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Extra- and Intracellular Proton-Binding Sites of Volume-Regulated Anion Channels

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Abstract. We have investigated the effects of extracellular and intracellular pH on single channel and macroscopic (macropatches) currents through volume-regulated anion channels (VRAC) in endothelial cells.

Protonation of extracellular binding sites with an apparent pK of 4.6 increased voltage independent of the single-channel amplitude. Cytosolic acidification had a dual effect on VRAC currents: on the one hand, it increased single channel conductance by ~20% due to protonation of a group with an apparent pK of 6.5 and a Hill coefficient of 2. On the other hand, it reduced channel activity due to protonation of a group with an apparent pK of 6.3 and a Hill coefficient of 2.1. This dual effect enhances the macroscopic current at a slightly acidic pH but inhibits it at more acidic pH. Cytosolic alkalization also reduced channel activity with a pK of 8.4 and a Hill coefficient of 1.9, but apparently did not affect single-channel conductance.

These data show that VRAC channels are maintained in an active state in a narrow pH range around the normal physiological pH and shut down outside this range. They also show that HEPES-buffered pipette solutions do not effectively buffer pH in the vicinity of the VRAC channels.

Key words: Endothelium — Volume-regulated anion channels — Open pore properties — Extracellular and intracellular pH — Volume regulation

Introduction

Anion channels regulated by cell volume (volume-regulated anion channels, VRAC) are ubiquitously expressed and involved in numerous cell functions, such as cell volume regulation (Nilius et al., 1996; Strange et al., 1996; Nilius et al., 1997*a;* Okada, 1997), transport of organic osmolytes, and possibly proliferation (for review *see* Nilius et al., 1996, 1997*a*). Although macroscopic VRAC current activation and regulation was studied in detail, data on proton modulation are rather scarce. It has been shown that intracellular alkalinity reduced the outwardly rectifying Cl^- current (ORCC) induced by hypotonicity in osteoclasts and neuroblastoma cells (Gerard et al., 1998; Sakai et al., 1999). In our previous work we demonstrated that VRAC of cultured endothelial cells is permeable to lactate and bicarbonate ions, and that the macroscopic whole-cell VRAC current is modulated by extra- and intracellular protons in the physiological pH range (Nilius et al., 1998). We have also reported that calixarenes inhibit VRAC in a pH-dependent manner by binding at a site inside the channel, most probably at histidine residues (Droogmans et al., 1998, 1999). The functional importance of these residues has recently been demonstrated for ClCl channels, where they are part of a pore forming segment (Fahlke et al., 1997).

Here we present data on the effects of protonation on VRAC at the single-channel level in cell-attached patches and on macroscopic current measured in cell-attached macropatches. These data describe modulation of the pore properties of VRAC likely resulting from direct binding of protons at sites reachable from the intra- and extracellular side. Most importantly, we show that VRAC can only be activated in a window of intracellular pH between approximately 6.5 and 8.0.

Materials and Methods

Cells

Cultured endothelial cells from bovine pulmonary artery (cell line CPAE, ATCC CCL-209) were grown in Dulbecco's modified Eagle's

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Table 1. Composition of extracellular solutions (mM)

Solution	NaCl	KCl	Ac or Bu	NH_4Cl	$CaCl_2$	$MgCl_2$	HEPES	Glucose
Krebs	150	6	—	—	1.5	1	10	10
HTS	—	100	—	—	1.5	1	10	10
HTS-pH_{low}	—	80	20	—	1.5	1	10	10
HTS-pH_{high}	—	80	—	20	1.5	1	10	10

Table 2. Composition of the intracellular (pipette) solutions

Solution	CsCl	Cs-asp	Cs-ac	$CaCl_2$	EGTA	$MgCl_2$	HEPES	Na_2ATP
Pip 7.2	100	40		1.93	5	1	10	4
Pip 6	100	40		0.3	10	1	10	4
Pip 7.2-ac	100	40		1.93	5	1	10	4
Pip 6-ac	100		40	0.3	10	1	10	4

medium with 2 mM L-glutamine, 100 $\mu g \cdot ml^{-1}$ streptomycin, 100 $U \cdot ml^{-1}$ penicillin and 20% fetal bovine serum at 37°C in a fully humidified atmosphere of 10% CO_2 in air. They were detached by exposure to 0.05% trypsin in a Ca^{2+}- and Mg^{2+}-free solution and plated on gelatin coated coverslips for electrophysiological experiments (for more details, *see* Nilius et al., 1997).

Solutions

The composition of the various extracellular solutions is summarized in Table 1. The pH of Krebs solution and hypotonic high-K solutions (HTS) were adjusted to 7.4 with KOH. For hypotonic high-K solutions with low pH (HTS-pH_{low}) and hypotonic high-K solutions with high pH (HTS-pH_{high}), the pH value was first adjusted to 7.4 with KOH, and then to the desired value using either 1 M KOH or 1 M HCl. Although the buffering capacity of HEPES is low outside the pH range 7–8, we preferred to use a single buffer to avoid potential direct effects of other buffers, since these are mostly anions that might affect VRAC channels in some other way related to its permeation and/or block.

The osmolality of Krebs solutions, as measured with a vapor pressure osmometer (Wescor 5500, Schlag, Gladbach, Germany), was 320 ± 5 mOsm. The osmolality of HTS solutions was 220 ± 5 mOsm and did not significantly change when 20 mM KCl was replaced by either K acetate (ac) K butyrate (bu) in HTS-pH_{low} or by ammonium chloride in HTS-pH_{high}. In some experiments we used the H^+ ionophore nigericin (0.5 μM) to change intracellular pH by adjusting the pH of the HEPES-buffered extracellular medium.

