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Ca^{2+}-Activated K^+ Channels from Cultured Renal Medullary Thick Ascending Limb Cells: Effects of pH

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Summary. Ca^{2+}-activated K^+ channels were studied in cultured medullary thick ascending limb cells (MTAL) using the patch-clamp technique. The purpose was to determine the effect of acidic pH on channel properties in excised patches of apical cell membrane. At pH 7.4, increasing Ca^{2+} on the intracellular side or applying positive voltages increases channel open probability. Reducing pH to 5.8 on the intracellular face of the channel decreases channel open probability at each voltage and Ca^{2+} concentration. Channel mean open times display two distributions and mean closed times display three distributions. Increasing Ca^{2+} or applying depolarizing voltages lengthens each of the mean open times and shortens each of the closed times. Lowering pH to 5.8 decreases the mean open times and increases mean closed times at each Ca^{2+} and voltage with the greatest effect on the mean closed times. In contrast, both single-channel conductance and channel kinetics are unaffected when pH is reduced to 5.8 on the extracellular face of the membrane. We conclude that protons interfere with Ca^{2+} binding to the gate of Ca^{2+}-activated K^+ channels reducing the probability of channel opening.

Key Words loop of Henle · potassium secretion · channels · acid/base balance · thick ascending limb · calcium

Introduction

In acute renal acidosis, potassium excretion from distal segments of the kidney is diminished (Malnic, de-Mello-Aires & Giebisch, 1971). The effects of pH are thought to be mediated directly at the apical membrane (Stanton, Guggino & Giebisch, 1982) by affecting apical potassium channels, which generate the membrane conductance. Ca^{2+}-activated K^+ channels are present on the apical membrane of distal segments of the kidney such as the rabbit cortical collecting tubule, the *Amphiuma* early distal tubule (*see* Hunter, Kawahara & Giebisch, 1986), cultured rabbit medullary thick ascending limb (MTAL) cells (Guggino et al., 1987*b*) and MDCK cells (Bolivar & Cereijido, 1987). In addition, it is known that vesicles isolated from outer renal medulla exhibit a Ca^{2+}-activated Rb^+ flux, which is blocked by Ba^{2+} and quinine (Burnham, Braw & Karlish, 1986). Although Ca^{2+}-activated K^+ channels do not appear to contribute to basal apical K^+ conductance (Frindt & Palmer, 1987), they represent a large reserve pathway for potassium excretion across the apical membrane since both the single-channel conductance and total number of these channels is large (Guggino et al., 1987*b*). Thus, they do represent a pathway that would clearly cause potassium excretion in the presence of agonists like norepinephrine, which increase intracellular Ca^{2+} (Goligorsky et al., 1986) or other hormones like aldosterone, which cause alkalinization of intracellular pH (Oberleithner, Kersting & Hunter, 1988). In addition, it has been shown that Ca^{2+}-activated K^+ channel activity is increased by membrane stretch and in hyposmotic solutions suggesting that these channels are important in volume regulation (Taniguchi & Guggino, 1989).

Ca^{2+}-activated K^+ channels in the apical membrane of cultured MTAL cells are blocked by barium, tetraethylammonium (TEA), quinine and charybdotoxin (Guggino et al., 1987*a*) suggesting that they are in the same class of channels as those present in the *t*-tubular membrane of skeletal muscle (Latorre & Miller, 1983). Because this channel may represent a pathway for K^+ secretion in the kidney, we tested the effect of pH on channel activity.

Previous reports by Cook et al. (1984) and Christensen & Zeuthen, (1987) have shown that voltage activation of Ca^{2+}-activated K^+ channels in beta pancreatic cells and the choroid plexus is inhibited by acid pH. We have determined that acid pH decreases both the Ca^{2+} and voltage-dependent activation of Ca^{2+}-activated K^+ channels in MTAL cells. Furthermore, we found that the inhibition of channel activity is mediated mainly by a pH-dependent inhibition of the mean closed time, suggesting that acid pH decreases the equilibrium-binding coefficient of Ca^{2+} to the closed state of the channel.

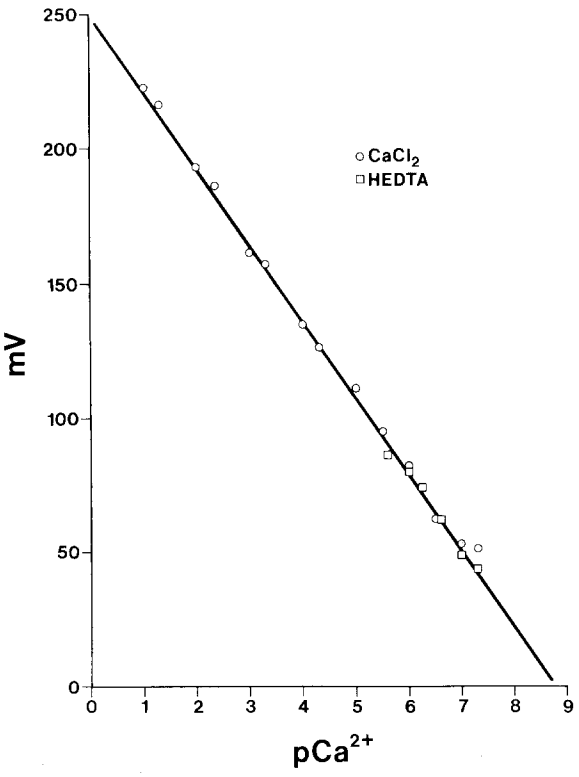

Fig. 1. Calibration of solutions with a Ca^{2+} electrode. Open circles show the voltage output of the electrode compared to the calculated calcium concentration. Solutions used in these experiments, open squares, had 10 mM H-EDTA added, then Ca^{2+} was added until the desired Ca^{2+} concentration was achieved

