Intracellular pH Modulates the Availability of Vascular L-type Ca\(^{2+}\) Channels

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**ABSTRACT** L-type Ca\(^{2+}\) channel currents were recorded from myocytes isolated from bovine pial and porcine coronary arteries to study the influence of changes in intracellular pH (pHi). Whole cell I\(_{Ca}\) fell when pHi was made more acidic by substituting HEPES/NaOH with CO\(_2\)/bicarbonate buffer (pHo 7.4, 36°C), and increased when pHi was made more alkaline by addition of 20 mM NH\(_4\)Cl. Peak I\(_{Ca}\) was less pHi sensitive than late I\(_{Ca}\) (170 ms after depolarization to 0 mV). pH\(_i\)-effects on single Ca\(^{2+}\) channel currents were studied with 110 mM BaCl\(_2\) as the charge carrier (22°C, pH\(_o\) 7.4). In cell-attached patches pHi was changed by extracellular NH\(_4\)Cl or through the opened cell. In inside-out patches pHi was controlled through the bath. Independent of the method used the following results were obtained: (a) Single channel conductance (24 pS) and lifetime of the open state were not influenced by pHi (between pH\(_i\) 6 and 8.4). (b) Alkaline pHi increased and acidic pHi reduced the channel availability (frequency of nonblank sweeps). (c) Alkaline pHi increased and acidic pHi reduced the frequency of late channel re-openings. The effects are discussed in terms of a deprotonation (protonation) of cytosolic binding sites that favor (prevent) the shift of the channels from a sleepy to an available state. Changes of bath pH\(_o\) mimicked the pHi effects within 20 s, suggesting that protons can rapidly permeate through the surface membrane of vascular smooth muscle cells. The role of pHi in Ca\(^{2+}\) homeostases and vasotonus is discussed.

**INTRODUCTION**

Elevation of pCO\(_2\) relaxes and reduction of pCO\(_2\) constricts pial arteries of the rabbit brain (e.g., Betz and Heuser, 1967; Wahl, 1985). The mechanism occurs at constant extracellular pH (pH\(_o\)) and is attributed to a change in intracellular pH (pHi). There are multiple mechanisms by which pH\(_i\) can modulate contractile state of the smooth muscle cells (smc). For example, intracellular acidosis (pHi < 7.2) reduces the Ca\(^{2+}\) sensitivity of the myofilaments (Rüegg, 1986) and reduces the Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR, Fabiato and Fabiato, 1978; Schulz, Thevenod, and Dehlinger-Kremer, 1989). In addition, acidic pHi suppresses the influx of extracellu-
lar \( \text{Ca}^{2+} \) with the consequence of a reduced SR \( \text{Ca}^{2+} \) load (van Breemen, Farinas, Garba, and McNaughton, 1972; Rinaldi, Cattaneo, and Cingolani, 1987).

In vascular smooth muscle cells (vsm), a large part of \( \text{Ca}^{2+} \) influx occurs through \( L \)-type \( \text{Ca}^{2+} \) channels. Modulation of \( L \)-type \( \text{Ca}^{2+} \) channels by \( \text{pHi} \) is the topic of the present paper. In initial experiments we dialyzed the cells with pipette solutions of \( \text{pH}_{6} \). Although this reduced whole cell \( \text{I}_{\text{Ca}} \) as reported in the literature (ventricular myocytes: Kurachi, 1982; Irisawa and Sato, 1986) the value of these experiments was limited because suppression of \( \text{I}_{\text{Ca}} \) due to intracellular acidosis could not be separated from the "run-down" of \( \text{I}_{\text{Ca}} \) that occurs in vsm within 5–10 min.

At constant \( \text{pH}_{6} \), \( \text{I}_{\text{Ca}} \) could be modulated easily and reversibly by \( \text{CO}_2 \) and \( \text{NH}_4\text{Cl} \). Changing from a HEPES/NaOH to a \( \text{CO}_2/\text{bicarbonate} \) buffer produces acidosis (Thomas, 1984) because \( \text{CO}_2 \) permeates rapidly through the plasma membrane while bicarbonate does not, leading to the formation and dissociation of \( \text{H}_2\text{CO}_3 \) in the cytosol and providing \( \text{H}^+ \) ions that may reduce \( \text{pHi} \) by ca. 0.2 units (Liu, Piwnica-Worms, and Lieberman, 1990, in cultured heart cells). Similarly, a solution of 20 mM \( \text{NH}_4\text{Cl} \) (pH 7.4) contains \( \sim 2 \) mM membrane permeable \( \text{NH}_3 \), and intracellular formation of \( \text{NH}_4^+ \) removes cytosolic protons and increases \( \text{pHi} \) by \( \sim 0.4 \) units (Blank, Silverman, Chung, Hogue, Stern, Hansford, Lakatta, and Capogrossi, 1992, rat ventricular cells).

Because it is uncertain whether the above \( \text{pHi} \) can be quantitatively extrapolated to vsm, a more direct control of \( \text{pHi} \) at the cytosolic site of the \( \text{Ca}^{2+} \) channel protein was desired. The inside-out patch seems to be an ideal preparation; however, excision of the patch is followed by a rapid run-down of \( L \)-type \( \text{Ca}^{2+} \) channel activity (Pelzer, Pelzer, and McDonald, 1990). An experimental compromise between control of \( \text{pHi} \) and run-down seems to be offered by the "open cell attached method" (Kameyama, Kakei, Sato, Shibasaki, Matsuda, and Irisawa, 1984; Horie, Irisawa, and Noma, 1987). The extracellular buffers can rapidly equilibrate through the crashed cell end with the cytosol and with the cytosolic side of the \( \text{Ca}^{2+} \) channel, and recordings from the cell attached patch can monitor the resulting change in single channel activity. In ventricular myocytes, the open-cell-attached method has shown that \( \text{pHi} \) acts predominantly through the availability of \( L \)-type \( \text{Ca}^{2+} \) channels (Kaibara and Kameyama, 1988).