The standard pipette solution for cell attached measurements contained (in mM): 100 CsCl, 1 $MgCl_2$, 1.5 $CaCl_2$, 10 HEPES, pH 7.4 adjusted with CsOH (210 ± 4 mOsm). Pipette solutions with higher pH were obtained by shifting the pH of this standard solution with CsOH. To obtain pipette solutions of lower pH, the standard pipette solution was mixed with the appropriate amount of a solution containing (mM) 100 CsCl, 1 $MgCl_2$, 1.5 $CaCl_2$ and 10 mM aspartic acid to keep the Cl^- concentration constant in all pipette solutions.

Table 2 shows the composition of the various pipette solutions used in the whole cell experiments.

Electrophysiology

CPAE cells grown on gelatin-coated coverslips were transferred to the experimental chamber and thoroughly washed with normal Krebs solution. At the beginning of patch-clamp recording the Krebs solution was replaced by HTS and cells were allowed to swell during 5–7 min. Cell-attached single-channel and macropatch membrane currents were measured with an EPC-7 (Heka-Electronics, Lambrecht/Pfalz, Germany) patch-clamp amplifier. Voltage protocols were controlled by a 386-based computer coupled to a DigiData 1200 (Axon Instruments, Foster City, CA) interface. Currents were filtered at 1 kHz and sampled at 2 kHz. Data acquisition and analysis were done using pCLAMP (Axon Instruments, Foster City, CA) and ASCD (Guy Droogmans) software. The hypotonic bath solution contained 100 mM K^+ to nullify the resting membrane potential. Assuming 140 mM $[K^+]_i$ in the cytosol, the expected membrane potential of a predominantly K^+-permeable membrane would be −8 mV. However, the resting membrane potential of CPAE cells scattered between −10 and −70 mV and is not solely determined by its K-conductance (Nilius et al., 1997*b*). Therefore, we did not make any further corrections for patch-membrane potentials. CPEA cells express a number of different ion channels (Nilius et al., 1997*b*). However, most of them are only revealed during a specific stimulation, while at resting conditions the cell membrane conductance is rather low and composed mainly of an inwardly rectifying K^+-conductance and a Cl^--conductance, presumably representing basal activity of VRAC-channels, since it can be inhibited by cell shrinkage. The pipette solution contained Cs^+ to block the inward rectifier. In very rare occasions we also observed VRAC-like channels in nonstimulated cells. In some patches, which were excluded from further analysis, a maxi-Cl channel was active.

Intracellular pH Changes and Intracellular pH Measurement

Intracellular pH was changed using acetate and ammonium based buffer solutions (Table 1). The rationale was that acetate can pass the membrane in non-ionized form and shift the internal pH by releasing a proton upon dissociation (Sharp & Thomas, 1981). This method was used by Tsai et al. (1995) to control intracellular pH of oocytes expressing an epithelial inwardly rectifying K-channel, ROMK1. A similar effect can be expected for ammonium-based buffers as well. Intracellular pH was assessed from the ratio of the fluorescence signals emitted at 510 nm in cells loaded with 2′,7′-biscarboxylethyl-5(6)carboxyfluorescein-tetra-acetoxymethyl ester (BCECF/AM, mo-

lecular Probes, Junction City, OR) and excited alternatively at wavelengths of 440 and 490 nm. More details about the loading and calibration procedure can be found in our recent paper (Nilius et al., 1998)

All experiments were done at room temperature (20–22°C).

Data Analysis

Dose-response data for proton block of single-channel amplitudes were fit to the equation:

$$y = a + \frac{b}{1 + 10^{(pK-pH)\cdot n}} \quad (1)$$

where y is the single-channel current or single-channel conductance at a given pH, Pk = −log(K), where K is the apparent dissociation constant for H^+, a is a limiting value of y, b is a titratable part of y, and n is the Hill coefficient.

The bell-shaped pH-dependence of the macropatch relative $N \cdot p_o$ (N = number of channels, p_o = open probability) was fit to the equation:

$$y = \frac{1}{[1 + 10^{(pK_a-pH)\cdot n_a}] \cdot [1 + 10^{(pH-pK_b)\cdot n_b}]} \quad (2)$$

pK_a and pK_b represent the apparent dissociation constants of the activation and deactivation binding sites of H^+ and n_a and n_b the corresponding Hill coefficients.

Data were analyzed in Origin 5.0 (MicroCal Software). Pooled data are given as mean ± SEM. Significance was tested at the 0.05 level.

