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 (pH_i). Whole cell I_{Ca} fell when pH_i was made more acidic by substituting HEPES/NaOH with CO_2/bicarbonate buffer (pH_o 7.4, 36°C), and increased when pH_i was made more alkaline by addition of 20 mM NH_4Cl. Peak I_{Ca} was less pH_i 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 pH_i was changed by extracellular NH_4Cl or through the opened cell. In inside-out patches pH_i was controlled through the bath. Independent of the method used the following results were obtained: (*a*) Single channel conductance (24 pS) and life time of the open state were not influenced by pH_i (between pH_i 6 and 8.4). (*b*) Alkaline pH_i increased and acidic pH_i reduced the channel availability (frequency of nonblank sweeps). (*c*) Alkaline pH_i increased and acidic pH_i 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 pH_i effects within 20 s, suggesting that protons can rapidly permeate through the surface membrane of vascular smooth muscle cells. The role of pH_i 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 (pH_i). There are multiple mechanisms by which pH_i can modulate contractile state of the smooth muscle cells (smc). For example, intracellular acidosis ($pH_i < 7.2$) 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 pH_i suppresses the influx of extracellu-

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lar Ca^{2+} with the consequence of a reduced SR Ca^{2+} load (van Breemen, Farinas, Garba, and McNaughton, 1972; Rinaldi, Cattaneo, and Cingoliani, 1987).

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

At constant pH_o, I_{Ca} could be modulated easily and reversibly by CO_2 and NH_4Cl. Changing from a HEPES/NaOH to a CO_2/bicarbonate buffer produces acidosis (Thomas, 1984) because CO_2 permeates rapidly through the plasma membrane while bicarbonate does not, leading to the formation and dissociation of H_2CO_3 in the cytosol and providing H^+ ions that may reduce pH_i by ca. 0.2 units (Liu, Piwnica-Worms, and Lieberman, 1990, in cultured heart cells). Similarly, a solution of 20 mM NH_4Cl (pH 7.4) contains ~2 mM membrane permeable NH_3, and intracellular formation of NH_4^+ removes cytosolic protons and increases pH_i by ~0.4 units (Blank, Silverman, Chung, Hogue, Stern, Hansford, Lakatta, and Capogrossi, 1992, rat ventricular cells).

Because it is uncertain whether the above pH_i can be quantitatively extrapolated to vsm, a more direct control of pH_i at the cytosolic site of the 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 Ca^{2+} channel activity (Pelzer, Pelzer, and McDonald, 1990). An experimental compromise between control of pH_i 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 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 pH_i acts predominantly through the availability of L-type 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 I_{Ca} to single channel current i_{Ca}

$$I_{Ca} = N_T \cdot P_F \cdot P_o \cdot i_{Ca} = N_F \cdot P_o \cdot i_{Ca} \quad (1)$$

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, $N_F = N_T \cdot P_F$ channels contribute to the current I_{Ca} whereas $N_T \cdot (1 - P_F)$ 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 ~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 Ca^{2+} channel agonist Bay K 8644 (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_F (Klöckner and Isenberg, 1991). To separate the pH_i-induced from the spontanous 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" $N \cdot P_o = N_T \cdot P_F \cdot P_o$. We have therefore investigated whether pH_i modulation of I_{Ca} is via the open channel current i_{Ca}, the open probability P_o (fast gating) or the channel availability P_F (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 $MgCl_2$, 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 $MgCl_2$, 3.6 $CaCl_2$, 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 $CaCl_2$ was increased this is indicated. In some experiments, Ca^{2+} channel current was studied using Na^+ ions as the charge carrier, in which case the bath solution contained neither Ca^{2+} nor Mg^{2+} but 1 mM EGTA. In experiments were CO_2 was used to change pH_i 25 mM $NaHCO_3$ was substituted for 25 mM NaCl and the solution was equilibrated with 5% CO_2 (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 $BaCl_2$ 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 Ca^{2+} 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_i was modified by diffusion of buffers through the open cell end. Ca^{2+} channel activity was stable for ~10 min which is long enough for studying the effect of one pH_i change on slow gating. The 10 min period, however, is too short to apply a series of different pH_i'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