A channel is called available if it opens upon the depolarizing clamp step. The effect of variable availability is illustrated by the relation between whole cell \( \text{I}_{\text{Ca}} \) to single channel current \( i_{\text{Ca}} \)

\[
\text{I}_{\text{Ca}} = N_T P_F P_o i_{\text{Ca}} = N_F P_o i_{\text{Ca}} \tag{1}
\]

The whole cell sarcolemma bears \( N_T \) channels, a fraction \( P_F \) of which open upon membrane depolarization. Because availability (\( P_F \)) is less than 1, \( N_F = N_T P_F \) channels contribute to the current \( \text{I}_{\text{Ca}} \) whereas \( N_T (1 - P_F) \) channels are "sleeping" (Ochi and Kawashima, 1990). The transition between the available and sleeping state is a slow gating between "modes" (Tsien, Bean, Hess, Lansman, Nilius, and Nowycky, 1986). Typically, records from vsm show for \( \sim 30 \) s active sweeps followed by 40–60 s where the sweeps are blank (Klöckner, Trieschmann, and Isenberg, 1989). The active sweeps show openings of short (0.5 ms, "mode 1") and long lifetime (7 ms, "mode 2") even in the absence of the \( \text{Ca}^{2+} \) channel agonist Bay K 8644 (Inoue, Xiong, Inoue, Xiong,
Kitamura, and Kuriyma, 1989). Bay K 8644 increased $P_o$ via the percentage of long openings but not through the channel availability $P_T$ (Klöckner and Isenberg, 1991). To separate the pH-induced from the spontaneous modulation of "slow gating" several hundred sweeps had to be recorded. The open probability $P_o$ describes with the "fast gating" how the available channel moves between the closed, open and inactivated states; during a depolarization, the $P_o$ increases to a peak and then falls with time. The number of channels per patch is not known in most of our experiments, therefore, we used instead of $P_o$ the "channel activity" $N'P_o = NT'PVPo$.

We have therefore investigated whether pH modulation of $I_{Ca}$ is via the open channel current $I_{Ca}$, the open probability $P_o$ (fast gating) or the channel availability $P_T$ (slow gating). Part of this work has been presented in abstract form (Isenberg and Klöckner, 1989).

**MATERIALS AND METHODS**

Whole cell recordings of $I_{Ca}$ were performed on isolated vascular myocytes from bovine pial and porcine coronary arteries as described in the preceding paper (Klöckner and Isenberg, 1994). Patch electrodes of ~3 MΩ resistance were filled with (in mM) 130 CsCl, 5 Cs⁺-oxalacetate, 5 Cs⁺-succinate, 5 Na⁺-pyruvate, 1 MgCl₂, 5 creatine, 10 EGTA, 10 HEPES/CsOH, pH 7.2 (compare Klöckner and Isenberg, 1985). The cells were continuously (2 ml/min) superfused by a prewarmed (36°C) extracellular solution containing (in mM) 150 NaCl, 5.4 KCl, 1.2 MgCl₂, 3.6 CaCl₂, 20 glucose, 10 buffer. pH was adjusted with HEPES/NaOH to 7.4, with MES/NaOH to more acidic pH between 5 and 7, and with Tris/HCl to more alkaline pH between 7.8 and 8.4. If the concentration of CaCl₂ was increased this is indicated. In some experiments, Ca²⁺ channel current was studied using Na⁺ ions as the charge carrier, in which case the bath solution contained neither Ca²⁺ nor Mg²⁺ but 1 mM EGTA. In experiments were CO₂ was used to change pH, 25 mM NaHCO₃ was substituted for 25 mM NaCl and the solution was equilibrated with 5% CO₂ (at 36°C).

Single channel recordings were performed at room temperature (22°C). The electrodes had ~4 MΩ resistance and were filled with 110 mM BaCl₂ plus 10 mM HEPES/KOH (pH 7.4). The membrane potential was zeroed with an "intracellular-medium" containing (in mM) 130 KCl, 5 K⁺-oxalate, 5 K⁺-succinate, 5 creatine, 10 EGTA, 10 HEPES/KOH (pH 7.4). In some experiments the pH of this medium was changed with MES/KOH or Tris/HCl (see above for extracellular solution). 400-ms voltage-clamp steps to 0 mV were applied from a holding potential of ~60 mV every 3 s. Single channel currents were recorded with an EPC7 amplifier (List Electronics, Darmstadt, Germany), filtered at 1 kHz, digitized at 5 kHz and stored on a PDP 11/73 minicomputer (Digital Equipment Corp., Marlboro, MA). Off line, blank records were subtracted to correct for capacitive and leakage currents. Open and close times were evaluated with a 1 ms bin width and a threshold of 50% of single channel current amplitude (from amplitude histograms). The distribution of blanks will be used to characterize the slow transition of the Ca²⁺ channel between the available and unavailable state (Tsien et al., 1986; Ochi and Kawashima, 1990).

