectively.

	Leak Ca^{2+} entry	Stimulated Ca^{2+} entry
Extracellular alkalosis	↑↑↑	↑↑↑
Extracellular acidosis	↔	↓↓↓
Intracellular alkalosis	↔	↓↓
Intracellular acidosis	↔	↓

In summary, our results strongly suggest that extracellular alkalosis increases both leak and store-depletion-activated trans-plasmalemmal Ca^{2+} entry due to the direct removal of protons from extracellular inhibitory sites, while intracellular alkalosis inhibits store-depletion-activated Ca^{2+} entry and thereby blunts the overall effect on Ca^{2+} influx. It has been proposed that an as yet unidentified diffusible messenger, termed Ca^{2+} influx factor, is produced upon depletion of Ca^{2+} stores, and activates a specific Ca^{2+} channel [42,43]. Further studies are required to clarify the role of such cellular signalling cascades and of specific cellular structures in the pH regulation of endothelial cell Ca^{2+} entry mechanisms.

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