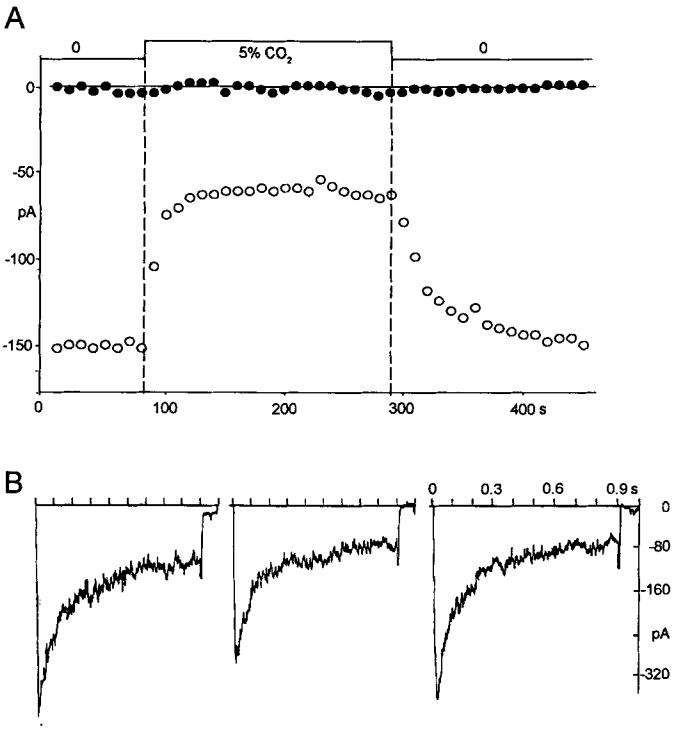

FIGURE 1. Change from HEPES/NaOH buffer to CO_2/bicarbonate buffer reduces Ca^{2+} channel currents. 36°C, pH_o at 7.4 throughout. (*A*) Time course of peak I_{Ca} (*circles*) and holding current (*dots*). Coronary vsm, 3.6 mM $[Ca^{2+}]_o$. (*B*) Reversible reduction of Na^+ current through Ca^{2+} channels. Pial vsm, in a Ca^{2+}- and Mg^{2+} free medium containing 1 mM EGTA. 900 ms steps from −65 to −20 mV, in HEPES/NaOH (*left*), 1 min after changing to CO_2/bicarbonate (*middle*) and 1 min after return to HEPES/NaOH buffer (*right*).

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 mM $[Ca^{2+}]_o$). Enlargement of I_{Ca} by high extracellular Ca^{2+} or Ba^{2+} concentrations was not possible because of

precipitating Ca^{2+} or Ba^{2+} carbonate. Therefore, the effects of CO_2/bicarbonate buffer on I_{Ca} could be measured in only a small number of vsm whose control I_{Ca} was large enough. Ca^{2+} channels carry Na^+ currents of large amplitude ($I_{Ca,Na}$) when the channel selectivity is reduced by a Ca^{2+} and Mg^{2+} free medium (for references see Kostyuk, 1980). In this medium, the change from HEPES/NaOH to CO_2/bicarbonate buffer reduced $I_{Ca,Na}$ within 30 s by 30% (Fig. 1 *B*). The reduction of $I_{Ca,Na}$ remained stable for at least 3 min. Upon return from CO_2/bicarbonate to HEPES-buffer, $I_{Ca,Na}$ recovered to control $I_{Ca,Na}$ within ~1 min. On average, pCO_2 (46 ± 5 Torr) plus 25