In the open-cell-attached patch method (Horie et al., 1987) the cell was in the intracellular medium and one end of the cell was crushed by a glass stylus. pH was modified by diffusion of buffers through the open cell end. Ca²⁺ channel activity was stable for ~10 min which is long enough for studying the effect of one pH change on slow gating. The 10 min period, however, is too short to apply a series of different pH's. Therefore, data averaged from several patches ($n$) were pooled and compared statistically as the mean ± standard error of the mean (SEM) with a $P$ of 0.05 (t test).
RESULTS

\(pH_i\) Effects on Whole Cell \(I_{Ca}\)

Bath applied \(CO_2\) reduces \(I_{Ca}\). At constant \(pH_o\) 7.4, the change from HEPES/NaOH buffer to \(CO_2/bicarbonate\) buffer reduced peak \(I_{Ca}\) to 55 ± 12% \((n = 6)\). In the presence of \(CO_2/bicarbonate\), reduction of \(I_{Ca}\) was maintained for up to 3 min (Fig. 1 A, circles). Upon return to HEPES/NaOH, peak \(I_{Ca}\) recovered to 80% of control within 30 s and completely within 2–3 min. An “overshooting” recovery was not observed. The addition or removal of \(CO_2/bicarbonate\) did not influence the holding current at -65 mV (Fig. 1 A, dots), suggesting that the effect was specific for \(I_{Ca}\).

Results similar to the one in Fig. 1 A were recorded in a total of 3 pial and 3 coronary vsm.

Usually peak \(I_{Ca}\) was between -10 and -20 pA \((2.0 \text{ mM } [Ca^{2+}]_o)\). Enlargement of \(I_{Ca}\) by high extracellular \(Ca^{2+}\) or \(Ba^{2+}\) concentrations was not possible because of
precipitating Ca\textsuperscript{2+} or Ba\textsuperscript{2+} carbonate. Therefore, the effects of CO\textsubscript{2}/bicarbonate buffer on \(I_{\text{Ca}}\) could be measured in only a small number of vsm whose control \(I_{\text{Ca}}\) was large enough. Ca\textsuperscript{2+} channels carry Na\textsuperscript{+} currents of large amplitude \((I_{\text{Ca,Na}})\) when the channel selectivity is reduced by a Ca\textsuperscript{2+} and Mg\textsuperscript{2+} free medium (for references see Kostyuk, 1980). In this medium, the change from HEPES/NaOH to CO\textsubscript{2}/bicarbonate buffer reduced \(I_{\text{Ca,Na}}\) within 30 s by 30% (Fig. 1B). The reduction of \(I_{\text{Ca,Na}}\) remained stable for at least 3 min. Upon return from CO\textsubscript{2}/bicarbonate to HEPES-buffer, \(I_{\text{Ca,Na}}\) recovered to control \(I_{\text{Ca,Na}}\) within ~1 min. On average, \(pCO\textsubscript{2} (46 \pm 5\) Torr) plus 25 mM bicarbonate reduced \(I_{\text{Ca,Na}}\) to 66 ± 10% of the control value in the HEPES-buffered solution (5 pial vsm). The depression of \(I_{\text{Ca,Na}}\) by CO\textsubscript{2}/bicarbonate buffer supports the hypothesis that intracellular acidification reduces Ca\textsuperscript{2+} channel current.

\textit{Bath application of NH\textsubscript{4}Cl increases \(I_{\text{Ca}}\).} Ammonium chloride (20 mM) increased peak \(I_{\text{Ca}}\) by 220% and “late” \(I_{\text{Ca}}\) by 600% (Fig. 2, A and B). The augmentation of \(I_{\text{Ca}}\) by NH\textsubscript{4}Cl was sustained as long as NH\textsubscript{4}Cl was present (tested for up to 3 min). During wash out of NH\textsubscript{4}Cl, \(I_{\text{Ca}}\) fell to the control value. NH\textsubscript{4}Cl-augmented \(I_{\text{Ca}}\) was strongly suppressed by 1 \(\mu\)M D600 (Fig. 2 C) suggesting that the current was indeed
a L-type $I_{Ca}$. On average, 20 mM NH$_4$Cl reversibly increased peak $I_{Ca}$ by 240 ± 80% and late $I_{Ca}$ by 410 ± 110% (eight coronary and seven pial vsm). A decay of $I_{Ca}$ during the 3 min application of NH$_4$Cl or an undershoot during wash out of NH$_4$Cl was not recorded ($n = 15$).

Fig. 2E compares the voltage-dependence of peak $I_{Ca}$ in absence (circles) and presence of 20 mM NH$_4$Cl (dots). There was no change of the threshold potential ($-40$ mV), of the potential of maximal $I_{Ca}$ (0 mV) or of the reversal potential (+40 mV). The curve in presence of NH$_4$Cl can be transformed into the control i-v curve by division with a voltage-independent factor (1.9). When the i-v curves were fitted with a voltage-dependent activation parameter (see preceding paper, Klöckner and Isenberg, 1994), half-maximal activation before and after NH$_4$Cl was found at similar potentials (shift of $5 ± 8$ mV, $n = 8$). Also, the position of the inactivation curve remained unmodified (insignificant shift by $-3.8 ± 7$ mV). Thus, it is unlikely that NH$_4$Cl augments $I_{Ca}$ through the voltage-dependent gating parameters.