Results

Effect of Extracellular Protons on Single VRAC Channels

Single channel events were consistently observed in cell-attached patches on cells swollen in hypotonic solution, but rarely in cells exposed to isotonic solution. Channel activity observed in pre-swollen cells disappeared if cells were exposed to isotonic solution (*data not shown*). In a few instances, single channel activity was induced in patches, which were silent in isotonic conditions, if the cell was perfused with hypotonic bath solution. Lowering Cl^- in the pipette solution from 100 to 50 mM reduced the current amplitude at +180 mV from 9.2 ± 1.1 to 4.7 ± 0.7 pA (n = 4). Complete substitution of Cl^- by aspartate further decreased the single channel current amplitude (i = 0.85 ± 0.2 pA at +180 mV), which is consistent with the previously reported permeation of aspartate through VRAC (Nilius et al., 1997*a*). Figure 1 shows selected traces at three different pipette Cl^- concentrations during a step from 0 to +180 mV and back to −100 mV, and the corresponding ensemble averaged currents. It is evident from these traces that the Cl^- substitution affects outward but not inward current amplitudes. They also clearly show inactivation of outward currents at positive potentials, which is a typical feature of VRAC channels (Voets et al., 1997). Replacing Cs^+ in the pipette solution with NMDG did not affect the single channel current amplitudes, indicating that the current is not carried by cations. These observations together with the outwardly rectifying property of these channels (*see below*) provide strong evidence that these single channel currents occur through VRAC and are activated by cell swelling. Channels with similar amplitude, rectification and gating kinetics were also observed in BC_3H1 cells (Voets et al., 1997), in human intestinal epithelial cells (Okada et al., 1994; Tsumura et al., 1996) and C6 glioma cells (Jackson & Strange, 1995).

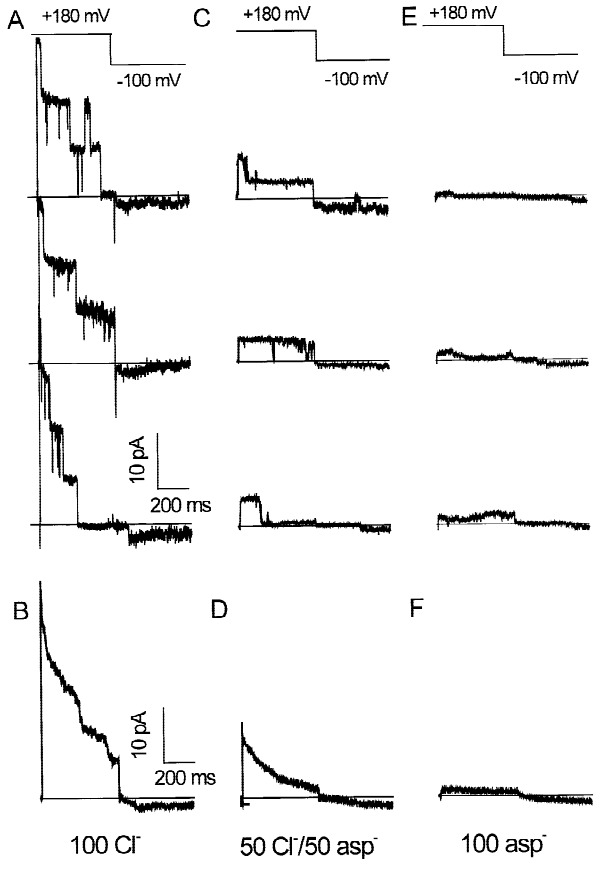

Fig. 1. Representative single-channel current traces recorded in cell-attached patch mode on cells pre-swollen with hypotonic solution at three different pipette Cl^- concentrations, i.e., 100 (*A*), 50 (*C*) and 0 (*E*), Cl^- being replaced with aspartate. Panels *B, D* and *F* represent the corresponding ensemble averaged currents.

Extracellular acidification by decreasing the pH of pipette solution to 3.5 led to a significant increase in single-channel current amplitude with no visible change in channel gating (Fig. 2*A* and *B*). The effect was most prominent on outward currents, whereas inward current were practically unchanged. The mean channel amplitude at +140 mV was 13.9 ± 0.3 pA (n = 6 patches) compared to 7.2 ± 0.1 pA (n = 6 patches) at pH 7.4.

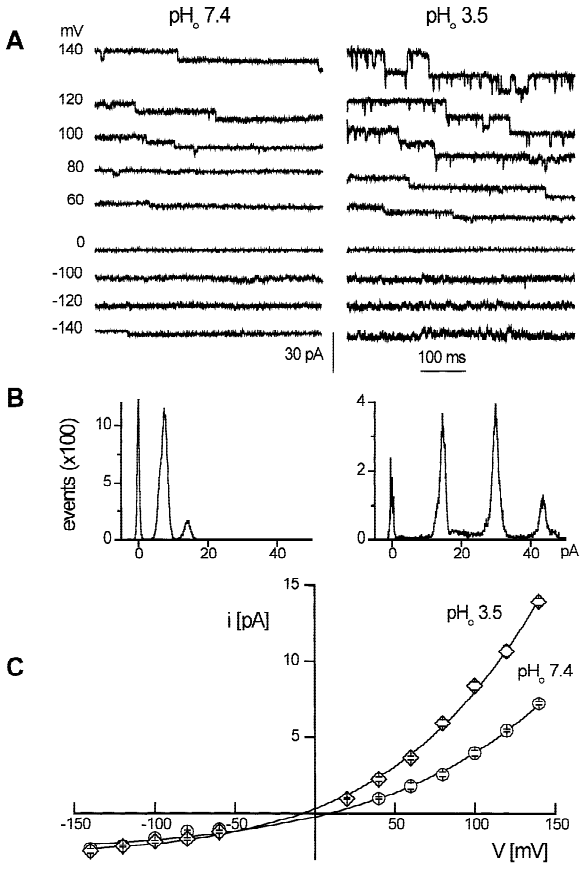

Fig. 2. Extracellular acidification increases outward current through single VRAC channels. (*A*) Representative cell-attached single-channel recordings at different test potentials using a pipette solution buffered at either pH 7.4 (left panel) or 3.5 (right panel). The potential was stepped from a holding potential of −50 mV to the various test potentials for 1 sec. (*B*) Current amplitude histograms from 21 (pH 7.4, left) and 14 (pH 3.5, right) records elicited by step-pulses from a holding potential of −50 to +140 mV. (*C*) Single-channel current-voltage relations at pH 7.4 (mean of 4–14 measurements; data from 6 patches) and pH 3.5 (mean of 5–12 measurements; data from 6 patches).