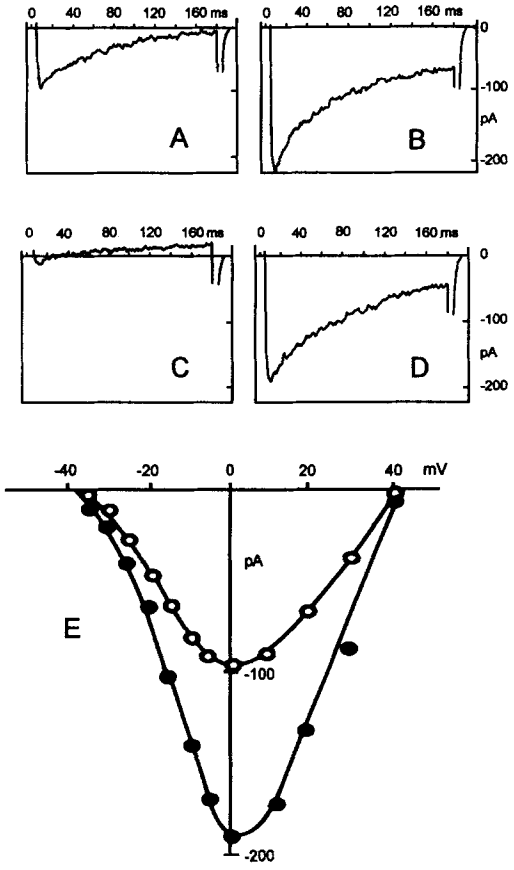

FIGURE 2. NH_4Cl augments Ca^{2+} channel currents. 3.6 mM $[Ca^{2+}]_o$, pH_o 7.4, 36°C. (*A–D*) Coronary vsm. 170-ms steps from −65 to 0 mV. (*A*) Control. (*B*) 1 min after addition of 20 mM NH_4Cl. (*C*) 1 μM D600 suppresses NH_4Cl augmented I_{Ca}. (*D*) Wash out of D600 for 1 min. (*E*) Pial vsm. Dependence of peak I_{Ca} (ordinate) on clamp step potential (abscissa). Circles before, dots 1–2 min after bath application of 20 mM NH_4Cl.

mM bicarbonate reduced $I_{Ca,Na}$ to 66 ± 10% of the control value in the HEPES-buffered solution (5 pial vsm). The depression of $I_{Ca,Na}$ by CO_2/bicarbonate buffer supports the hypothesis that intracellular acidification reduces Ca^{2+} channel current.

Bath application of NH_4Cl increases I_{Ca}. Ammonium chloride (20 mM) increased peak I_{Ca} by 220% and "late" I_{Ca} by 600% (Fig. 2, *A* and *B*). The augmentation of I_{Ca} by NH_4Cl was sustained as long as NH_4Cl was present (tested for up to 3 min). During wash out of NH_4Cl, I_{Ca} fell to the control value. NH_4Cl-augmented I_{Ca} was strongly suppressed by 1 μM D600 (Fig. 2 *C*) suggesting that the current was indeed

a L-type I_{Ca}. On average, 20 mM NH_4Cl 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_4Cl or an undershoot during wash out of NH_4Cl was not recorded (n = 15).

Fig. 2 *E* compares the voltage-dependence of peak I_{Ca} in absence (*circles*) and presence of 20 mM NH_4Cl (*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_4Cl 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_4Cl 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_4Cl augments I_{Ca} through the voltage-dependent gating parameters.

Single Ca^{2+} Channel Currents

NH_4Cl increases NP_o. 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_4Cl (20 mM, 1 min) the currents occurred more frequently, and occasionly two channels were active simultaneously (Fig. 3). Computer-evaluation indicated that NH_4Cl increased NP_o 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_4Cl modulated the Ca^{2+} channel through an intracellular messenger, most likely through a reduction of protons.

Bath application of 20 mM NH_4Cl 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. 4 *B* shows that the mean current transports, in the presence of NH_4Cl 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_4Cl increased the channel activity $N \cdot P_o$ from 0.24 to 0.79. In amplitude histograms NH_4Cl-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. 6 *B*). NH_4Cl 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_4Cl.