**Single Ca$^{2+}$ Channel Currents**

NH$_4$Cl increases $N_{Po}$. Currents through single Ca$^{2+}$ channels were recorded from cell attached patches. At $-50$ mV (approximately the resting potential of pial vsm) the single Ca$^{2+}$ channel activity was low, i.e., single channel currents ($-1.6$ pA) were recorded only three times during a 1 min period. In the presence of NH$_4$Cl (20 mM, 1 min) the currents occurred more frequently, and occasionally two channels were active simultaneously (Fig. 3). Computer-evaluation indicated that NH$_4$Cl increased $N_{Po}$ from 0.005 to 0.018, i.e., by a factor of 3.6. Because the patch was isolated from the bath, the result supports the hypothesis that NH$_4$Cl modulated the Ca$^{2+}$ channel through an intracellular messenger, most likely through a reduction of protons.

Bath application of 20 mM NH$_4$Cl also augmented the Ca$^{2+}$ channel activity during clamp steps to 0 mV. The augmentation was similar in pial ($n = 5$) and coronary vsm ($n = 4$) and did not depend on the presence of Bay K 8644. Fig. 4 shows the effects on a pial vsm in the absence of Bay K 8644. Fig. 4B shows that the mean current transports, in the presence of NH$_4$Cl a three times higher charge (time integral, $-260$ fA·s per 0.4 s sweep) than during control ($-80$ fA·s per 0.4 s sweep). Division of the charge by the single channel current ($I_{Ca} = -1.1$ pA) yields the result that NH$_4$Cl increased the channel activity $N_{Po}$ from 0.24 to 0.79. In amplitude histograms NH$_4$Cl-augmented channel activity is indicated not only by the larger Gaussian areas at $-1.1$ and $-2.2$ pA but also by the appearance of a third and fourth current level (see Fig. 6B). NH$_4$Cl had no influence on the amplitude of the unitary current, correspondingly the conductance was $24 ± 2$ pS (110 mM BaCl$_2$) at control and $24 ± 3$ pS in presence of NH$_4$Cl.

The mean current data showed that NH$_4$Cl increased the peak of $I_{Ca}$ 2.3-fold and the late current 10-fold. The life time of the open state (Fig. 4D) did not change, i.e., time constants of the double exponential distributions were very similar (1.5 and 7.1 ms before and 1.4 and 8.0 ms after addition of NH$_4$Cl, no Bay K 8644). Hence, the increase in the late current suggests that the channel re-opened at later times more frequently when NH$_4$Cl was present (Fig. 4A). NH$_4$Cl reduced the number of blank records from $82 ± 6$% to $66 ± 8$% (1,000 sweeps from nine cells, 1 μM Bay K 8644 present).
Ca\textsuperscript{2+} channels in open cell-attached patches. Fig. 5A shows that Ca\textsuperscript{2+} channel activity continues when one end of the coronary vsm is crushed mechanically (1 \mu M Bay K 8644 present). Through the ~5 \mu m wide opening the solution’s buffer is thought to slowly equilibrate with the cytosol and the inner site of the Ca\textsuperscript{2+} channel. Bath pH 7.4 moderately increased NPo and reduced the frequency of blanks (Fig. 5A). pH 8.4 suppressed the blanks and increased NPo threefold; individual sweeps showed up to five superimposed current levels where at control pH only two levels were recorded (not illustrated). The subsequent exposure to pH 6.2 strongly reduced NPo and increased the number of blanks.

Fig. 5, B and C, shows an experiment from a patch attached to an opened pial vsm (1 \mu M Bay K 8644 present). The cell was opened in bath pH 8.4. The change to pH 6.0 completely suppressed the channel activity. Upon return to pH 8.4 channel activity rapidly recovered. Fig. 5, B and C stands for a total of three experiments with

**Figure 3.** Augmentation of Ca\textsuperscript{2+} channel activity by bath application of NH\textsubscript{4}Cl (20 mM, 1 min). Patch potential held continuously at −50 mV. Cell-attached patch from pial vsm in extracellular solution, pH\textsubscript{e} 7.4, 22°C, no Bay K 8644. Patch electrode solution of 10 mM BaCl\textsubscript{2}, 140 mM NaCl, 10 HEPES/KOH, pH 7.4, provided screening of surface charges comparable to extracellular solution with 2 mM [Ca\textsuperscript{2+}]\textsubscript{o} (Ganitkevich and Isenberg, 1990). Note: the non equal size of the currents (downward deflection) is due to the pen-recorder that attenuated short events more than long lasting ones.

only one current level at both control and alkaline pH\textsubscript{e}. In 17 of 40 patches, however, one current level was recorded at pH 7.4 but two or more levels at pH 8.4.

pH-modulation of Ca\textsuperscript{2+} channel currents was also studied in the absence of Bay K 8644. Fig. 6 shows results from a pial vsm. The change from pH 7.4 to 8.4 increased the mean current. From the time integral of the mean current one estimates that NPo increases from 0.33 to 1.48. The amplitude histogram (B) shows that the contribution of the closed state (peak at 0 pA) is reduced while the contribution of the open state is complementarily increased, partially due to the appearance of 4 instead of 2 current levels. pH 8.4 did not significantly change the life time of the open state, i.e., time constants of 0.85 and 6.4 ms (pH 7.4) and of 0.7 and 6.7 ms (pH 8.4) were similar. pH 8.4 increased the peak of the mean current by 240% but the late current by 2,500%. The single channel analysis attributes the preferential increase in late current to late re-openings of the channel, events that were almost absent during control but frequently recorded during alkalosis.
pH more acidic than 7.2 reduced the Ca$^{2+}$ channel activity. At pH 6.9 NPs was ~50% of the control recorded from the nonopened cell. NPs was lower because of less frequent late re-openings and a higher percentage of blank sweeps. The single channel conductance and the life time of the open state remained essentially
constant. When pH was reduced to 6.4 or 6.0, the nonblank sweeps occurred at such a low frequency that statistical evaluation was not possible.