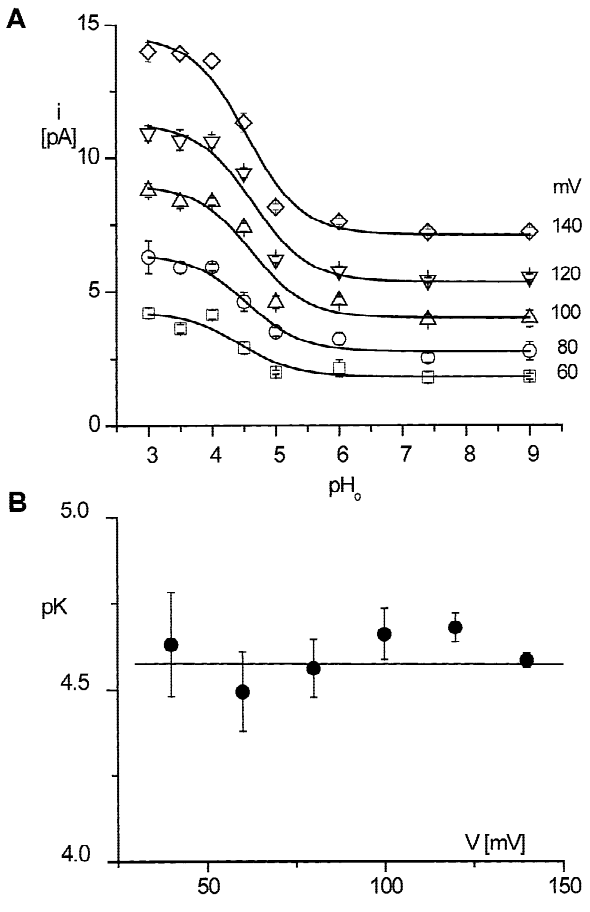

Fig. 3. Concentration- and voltage-dependence of the proton effects on single VRAC channels. (*A*) Concentration-response relations between single-channel current amplitudes measured at the indicated test-potentials and pH of pipette solution. Each point is a mean of 4–14 measurements from 5–7 different patches. The curves represent fits to Eq. (1) for a Hill coefficient $n = 2$ (*see* Materials and Methods). (*B*) Voltage-dependence of the corresponding apparent dissociation constants for H^+ obtained from the fits in *A*. The vertical bars represent the errors in the estimated values as obtained from the weighed fit to the data in panel *A*.

In contrast, the current amplitudes at −140 mV of −2.5 ± 0.1 pA ($n = 6$ patches) at pH 3.5 and of −2.3 ± 0.1 pA ($n = 6$ patches) at pH 7.4 were not significantly different. The outward current amplitudes were obtained from amplitude histograms, those at negative potentials were more difficult to assess because of the high open channel probability at these potentials. They were therefore derived from single channel current events during recovery from inactivation, i.e., channel inactivation was induced by a preceding step to positive potentials, and channel activity was recorded during a second step to negative potentials. The corresponding *I-V* relations of these single channel currents, as obtained from 6 patches, are shown in Fig. 2*C*, which clearly shows the outwardly rectifying property of the current and the reduction of outward current at higher pH. Figure 3*A* shows the titration curves of single-channel outward currents at different test potentials. At all test potentials, current amplitudes were pH-insensitive for pH values ranging from 6 to 9. At pH values lower than 6, the channel currents gradually increased and reached a plateau at pH values less than 4. All curves could be fitted to Eq. (1) using a Hill coefficient of $n = 2$, which is consistent with the binding of at least two protons at the external mouth of the channel. This binding was voltage-independent, as its pK value of 4.65 at +40 mV was nearly identical to that of 4.58 at +140 mV (Fig. 3*B*). This finding suggests that carboxyl groups (presumably Asp and/or Glu) are involved in the regulation of the single-channel conductance of VRAC by extracellular pH.

Effect of Intracellular Protons on Single VRAC Channels

VRAC channels showed fast and irreversible run down in excised inside-out patches. We have therefore used an indirect way to study the effects of changes in intracellular pH on single-channel VRAC currents, which in addition does not significantly disturb the cytosolic environment, i.e., permeable acetate- and ammonium-based buffers combined with parallel measurements of pH_i using a pH-sensitive fluorescent dye. Since both intracellular acidification and alkalization reduced channel activity (*see below*), we first established an on-cell macropatch at normal pH that contains a large number of VRAC channels, switched then to the desired pH and recorded channel activity shortly before activity completely ceased. Figure 4*A* shows such traces of single-channel outward current measured at +140 mV on a cell perfused with an acetate buffer at pH 6 or 7.4 and an ammonium buffer at pH 9 (the pipette solution was HEPES-buffered at pH 7.4). The corresponding amplitude histograms are shown at the right of the current traces. It is obvious that the current amplitude at pH 6.0 is significantly larger than at pH 7.4 using the same buffer or an ammonium-buffered bath solution of pH 9.0, indicating that intracellular protonation increases single-channel conductance. Titration curves, representing the dependence of single channel conductance on measured intracellular pH (Fig. 4*B*) could be fitted to Eq. (1), giving a pK of 6.5 and $n = 2$. These findings are consistent with binding of two protons at the internal mouth of the channel. Because of the rather limited effects of changes in pH_i we did not analyze the voltage-dependence of intracellular protonation in more detail.