The mean current data showed that NH_4Cl increased the peak of I_{Ca} 2.3-fold and the late current 10-fold. The life time of the open state (Fig. 4 *D*) 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_4Cl, no Bay K 8644). Hence, the increase in the late current suggests that the channel re-opened at later times more frequently when NH_4Cl was present (Fig. 4 *A*). NH_4Cl 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^{2+} channels in open cell-attached patches. Fig. 5 *A* shows that Ca^{2+} channel activity continues when one end of the coronary vsm is crushed mechanically (1 μM Bay K 8644 present). Through the ~5 μm wide opening the solution's buffer is thought to slowly equilibrate with the cytosol and the inner site of the Ca^{2+} channel. Bath pH 7.4 moderately increased NP_o and reduced the frequency of blanks (Fig. 5 *A*). pH 8.4 suppressed the blanks and increased NP_o 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 NP_o and increased the number of blanks.

Fig. 5, *B* and *C*, shows an experiment from a patch attached to an opened pial vsm (1 μM 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^{2+} channel activity by bath application of NH_4Cl (20 mM, 1 min). Patch potential held continuously at −50 mV. Cell-attached patch from pial vsm in extracellular solution. pH_o 7.4, 22°C, no Bay K 8644. Patch electrode solution of 10 mM $BaCl_2$, 140 mM NaCl, 10 HEPES/KOH, pH 7.4, provided screening of surface charges comparable to extracellular solution with 2 mM $[Ca^{2+}]_o$ (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_i. In 17 of 40 patches, however, one current level was recorded at pH 7.4 but two or more levels at pH_i 8.4.

pH-modulation of Ca^{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 NP_o 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 complementaryly 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.