The pH effects (between 6.4 and 8.4) on 40 open cell attached patches can be summarized as follows: (a) The conductance of the open channel (24 pS) was pH insensitive. (b) The life time of the open state was pH insensitive. (c) $N_{Po}$ was increased by acidosis and reduced by alkalosis. (d) The frequency of blank sweeps increased during acidosis and fell during alkalosis. (e) Channel re-openings occurred at higher probability when pH was more alkaline. (f) The probability for superimposed openings increased during alkalosis.

**pH-effects in inside-out patches.** Intracellular protons could have influenced the channel activity by interacting either with the channel protein ($\alpha_1$ or other subunits), or, more indirectly, with cytosolic kinases and phosphatases. This question was tested
in cell-free inside-out patches where the cytosolic constituents are thought to be washed off and where solutions of adjusted pH_i should have direct access to the cytosolic side of the channel protein. In the inside-out configuration the single channel currents had the usual amplitude (~1.2 pA) and life time (between 5 and 8 ms, 1 μM Bay K 8644 present). The channel activity was strongly suppressed by 1 μM D600, suggesting that the channel was of the l-type. In the inside-out configuration, Ca^{2+} channels rapidly "run down," i.e., NP_o decayed with a half time of 1.4 ± 0.5 min and disappeared within 4–5 min. Only in four out of the 50 trials did the activity last

![Figure 6](image_url)

**Figure 6.** Analysis of Ca^{2+} channel activity at pH 7.4 (left) and 8.4 (right). Opened cell attached patch, coronary myocyte. No Bay K 8644 present. (A) Mean current from 80 tracings, current integral is labeled. (B) Amplitude histogram. Bin size 0.1 pA. Peak at 0 pA indicates closed channel. Number of current levels increases from 2 to 4. (C) Distribution of the open times. Data were fitted with two exponentials, amplitude and time constants are indicated.
for 4 min or longer. In these four patches the pH effects were tested, and reduction of pH from 7.2 to 6.0 blocked the single channel currents within 20 s corresponding to the rate of the solution change (Fig. 7A). pH 8.4 increased NPₒ to 200% of the control value at pH 7.2. The second change to pH 6.0 suppressed the channel activity again, however, because the activity did not recover a possible pHₒ effect cannot be distinguished from run-down. The results suggest that protons, applied to the cytosolic site of an inside-out patch, modulate the Ca²⁺ channel activity in a similar way as in cell-attached patches. One may interprete the result to suggest that

Figure 7. pH-modulation of Ca²⁺ channel currents in an inside-out patch from a coronary vsm. The electrode was filled with 110 mM BaCl₂ and 1 µM Bay K 8644. 400-ms steps from -60 to 0 mV, 0.5 Hz. (A) Channel activity N-Pₒ under influence of pH. Original tracings were taken at pH 7.2 (B) and immediately after the change from pH 7.2 to 6.0 (C).
soluble cytosolic enzymes are not involved in the proton modulation of the Ca\(^{2+}\) channel. However, such a conclusion is not warranted (see Discussion).

\(pH_o\) modulates \(Ca^{2+}\) channel activity through changes in \(pH_i\). A recent paper has shown that \(pH_i\) in mesenteric smc changed rapidly when \(pH_o\) was altered, within 1 min \(pH_i\) reached a steady value that was ~70% the \(pH_o\) (Austin and Wray, 1993). The result predicts that changes in \(pH_o\) modify \(pH_i\) and thereby \(NP_o\). To make the experimental conditions comparable with those of the preceding paper (Klöckner and Isenberg, 1994), pipette solutions of 50 mM BaCl\(_2\) plus 1 \(\mu\)M Bay K 8644 were adjusted to pH 5.0. At a bath pH 7.0 \(NP_o\) was 0.11. Increasing \(pH_o\) to 9.0 increased \(NP_o\) to 1.2 and suppressed the blank sweeps within 40 s (Fig. 8 B). The amplitude histogram showed three instead of two current levels. Bath pH 9.0 changed neither the amplitude of unitary current (multiples of ~0.55 pA) nor the life time of the open state (Fig. 8, E and F: 8.7 vs 8.8 ms). When \(pH_o\) was reduced to 5.0, within 6 s the superimposed current levels fell from three (first three sweeps in Fig. 8 A) to two and one (sweep 4 to 7, or 8 to 14 s). Then, the \(Ca^{2+}\) channels no longer opened at all. The rapid modulation of \(Ca^{2+}\) channel activity by bath pH supports the above idea that protons can easily permeate through the sarcolemma and that changes in \([H^+].i\) and \(NP_o\) contribute to the effects of \(pH_o\) on the whole cell current that were described in the preceding paper.