It should be noted that at severe acidification and alkalinization the open probability of the channels was still high at moderate test-potentials, and closures were only observed at relatively high voltages of 100–140 mV. We were not able to further explore the effects on channel kinetics quantitatively because of the slow gating of the channels. The limited effects of pH on single channel amplitude and kinetics of gating nevertheless suggest that intracellular pH affects the number of active channels rather than their open-channel probability.

Effect of Intracellular Protons on Macropatch VRAC Currents

The high density of VRAC channels in swollen CPAE cells enabled us to record membrane currents of 100–1000 pA (at −140 mV) in macropatches obtained with low-resistance pipettes (0.5–1 MΩ). These macropatch currents (Fig. 5*A*, top panel) show the characteristic outward rectification and voltage-dependent inactivation of

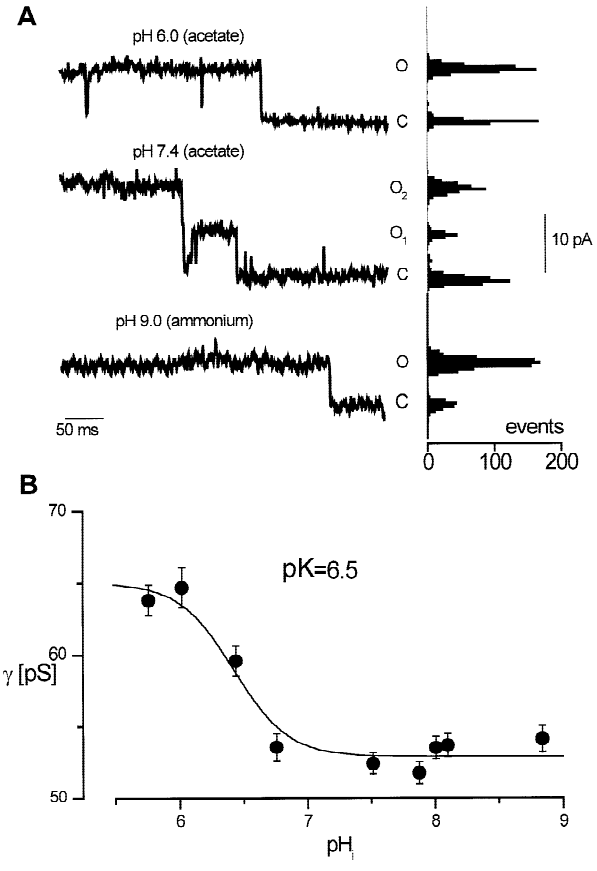

Fig. 4. Effect of intracellular pH on the current through single VRAC channels. (*A*) Representative on-cell recordings of VRAC channels at +140 mV from cells perfused with bath solutions buffered using the indicated membrane-permeable buffers (*see* Table 1). Corresponding current amplitude histograms are shown at the right of each trace. Traces at pH 6 and 9 were obtained from multi-channel patches before channel activity disappeared completely. (*B*) Single-channel conductance measured at 140 mV as a function of the actually measured intracellular pH (BCECF fluorescence). Each point represents the mean of 14–28 measurements from 3–5 different patches. The solid line represents the fit to Eq. (1) with pK and n of respectively 6. 5 and 2.

whole-cell VRAC currents (Nilius et al., 1998). However, intracellular acidification to pH 6 (Fig. 5*A*, middle panel) strongly decreased the amplitude of the macropatch-current, which is not only in contrast with the increased single-channel current amplitude at this intracellular pH as described above, but also with our previously reported data of a slight potentiation of the whole-cell macroscopic current with pipette solutions buffered at pH 6 with HEPES (Nilius et al., 1998). The effect of intracellular acidification was only partially reversible. The corresponding *I-V* curves in Fig. 5*B* before administration of low pH_i and after washout clearly show the characteristic outward rectification of VRAC currents.

The time course of the pH_i-dependent current reduction was rather slow and required several minutes to