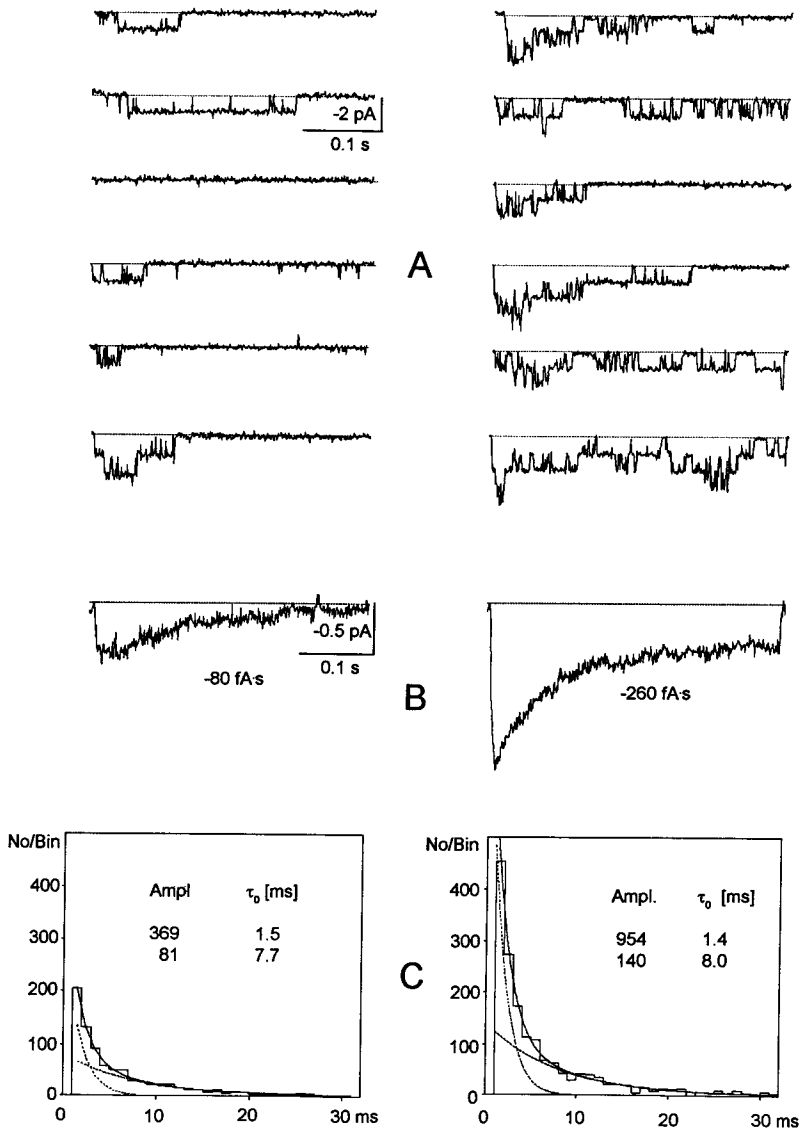

FIGURE 4. Analysis of NH_4Cl (20 mM) increased Ca^{2+} channel activity. Left before, right 1–2 min after application of NH_4Cl. Cell attached patch, pial vsm, 22°C, no Bay K 8644. (*A*) Single channel activity during 400-ms pulses from −60 to 0 mV (0.2 Hz). (*B*) Mean currents from 80 sweeps, the charge is labeled. (*C*) Distribution of the open time. Data were fitted with two exponentials, amplitude and time constants are indicated. Note: according to Tsien et al., (1986), the long openings characterize gating mode 2 that occurs in vsm spontaneously.

pH_i more acidic than 7.2 reduced the Ca^{2+} channel activity. At pH 6.9 NP_o was ~50% of the control recorded from the nonopened cell. NP_o 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*) NP_o was increased by acidosis and reduced by alkalosis. (*d*) The frequency of blank sweeps

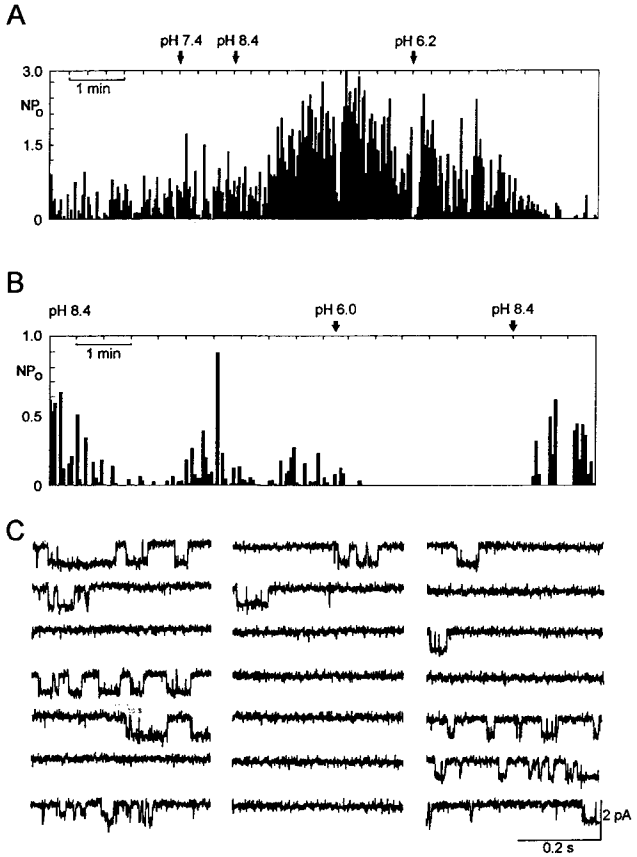

FIGURE 5. Ca^{2+} channel activity of an opened cell attached patch (1 μM Bay K 8644). 400 ms pulses from −60 to 0 mV. (*A*) Coronary vsm. NP_o (0 mV) is plotted as function of time. Cell was opened at arrow "7.4." Through the 5 μm wide cell opening, pH_o may have equilibrated with pH_i within ~2 min. (*B* and *C*) Pial vsm. Patch containing only one channel. Cell was opened 1 min before the start of the record. (*B*) Time dependence of NP_o. (*C*) Consecutive sweeps at pH 8.4 during the change to pH 6.0 and during return to pH 8.4.