**DISCUSSION**

This study on vsm from pial and coronary artery has shown that intracellular acidosis reduces and alkalosis increases \(I_{Ca}\), confirming earlier reports on cardiac \(I_{Ca}\) (Irisawa and Sato, 1986). In the first part of this study, whole cell \(I_{Ca}\) was modulated by acidosis due to elevated pCO\(_2\) and by alkalosis due to 20 mM NH\(_4\)Cl. Both interventions have been reported to change \(pH_i\) only transiently, the normalization of \(pH_i\) being related to the slow permeation of HCO\(_3^-\) or NH\(_4^+\), respectively (Thomas, 1984). For example, 20 mM NH\(_4\)Cl increased \(pH_i\) of ventricular cells from 7.48 within 30 s to 7.95 and then \(pH_i\) returned to 7.48 within 20 min (Blank et al., 1992). In this study, the changes of \(I_{Ca}\) were stable during the 3 min of observation, presumably, a substantial uptake of NH\(_4^+\) or HCO\(_3^-\) and recovery of \(pH_i\) did not occur during this period. Because no recovery of \(I_{Ca}\) was recorded in presence of NH\(_4\)Cl, washout of NH\(_4\)Cl is expected to return \(I_{Ca}\) without an undershoot of \(pH_i\).

In contrast to \(pH_o\), \(pH_i\) modulated whole cell \(I_{Ca}\) without changing the voltage dependent gating (peak \(I_{Ca}\), steady state inactivation). This result from coronary and pial vsm differs from the 10 mV shift for 2 \(pH\) units that was reported for cardiac myocytes (Kaibara and Kameyama, 1988); a low charge density at the inner site of the sarcolemma of vsm may account for the discrepancy.

When single channel analysis was performed on cell-attached patches, \(pH_i\) was modified indirectly via NH\(_4\)Cl and \(pH_o\). \(pH_i\) was more directly controlled in inside-out and in open-cell-attached patches. The results obtained by the four different methods were similar. That is, intracellular alkalosis increased and acidosis reduced the ensemble average currents. The effects were not mediated by the single channel conductance or the life time of the open state. However, \(pH_i\) had a strong effect on \(NP_o\). Part of this effect was due to the channel availability \(P_e\) that was estimated from the frequency of nonblank records. In comparison to cell-attached
Figure 8. Bath pHo modifies Ca²⁺ channel currents as if pHᵢ followed pHₑ. Cell attached patch. Coronary myocyte. Electrode solution with 50 mM BaCl₂ and 1 µM Bay K 8644 adjusted to pH 5.0. 400 ms steps from -65 to 0 mV. (A) Single channel currents during the change of bath pH from 9.0 to 5.0. (B) Channel activity NPo. (C) Amplitude histograms at bath pH 7.2 (C) and 9 (D). The distribution of closed channels has been subtracted. (E and F) Open time distributions fitted with single exponentials. (E) Bath pH 7.2, amplitude N = 30, time constant 8.8 ms. (F) Bath pH 9.0, amplitude 250, time constant 8.8 ms.

control records, pHᵢ 8.4 increased Pᵢ ≈ threefold, whereas pHᵢ 6.9 reduced Pᵢ to 50% and pHᵢ 6.0 blocked Pᵢ. The effects of pHᵢ on Pᵢ did not depend on the presence or absence of Bay K 8644. The effect of pHᵢ on Pᵢ was reflected in the change of the number of superimposed current levels. Using the model of Ochi and Kawashima (1990), this can be interpreted as more alkaline pHᵢ promoting the slow gating from a "sleeping" into an available state.

The possibility that the protons modified the Ca²⁺ channel activity not "directly"
but through a modification of the Bay K 8644 effect on the channel seems to be unlikely. Bay K 8644 has a pK' value of > 10, hence different to e.g., amlodipine, the uncharged fraction of the molecule does not change if the pH is increased from 6.4 and 8.4 (S. Goldmann, Bayer AG, Wupperthal, Germany). Therefore, a change in the local concentration of Bay K 8644 due a pH-induced screening of surface charges can be excluded. Because the positive inotropic effect of Bay K 8644 was not significantly modified by changes in pH o or pH i (atrial preparations: Ghysel-Burton and Goodfraind, 1990) we like to extrapolate that other, unknown interactions between Bay K 8644 and Ca2+ channel activity are unlikely. Finally, a series of whole-cell and single-channel experiments was repeated in the absence of Bay K 8644, and the results were not distinguishable.

Our results suggest that pH i also modulates the fast gating as it is reflected in Po(V,t). We found that the peak of mean current was less pH i sensitive than the late current. Because the life time of the open state was pH i-insensitive, this result suggests that the L-type Ca2+ channel re-opens at higher probability (P o) when pH i is more alkaline. Vice versa, a higher concentration of intracellular protons seems to suppress the re-openings and thereby to promote the decay of I Ca. Whether this phenomenon is linked to the "Ca2+ mediated inactivation" of Ca2+ channels cannot be answered by the present experiments.

The pH i effects on ica in cell-free inside-out and in cell-attached patches were similar. Because the cytosol is rapidly washed off from inside-out patches the persistence of the pH i effect could suggest that the protons directly interact with the pore-forming protein (a1 subunit), with one of the other subunits, or with membrane constituents in the close neighborhood to the channel. Assuming that the protons bind to the a1 subunit, the pH i-insensitivity of the voltage-dependent gating argues that protons bind outside the electrical field of the membrane. The long cytosolic carboxy terminal of the a1 subunit would be a suitable candidate. However, in 46 of 50 inside-out patches the Ca2+ channel activity rapidly ran down. One may speculate that the only four successful experiments are those where the cytosolic factors were not washed out (Kameyama, Kameyma, Nakayama, and Kaibara, 1988). With this speculation, pH i could have modulated NPo through the soluble cytosolic factor. Hence, the interpretation of the results from inside-out patches is not unique.