Materials and Methods

Cell Culture

Clone A3 of rabbit medullary thick ascending limb cells used in this study were established by Green et al. (1985). The cells were grown in culture as previously described (Guggino et al., 1986), and used for patch-clamp experiments 2 to 7 days after seeding when the cells were in small clumps or near confluence. Excised patch-clamp experiments were performed at room temperature.

Patch-Clamp Techniques

Pipettes made from flint glass were fabricated as described in detail by Hamill et al. (1981). Pipettes were pulled in two steps on a Kopf (Tujunga, CA) pipette puller, coated with Sylgard (Dow Corning, MI), and fire polished on a microforge assembled from a Leitz microscope (Bunton Inst., Rockville, MD). Pipettes routinely had an open-tip resistance of 5–8 MΩ. Contact between the patch clamp and bath or pipette solution was via silver chloride wires.

Seals were made by gently lowering the patch pipette onto the surface of a cell with a Leitz micromanipulator (Bunton Inst., Rockville, MD), until a small increase in resistance was observed, then giga-seals between 5–10 GΩ were obtained with gentle mouth suction. Inside-out patches were verified at the beginning of each experiment by a decrease in amplitude in the presence of 20 mM TEA but not 1 mM TEA.

Membrane currents were recorded with an extracellular patch clamp (Yale Physiology Department, Mark V, New Haven, CT). The signal from the patch clamp was filtered at 3 kHz on an eight-pole Bessel filter (Frequency Devices, Haverhill, MA), digitized by a pulse code modulator (Model VP101, JVC, Tokyo, Japan) and recorded on a video tape recorder (Model BR6400U, JVC, Tokyo, Japan). Outward currents from inside-out excised patches are designated as upward deflections or positive current. Voltages are applied voltages, designated as the voltages applied at the intracellular face of the membrane with respect to ground in the pipette. Data are reported as mean +/− SE (n = number of observations).

Calibration of Calcium

Free Ca^{2+} was calibrated with a Ca^{2+} electrode (F2210 Ca, Radiometer). Calibration of the electrode was in the presence of $CaCl_2$ standards containing ultrapure KCl (Gold Label, Aldrich) dissolved in deionized water. The electrode responded linearly with a slope of 28.4 mV/pCa^{2+} (Fig. 1). Solutions for these experiments containing 10 mM EGTA were made by titrating with Ca^{2+} to the desired free-Ca^{2+} measured from the voltage output from the electrode. In addition, the pH was controlled by the addition of 10 N KOH.

Solutions

In all experiments, the pipette was filled with (in mM): 135 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, and 15 HEPES adjusted to pH 7.4 with NaOH. The bath solution contained 135 KCl, 5 NaCl, and 15 HEPES, 10 EGTA titrated with $CaCl_2$ and adjusted between pH 5.8 to 7.4 with KOH.

Data Analysis

For analysis, the video data were transferred to a computer (Micro-PDP/11, Digital Equipment Corp., Maynard, MA) at a sampling rate of 44 kHz. Open and closed events were discriminated at 50% of the maximal open amplitude. The open probability was expressed as the ratio of open time to total recording time. The mean open times and mean closed times were determined by plotting frequency histograms of open times or closed times *vs.* time. The distributions were fitted to an exponential equation with one to three terms using the SAAM program (Berman, Shahn & Weiss, 1962; Berman & Weiss, 1978). For Figs. 1, 2*B*, 3*B*,*C*, 4, 5, 7 and 8, straight lines through the data were estimated by eye. Figures 1–8 are representative experiments of 10 experiments. Comparisons of channel properties depicted in the figures were always made on the same channel at different pH values.

Results

Influence of pH on Single-Channel Conductance and Opening Probability

Figure 2*A* shows a tracing of a Ca^{2+}-activated K^+ channel at two different pH values. Note that low pH does not affect single-channel amplitude sug-

gesting that there is no change in single-channel conductance (Fig. 2*B*). This lack of effect on conductance implies that a pH change as low as 5.8 does not interfere with K^+ conduction through the pore. In contrast, Fig. 2*A* demonstrates that the major effect of protons is to reduce the opening probability of the channel.