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 (α_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

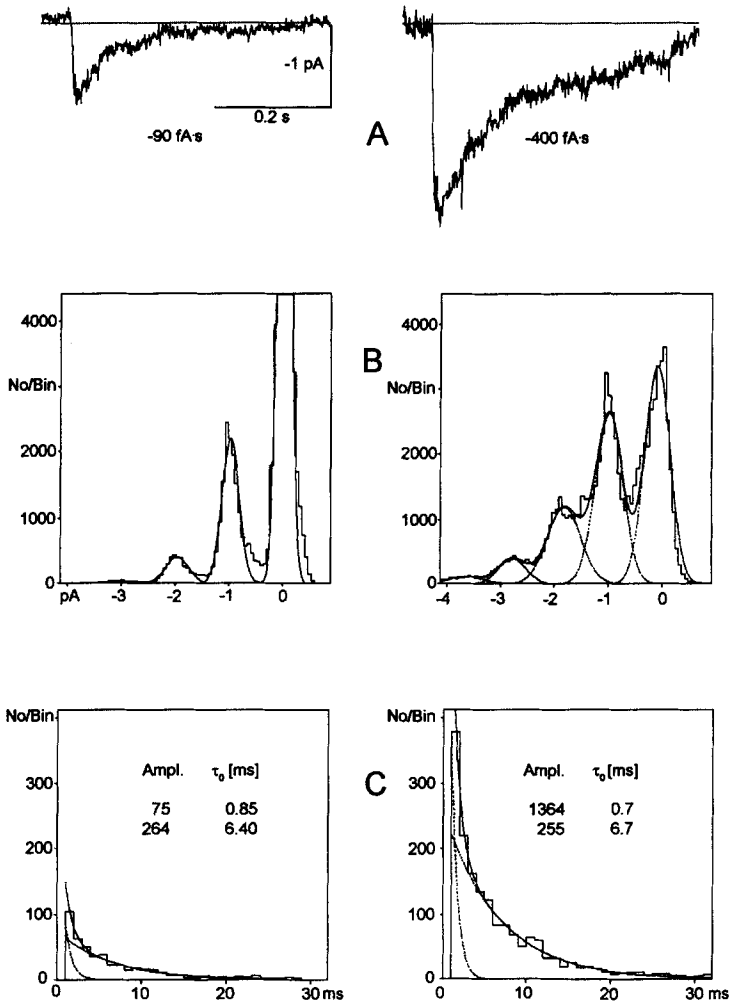

FIGURE 6. Analysis of Ca^{2+} channel activity at pH_i 7.4 (*left*) and 8.4 (*right*). Opened cell attached patch, coronary myocyte. No Bay K 8644 present. (*A*) Mean current from 80 tracings, current integral is labeled. (*B*) Amplitude histogram. Bin size 0.1 pA. Peak at 0 pA indicates closed channel. 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.

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

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. 7 *A*). pHi 8.4 increased NP_o to 200% of the control value at pH 7.2. The second change to pH 6.0 suppressed the channel