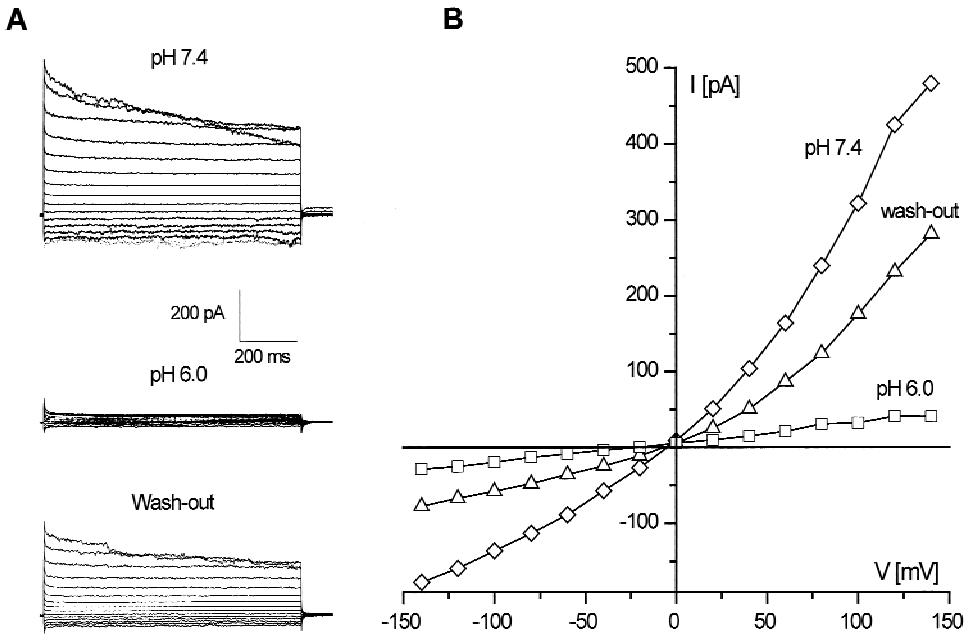

Fig. 5. Effect of intracellular acidification on VRAC currents through a macropatch in cell-attached mode. (*A*) Current traces elicited by 1 sec steps from a holding potential of −50 to ±140 mV in 20 mV increments. Upper traces were recorded at pH 7.4, the middle traces after about 5 min perfusion with acetate buffer at pH 6.0, and the lower traces after washout. (*B*) Current-voltage (*I-V*) relationships in normal solution (diamonds), after inhibition of the macropatch VRAC current by low intracellular pH (triangles), and after washout (squares). Data points represent current levels measured at the beginning of the voltage step from the records in *A.*

complete (Fig. 6*A*). The effect was also voltage-independent, as can be assessed from the minor effect of intracellular acidification on the ratio of the currents measured at +100 and −100 mV (Fig. 6*A*, bottom) and which could possibly be due to a contamination with background currents. A similar effect was observed if the cytosol was alkalinized during perfusion with a bath solution buffered at pH 9 with 20 mM ammonium chloride (Fig. 6*B*). Current inhibition developed faster during alkalization than during intracellular acidification and was fully reversible upon return to normal pH. The voltage dependence of current inhibition (Fig. 6*B*, lower panel) might be slightly more pronounced than during acidification, but this feature has not been further investigated.

Figure 6*C* summarizes normalized current values at +140 mV from various patches as a function of the measured intracellular pH. The various current amplitudes were first normalized to their value at pH 7.4 for the same patch and then converted to $N \cdot p_o$ values using the single-channel current amplitudes from Fig. 4*B*. From our single-channel recordings (*see above*) it appeared that N rather than p_o was affected by intracellular pH, indicating that the bell-shaped dependence of $N \cdot p_o$ mainly represents the effect of intracellular pH on the number of active channels. The pH dependence could be fitted by assuming the presence of two intracellular regulatory sites (Eq. (2)). Protonation of a site(s) with an apparent pK_a of 8.4 and $n_a = 1.9$ enhances channel activity ($N \cdot p_o$), whereas protonation of another site with an apparent pK_b of 6.3 and $n_b = 2.1$ reduces it.

Whole-Cell VRAC Currents and Intracellular pH

These changes in $N \cdot p_o$ are at variance with our previously published whole cell data (Nilius et al., 1998) where we observed a slightly enhanced current density in cells dialyzed with a HEPES buffered pipette solution at pH 6. We have repeated these experiments and were still able to record large VRAC currents in a cell dialyzed with a HEPES buffered pipette solution at pH 6 and superfused with a HEPES buffered extracellular solution (Fig. 7*A*). In addition, extracellular acidification by itself did not significantly affect the whole-cell VRAC current, nor did the addition of acetate or butyrate (20 mM) at pH 7.4 (*not shown*). Adding these permeable buffers at pH 6 caused however a fast and nearly complete inhibition of the whole cell current. This effect could be reversed in the presence of 20 mM acetate by changing the pH to 7.4.

In another experiment (*data not shown*), we dialyzed cells with pipette solutions in which 40 mM aspartate was replaced by 40 mM acetate and found large VRAC currents if that solution was buffered at pH 7.4 but only