This study did not quantify the pH i effects on NPo. Because the channel activity could be recorded only over a short period of time, one would have to average data from different patches. Averaging requires the normalization of N-Po = N-Po-Po by the unknown number of channels in the patch (N). In 37 of 40 experiments, there was more than one channel in the patch, suggesting that vsm have Ca2+ channels in clusters. One could assume Po = 1 for pH i = 8.4 and normalize the data by NPo during a final exposure to pH i 8.4. However, many experiments could not include such a final step, and, the run down of NPo with time makes such a normalization questionable.

Normalization was possible in cardiac myocytes, where patches with only one Ca2+ channel were obtained more frequently (Kaibara and Kameyma, 1988). The effect of
intracellular protons on normalized channel activity ($P_o^*$) was quantified with

$$P_o^* = 1/[1 + ([H^+]_i/K')]$$  \hspace{2cm} (2)

Kaibara and Kameyama found an apparent dissociation constant $K'$ of 0.27 μM corresponding to a pK' of 6.7 which is close to the pK' value of histidine (6.3–6.7). Therefore, histidine-residues were suggested as the potential proton binding sites. The carboxy terminal of the α1 subunit of the l-type Ca²⁺ channel contains 18 histidine-residues (Ca²⁺ channel from rat aorta, Koch, Ellinor, and Schwartz, 1990) and could be a suitable candidate. The proposal is in line with our own preliminary results with diethylpyrocarbonat; the drug deprotonates histidine even at acidic pHᵢ, and indeed partially recovered NPₒ at pHᵢ 6 (three of six patches). However, the deprotonation of histidine-residues cannot easily explain the effects of alkaline pHᵢ. A change of pHᵢ from 7.4 to 8.4 that increases the fraction of deprotonated histidines from 83 to 98% should account for the 300% increase in NPₒ.

In this study, the single channel currents were recorded at 22°C, with 110 mM BaCl₂ as the charge carrier and with clamp steps to 0 mV. One wonders, whether the results can be extrapolated to more physiological conditions with 2 mM [Ca²⁺]ₒ and a membrane resting potential of ca –50 mV (own current clamp measurements; see also Kuriyama, Ito, Suzuki, Kitamura, and Itoh, 1982, for porcine coronary arteries). Fig. 3 demonstrated that intracellular alkalosis due to NH₄Cl indeed augments the Ca²⁺ channel activity at –50 mV, provided the pipette solution contained 10 mM Ba²⁺ which screens the external surface charges to a similar extent as 2 mM Ca²⁺ (Ganitkevich and Isenberg, 1990).

The result of Fig. 3 supports the view that intracellular protons can regulate Ca²⁺ channels at more physiological conditions. Because the activity of Ca²⁺ channels controls Ca²⁺ influx and contractility, one can consider protons as another second messenger involved in the regulation of contraction of vsm (compare Siskind, McCoy, Chobanian, and Schwartz, 1989). Because pHᵢ of vsm is regulated by vasopressin, angiotensin II and other hormones, the proton pathway could be of major physiological significance (for references see Bers, Canessa, Vallega, and Alexander, 1987).

Traditionally, respiratory pH effects on vascular tone have been distinguished from metabolic ones (Rooke and Sparks, 1981). On the first view, one may relate the effects of respiratory pCO₂ changes to the pHᵢ mediated modulation in Ca²⁺ channel availability, and the metabolic changes in pHₒ to the modulation of fast gating and single channel conductance described in the preceding paper (Klöckner and Isenberg, 1994). However, we have shown that “metabolic” changes in bath pHₒ rapidly modulate the channel activity in cell attached patches. The results suggest that the protons can easily permeate through the sarcolemma (Austin and Wray, 1993). The effect of pHₒ on NPₒ could not be reproduced in myocytes from the urinary bladder or ventricle of the guinea-pig (unpublished results), hence it seems to be specific for vascular myocytes. In conclusion, pHₒ changes in blood or extracellular space modulate the Ca²⁺ channel activity of vascular myocytes through both extra- and intracellular mechanisms.

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REFERENCES

Austin, C., and S. Wray. 1993. Extracellular pH signals affect rat vascular tone by rapid transduction into intracellular pH changes. Journal of Physiology. 466:1–8.

Bers, B. C., M. Canessa, G. Vallega, and R. W. Alexander. 1988. Agonist-mediated changes in intracellular pH: role in vascular smooth muscle cell function. Journal of Cardiovascular Pharmacology. 12:104–114.

Betz, E., and D. Heuser. 1967. Brain blood flow and acid-base equilibrium. Journal of Applied Physiology. 23:726–733.

Blank, P. S., H. S. Silverman, OK. Y. Chung, B. A. Hogue, M. D. Stem, R. G. Hansford, E. G. Lakatta, and M. C. Capogrossi. 1992. Cytosolic pH measurements in single cardiac myocytes using carboxy-seminaphthorhodafluor-1. American Journal of Physiology. 263:H276–H284.

Fabriato, A., and F. Fabiato. 1978. Effect of pH on the myofilaments and the sarcoplasmic reticulum of skinned cells from cardiac and skeletal muscles. Journal of Physiology. 276:233–255.

Ganitkevich, V. Ya., and G. Isenberg. 1990. Contribution of two types of calcium channels to membrane conductance of single myocytes from guinea-pig coronary artery. Journal of Physiology. 426:19–42.

Ghysel-Burton, J., and T. Godfraind. 1990. Role of Na-H exchange in the inotropic action of Bay K 8644 and of ouabain in guinea-pig isolated atria. British Journal of Pharmacology. 100:717–722.