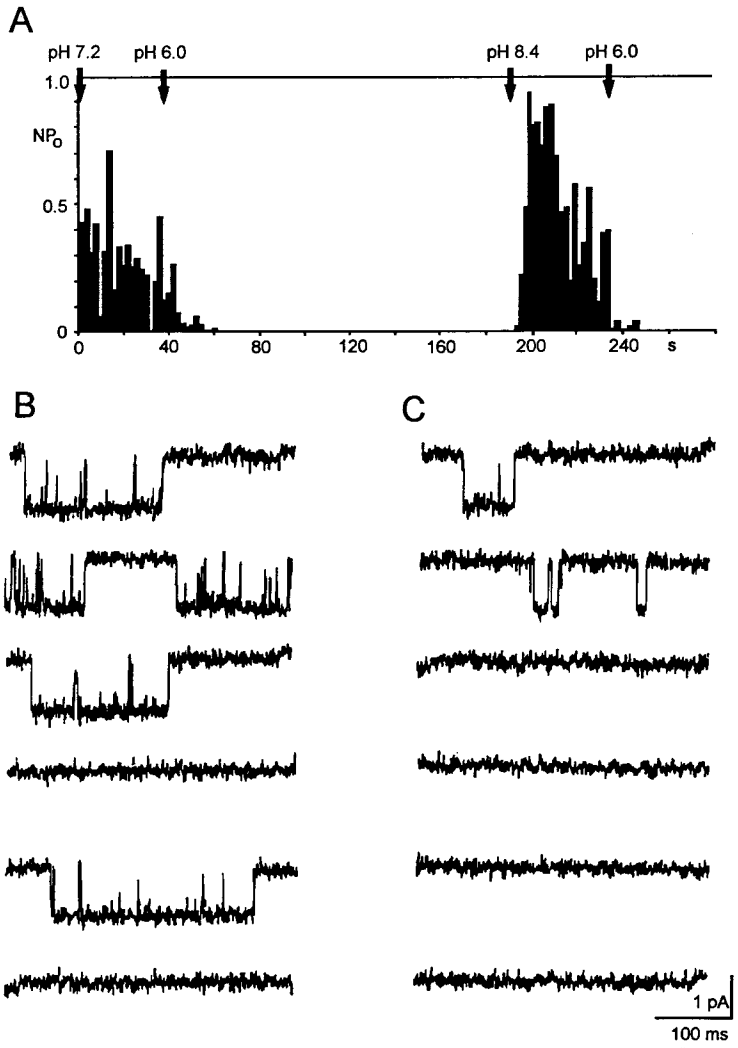

FIGURE 7. pH-modulation of Ca^{2+} channel currents in an inside-out patch from a coronary vsm. The electrode was filled with 110 mM $BaCl_2$ and 1 μM Bay K 8644. 400-ms steps from −60 to 0 mV, 0.5 Hz. (*A*) Channel activity $N \cdot P_o$ under influence of pH. Original tracings were taken at pH_i 7.2 (*B*) and immediately after the change from pH 7.2 to 6.0 (*C*).

activity again, however, because the activity did not recover a possible pH_i effect cannot be distinguished from run-down. The results suggest that protons, applied to the cytosolic site of an inside-out patch, modulate the Ca^{2+} channel activity in a similar way as in cell-attached patches. One may interprete the result to suggest that

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 μM 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_4Cl. 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_4Cl 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_4Cl, washout of NH_4Cl 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_i 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_4Cl 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_F that was estimated from the frequency of nonblank records. In comparison to cell-attached