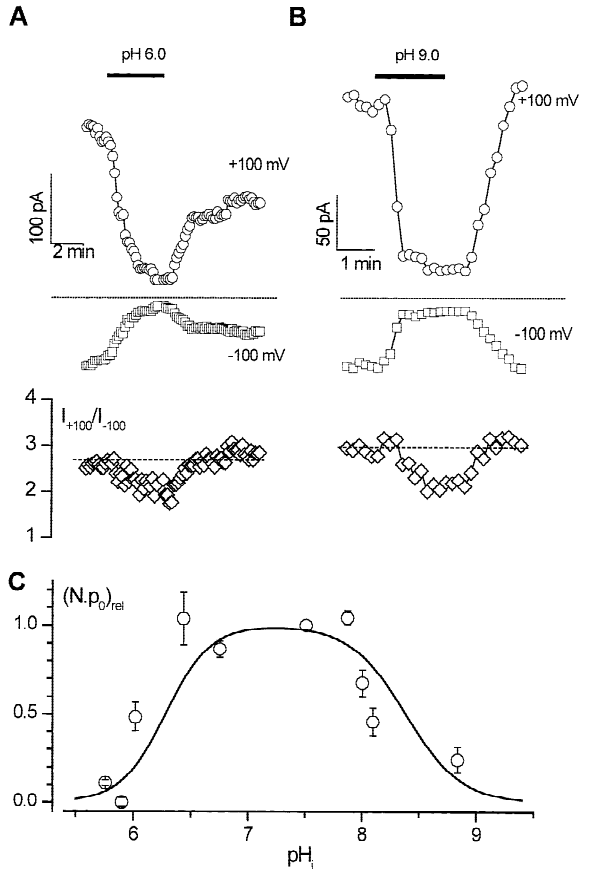

Fig. 6. The time course of VRAC current inhibition by changes in intracellular pH. (*A*) Peak current amplitudes measured in a cell-attached macropatch during test pulses to ±100 mV of 500 msec duration applied every 10 sec as a function of time. The horizontal bar indicates the administration of low-pH acetate-bath solution buffered at pH 6.0. The bottom panel represents the time course of the ratio of both current amplitudes, indicating that the current inhibition is largely voltage-independent. (*B*) A similar experiment as in (*A*) but with an ammonium-bath solution buffered at pH 9. (*C*) Current amplitudes at +140 mV from 4–6 macropatches normalized to the corresponding current amplitude at pH 7.4 (HEPES buffer) and corrected for changes in single channel conductance as a function of the actually measured pH. The solid line represents the best fit to Eq. (2) with parameters: $pK_a = 8.4$ and $n_a = 1.9$, $pK_b = 6.3$ and $n_b = 2.1$.

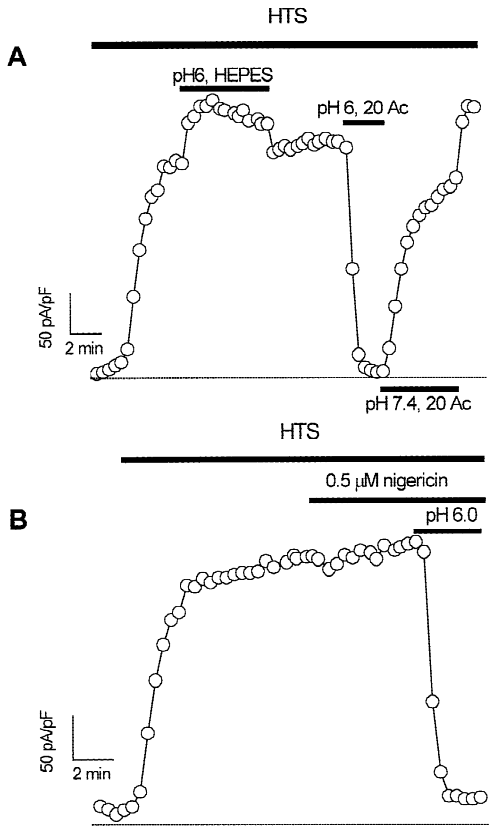

Fig. 7. Modulation of whole-cell VRAC currents under different buffering conditions. Time course of the VRAC current at +100 mV activated in a HEPES-buffered 25% hypotonic solution at pH 7.4 in a cell dialyzed with a HEPES buffered pipette solution at pH 6 (indicated by top bar). (*A*) Changing the extracellular pH to 6 has no pronounced effect on the current, whereas the same change in pH but in the presence of 20 mM acetate completely abolishes the current. The current slowly recovers in the continued presence of acetate after returning to pH 7.4. (*B*) Application of 0.5 μM nigericin at pH 7.4 does not affect VRAC, but a subsequent reduction of extracellular pH to 6 drastically inhibits it.

minute currents if it was buffered at pH 6. These data are in agreement with the macropatch results, but discrepant with the whole-cell currents with HEPES-buffered pipette solutions at pH 6. These data are therefore compatible with either a direct effect of the non-ionized form of intracellular acetate, which is more abundant at pH 6, on VRAC or with distinct buffering properties of HEPES and acetate (or butyrate) near the plasma membrane. We can also not rule out a direct effect of the permeable buffers on VRAC, and have therefore used in another experiment the H^+ ionophore nigericin to abolish the transmembrane proton gradient when VRAC was activated at pH 7.4 in a cell dialyzed with an HEPES buffered pipette solution at pH 6 (Fig. 7*B*). Application of 0.5 μM nigericin did not affect the current, but a subsequent reduction of extracellular pH to 6 almost completely abolished it.

Taken together, these results imply that a HEPES buffered pipette solution is not able to buffer the local pH in the intracellular vicinity of the VRAC channel as effectively as in case when the cell was superfused with low-pH bath in the presence of permeable buffers or during a proton load of the cell induced by nigericin.

Discussion

The present study demonstrates that VRAC channels are sensitive to both extracellular and intracellular pH. In contrast to sodium- and potassium channels, for which

protonation of an extracellular binding site was shown to suppress single-channel conductance (Zhang & Siegelbaum, 1991; Sabirov et al., 1997), binding of H^+ to at least two extracellular sites increases the conductance of VRAC channels in a voltage-independent manner. The pK value of these sites of 4.6 suggests that the extracellular proton sensor is composed of carboxyl residues of Asp and/or Glu. It is rather unlikely that endothelial cells will ever face such low pH values, but other cell types, such as osteoclasts, also express VRAC (Kelly et al., 1994) and function normally in a low-pH environment. In such case extracellular H^+-regulation may have a certain physiological significance. The biophysical basis of the effect is probably an increase in the positive electrostatic potential at the outer entrance of the pore that increases the local anion concentration, and therefore the probability of ion passage through the pore. A similar increase in single-channel conductance at low extracellular pH has been described previously for an anion-selective channel formed by Staphylococcal alpha-toxin in lipid bilayers (Krasilnikov & Sabirov, 1989) and a sarcoplasmic chloride channel reconstituted in lipid bilayers (Townsend & Rosenberg, 1995).