Horie, M., H. Irisawa, and A. Noma. 1987. Voltage dependent magnesium block of adenosine-triphosphate-sensitive channels in guinea-pig ventricular cells. Journal of Physiology. 387:251–272.

Inoue, Y., Z. Xiong, K. Kitamura, and H. Kuriyama. 1989. Modulation produced by nifedipine of the unitary Ba current of dispersed smooth muscle cells of the rabbit ileum. Pflügers Archiv. 414:534–542.

Irisawa, H., and R. Sato. 1986. Intra- and extracellular actions of protons on the calcium current of isolated guinea-pig ventricular cells. Circulation Research. 59:548–555.

Isenberg, G., and U. Klöckner. 1989. Intracellular protons modulate availability of the 1-type Ca-channel of porcine coronary myocytes. Journal of Physiology. 417:80a. (Abstr.)

Kaibara, M., and M. Kameyama. 1988. Inhibition of the calcium channel by intracellular protons in single ventricular myocytes of the guinea-pig. Journal of Physiology. 403:621–640.

Kameyama, M., M. Kakei, R. Sato, T. Shibasaki, H. Matsuda, and H. Irisawa. 1984. Intracellular Na⁺ activates a K⁺ channel in mammalian cardiac cells. Nature. 309:354–356.

Kameyama, M., A. Kameyama, T. Nakayama, and M. Kaibara. 1988. Tissue extract recovers cardiac calcium channels from “run-down.” Pflügers Archiv. 412:328–330.

Klöckner, U., U. Trieschmann, and G. Isenberg. 1989. Action potentials and net membrane currents of smooth muscle cells (urinary bladder of the guinea-pig). Pflügers Archiv. 405:340–348.

Klöckner, U., and G. Isenberg. 1991. Myocytes isolated from porcine coronary arteries: reduction of currents through L-type Ca-channels by verapamil-type Ca-antagonists. Journal of Physiology and Pharmacology. 42:163–179.

Klöckner, U., and G. Isenberg. 1994. Calcium channel current of vascular smooth muscle cells: extracellular protons modulate gating and single channel conductance. Journal of General Physiology. 103:665–678.

Klöckner, U., U. Trieschmann, and G. Isenberg. 1989. Modulation of calcium and potassium channels in isolated vascular smooth muscle cells. Drug Research. 39:120–126.

Koch, W. J., P. T. Ellinor, and A. Schwartz. 1990. cDNA cloning of a dihydropyridine-sensitive calcium channel from rat aorta. The Journal of Biological Chemistry. 256:17786–17791.

Kostryuk, P. G. 1980. Calcium ionic channels in electrically excitable membrane. Neuroscience. 5:945–959.
Kurachi, Y. 1982. The effect of intracellular protons on electrical activity of single ventricular cells. Pflügers Archiv. 394:264–270.

Kuriyama, H., Y. Ito, H. Suzuki, K. Kitamura, and T. Itoh. 1982. Factors modifying contraction-relaxation cycle in vascular smooth muscles. American Journal of Physiology. 243:H641–H662.

Liu, S., D. Piwnica-Worms, and M. Lieberman. 1990. Intracellular pH regulation in cultured embryonic chick heart cells. Journal of General Physiology. 95:1247–1269.

Ochi, R., and Y. Kawashima. 1990. Modulation of slow gating process of calcium channels by isoprenaline in guinea-pig ventricular cells. Journal of Physiology. 424:187–204.

Pelzer, D., S. Pelzer, and T. F. McDonald. 1990. Properties and regulation of calcium channels in muscle cells. Reviews of Physiology, Biochemistry and Pharmacology. 114:107–207.

Rinaldi, G. J., E. A. Cattaneo, and H. E. Cingolani. 1987. Interaction between calcium and hydrogen ions in canine coronary arteries. Journal of Molecular and Cellular Cardiology. 19:773–784.

Rooke, T. W., and H. W. Sparks. 1981. Effect of metabolic versus respiratory acid-base changes in isolated coronary arteries. Experientia. 37:982–983.

Rüegg, J. C. 1986. Calcium in Muscle Activation: A Comparative Approach. Springer Verlag, Berlin.

Schulz, I., F. Thevenod, and M. Dehlinger-Kremer. 1989. Modulation of intracellular free Ca$^{2+}$ concentration by IP$_3$-sensitive and IP$_3$-insensitive non-mitochondrial Ca$^{2+}$ pools. Cell Calcium. 10:325–336.

Siskind, M. S., C. E. McCoy, A. Chobanian, and J. H. Schwartz. 1989. Regulation of intracellular calcium by cell pH in vascular smooth muscle cells. American Journal of Physiology. 256:C234–C240.

Thomas, R. C. 1984. Experimental displacement of intracellular pH and the mechanism of its subsequent recovery. Journal of Physiology. 354:2P–22P.

Tsien, R. W., B. P. Bean, P. Hess, J. B. Lansman, B. Nilius, and M. C. Nowycky. 1986. Mechanism of calcium channel modulation by beta-adrenergic agents and dihydropyridine calcium agonists. Journal of Molecular and Cellular Cardiology. 18:691–710.

van Breemen, C., B. R. Farinas, P. Garba, and E. D. McNaughton. 1972. Excitation-contraction coupling in rabbit aorta studied by the lanthanum method for measuring cellular calcium influx. Circulation Research. 30:44–54.

Wahl, M. 1985. Local chemical, neural, and humoral regulation of cerebrovascular resistance vessels. Journal of Cardiovascular Pharmacology. 7:S36–S46.