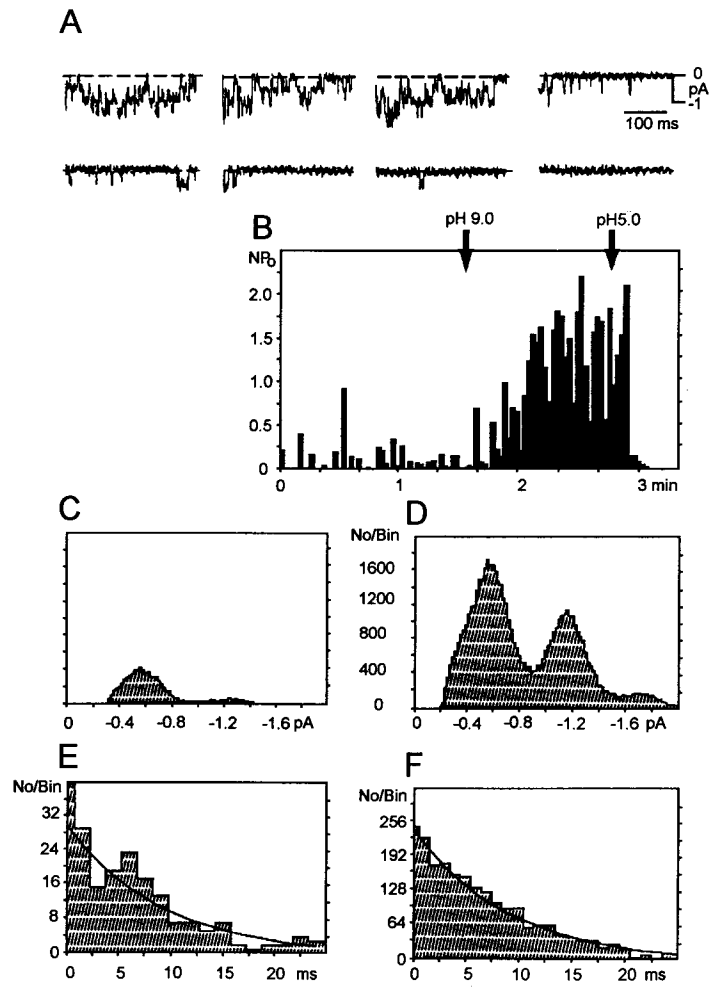

FIGURE 8. Bath pH_o modifies Ca^{2+} channel currents as if pH_i followed pH_o. Cell attached patch. Coronary myocyte. Electrode solution with 50 mM $BaCl_2$ and 1 μM Bay K 8644 adjusted to pH 5.0. 400 ms steps from −65 to 0 mV. (*A*) Single channel currents during the change of bath pH from 9.0 to 5.0. (*B*) Channel activity NP_o. (*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, pH_i 8.4 increased P_F ~ threefold, whereas pH_i 6.9 reduced P_F to 50% and pH_i 6.0 blocked P_F. The effects of pH_i on P_F did not depend on the presence or absence of Bay K 8644. The effect of pH_i on P_F 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 pH_i promoting the slow gating from a "sleeping" into an available state.

The possibility that the protons modified the Ca^{2+} 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 Ca^{2+} 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 $P_o(V,t)$. 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 Ca^{2+} 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 "Ca^{2+} mediated inactivation" of Ca^{2+} channels cannot be answered by the present experiments.

The pH_i effects on i_{Ca} 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 (α_1 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 α_1-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 α_1 subunit would be a suitable candidate. However, in 46 of 50 inside-out patches the Ca^{2+} 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, Kameyama, Nakayama, and Kaibara, 1988). With this speculation, pH_i could have modulated NP_o 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 NP_o. 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 $N \cdot P_o = N_T \cdot P_F \cdot P_o$ by the unknown number of channels in the patch (N_T). In 37 of 40 experiments, there was more than one channel in the patch, suggesting that vsm have Ca^{2+} channels in clusters. One could assume $P_F = 1$ for $pH_i = 8.4$ and normalize the data by NP_o during a final exposure to pH_i 8.4. However, many experiments could not include such a final step, and, the run down of NP_o with time makes such a normalization questionable.

Normalization was possible in cardiac myocytes, where patches with only one Ca^{2+} channel were obtained more frequently (Kaibara and Kameyma, 1988). The effect of

intracellular protons on normalized channel activity (P_o^*) was quantified with

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

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 α_1 subunit of the L-type Ca^{2+} channel contains 18 histidine-residues (Ca^{2+} 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 pH_i, and indeed partially recovered NP_o at pH_i 6 (three of six patches). However, the deprotonation of histidine-residues cannot easily explain the effects of alkaline pH_i. A change of pH_i from 7.4 to 8.4 that increases the fraction of deprotonated histidines from 83 to 98% should account for the 300% increase in NP_o.

In this study, the single channel currents were recorded at 22°C, with 110 mM $BaCl_2$ 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 $[Ca^{2+}]_o$ 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 NH_4Cl indeed augments the Ca^{2+} channel activity at −50 mV, provided the pipette solution contained 10 mM Ba^{2+} which screens the external surface charges to a similar extent as 2 mM Ca^{2+} (Ganitkevich and Isenberg, 1990).

The result of Fig. 3 supports the view that intracellular protons can regulate Ca^{2+} channels at more physiological conditions. Because the activity of Ca^{2+} channels controls Ca^{2+} 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 pH_i 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 pCO_2 changes to the pH_i mediated modulation in Ca^{2+} channel availability, and the metabolic changes in pH_o 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 pH_o 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 pH_o on NP_o 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, pH_o changes in blood or extracellular space modulate the Ca^{2+} channel activity of vascular myocytes through both extra- and intracellular mechanisms.

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