The effects of intracellular protons on VRAC are more complex. Protonation of a group with an apparent pK of 6.5 enhances single channel conductance. On the other hand, the bell-shaped pH dependence of $N \cdot p_o$ suggests a dual effect of cytosolic pH on channel gating. Protonation of a group with an apparent pK of 8.4 enhances, whereas protonation of a site with a pK of 6.3 reduces $N \cdot p_o$, which results in a peculiar pH dependence with a maximum around normal pH and a nearly complete abolishment of channel activity at 1 to 2 pH-units more acidic or alkaline pH. It is not clear whether the sites that affect single channel conductance (pK of 6.5) and channel activity (pK of 6.3) represent the same groups or not. In any case, the pK value of this group (groups) suggests either His or Lys. Note that for a cloned inward rectifier K^+-channel, ROMK1, the intracellular inactivating proton-sensitive site with a pK of 6.79 (Tsai et al., 1995) has recently been identified as a Lys residue at position 80 (Fakler et al., 1996).

The descending part of the pH-dependence at alkaline pH (Fig. 6*B*) gives evidence for yet another proton binding site ($pK = 8.4$, $n = 1.9$) which, in contrast to the acidic one, increases channel activity upon H^+ binding, but has no effect on single-channel current amplitude. This residue could be Lys, Tyr or Arg.

We have described in a recent publication the effects of extra- and intracellular pH on whole-cell VRAC currents measured using ruptured patches (Nilius et al., 1998). Alkalization was found to inhibit the whole-cell VRAC current, consistent with the present data in cell-attached patches. However, we also observed macroscopic current measured in whole-cell mode with an internal pipette solution buffered at pH 6.0 which were consistently larger than the current measured under the same conditions with a pipette solution buffered at pH 7.4. This apparent contradiction with the present data could be due to washout of an intracellular component responsible for the H^+-dependent inhibition of VRAC. However, this explanation is rather unlikely since inhibition of whole-cell VRAC currents also occurred in cells dialyzed with an 40 mM acetate pipette solution buffered at pH 6.0. The experiments with nigericin also exclude a direct inhibitory effect of acetate. The divergent whole-cell results with HEPES and acetate buffered pipette solutions at pH 6 can be explained if we assume that the faster diffusion rate of the permeable buffers provides a more effective pH buffering near the cell membrane than the HEPES buffered pipette solution. It is feasible that near the plasma membrane the restricted diffusion of HEPES is at least partially counterbalanced by the intrinsic buffering capacity and transmembrane H^+ extrusion mechanisms, which result in a less acidic submembrane pH compared to that of the pipette solution. This less acidic local pH could explain the increase in VRAC current, which was also observed during slight acidification in macropatches.

Protons affect anion-selective channels in many different ways. Moderate acidification to pH 5–6 inhibits, and alkaline pH enhances the macroscopic conductance of native muscle Cl^- channels (Hagiwara & Takahashi, 1974; Palade & Barchi, 1977) and a cloned counterpart of these channels, ClC1 (Rychkov et al., 1996, 1997) and strongly affects their gating (Vaughan et al., 1991). Inhibition by acidic extracellular pH was also shown for mini Cl-channels from the basolateral surface of parietal cells (Kajita et al., 1995), Ca^{2+} activated Cl^- currents (CaCC) in colon carcinoma (T84) epithelial cells (Arreola et al., 1995), and for cloned ClC4 and ClC5 channels (Friedrich et al., 1999; Mo et al., 1999). In contrast, a chloride channel from *Torpedo* Electroplax (Hanke & Miller, 1983) and cloned ClC2 channels (Jordt & Jentsch, 1997) were activated by low extracellular pH due to change in open probability. Insensitive to extracellular pH, CFTR-channels can be turned to highly pH-sensitive by R352H mutation (Guinamard et al., 1995). Intracellular acidification inhibited CFTR-type Cl channel in epithelial cells from sweat ducts (Reddy et al., 1998) and in mouse thick ascending limb (Guinamard et al., 1995) by interference with phosphorylation of R-domain. The distinct pH-sensitivity profile of VRAC allows distinguishing it from other types of Cl^- channels and may be a valuable tool for its molecular identification. From this point of view, ClC2, ClC4 and ClC5 have a quite different pH-sensitivity and should be excluded as molecular candidates for the VRAC channel of CPAE cells. Unfortunately, the interaction of the most

promising candidate ClC3 with protons has not been studied yet.

In conclusion, the VRAC channel is maintained in an active state in a narrow pH range around the normal physiological pH, and is shut down whenever the cytoplasm gets more acidic or alkaline. A specific feature of the pH-dependence of the VRAC current is that its maximum is located at a pH slightly below the normal basal value, which means that moderate acidification increases ionic flow through this channel. The concomitant enhanced HCO_3^--influx through VRAC may thereby counteract the original acidification, as suggested previously (Nilius et al., 1998). The descending part of pH-dependence at low pH may serve as a delimiter (or negative feedback) which shuts the channels at sufficiently low pH and prevents cell death due to extreme acidification.

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