Interactions of H^+ on Ca^{2+}-Induced Gating of Ca^{2+}-Activated K^+ Channels in MTAL Cells

The effect of low pH on opening probability illustrated in Fig. 2*A*, suggests that the primary effect of H^+ is on channel gating. In order to examine in detail the effect of low pH on channel gating, open probability was measured at various Ca^{2+} concentrations (*see* Fig. 3*A*). The data shows that at pH 7.4, the Ca^{2+} concentration needed for an open probability of 0.5 is 3 μM, whereas 8 μM Ca^{2+} is required at pH 5.8. A measure of the ability of Ca^{2+} to open the channel was obtained by calculating from the data given in Fig. 3*A* the affinity of Ca^{2+} for its binding site. The affinity diminishes from 7 μM at pH 7.4, to 13 μM at pH 6.8 and to 40 μM at pH 5.8 (Fig. 3*B*) demonstrating that at higher H^+ concentrations much more Ca^{2+} is required to open the channel. The Hill coefficient at pH 7.4 (Fig. 3*C*) is 2.7 suggesting that more than one Ca^{2+} binding site is involved in regulating channel opening probability. Lowering pH reduces the Hill coefficient (Fig. 3*C*), further evidence that protons are interacting with Ca^{2+} binding to the gating mechanism.

In contrast, pH changes to as low as 5.8 at the external membrane do not affect gating if intracellular pH is maintained at pH 7.4. Thus, our data show clearly that the primary effect of raising the proton concentration is to decrease the ability of Ca^{2+} in opening the channel.

To study the kinetics of this pH effect in more detail, we analyzed both open-time and closed-time distributions at pH 7.4 and 5.8. At pH 7.4, when the channel shows a low probability of opening (e.g., at 2.5 μM Ca^{2+}) there are clearly two mean open times (*see* Fig. 4), a short and a longer distribution. As Ca^{2+} concentration on the intracellular face of the membrane is increased, the duration of each class of opening events lengthens. The mean open time of the shorter distribution increases faster than the longer one until at higher Ca^{2+} concentrations (when the channel reaches near maximal open probability) only one distribution is evident. This is important because at the maximum open probability the channel is not open continuously, but, as is evident from an examination of the closed-time distribution (*discussed below*), the openings are still interrupted by brief closing events.

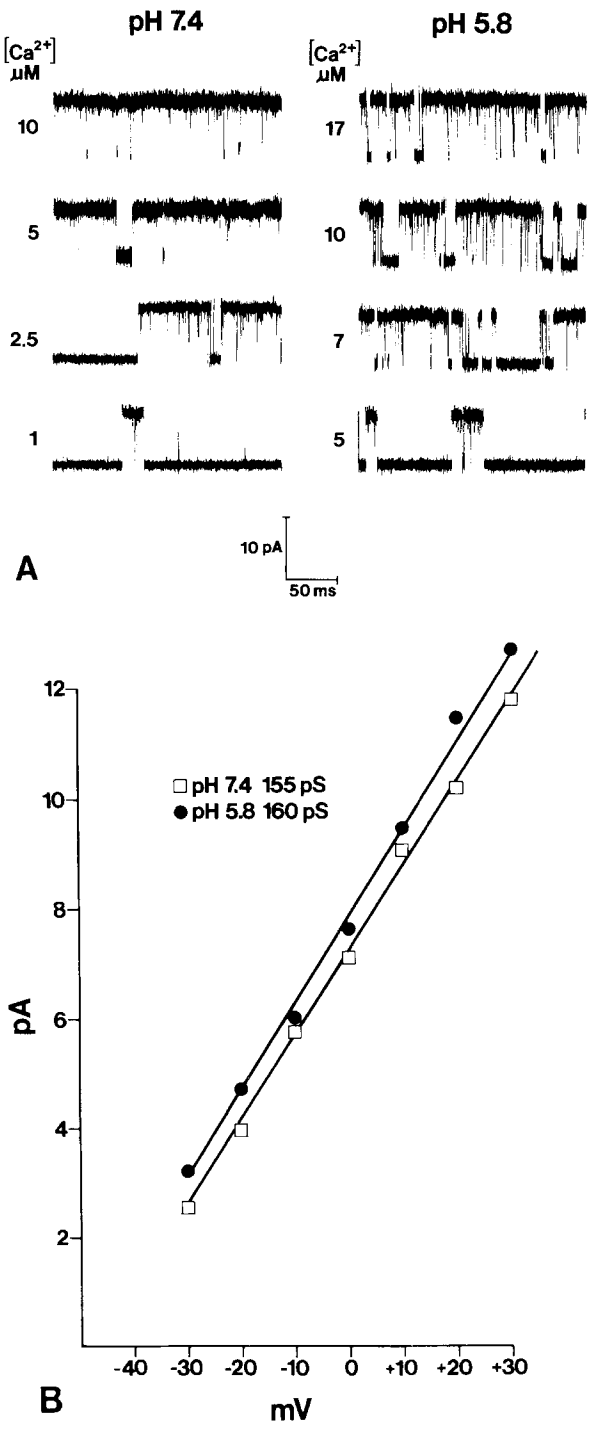

Fig. 2. (*A*) The effect of pH 7.4 and 5.8 on single-channel events at various Ca^{2+} concentrations at the internal face of a channel. The channel opening probability at pH 7.4 is much greater than at pH 5.8. Channel recordings were chosen to show that it takes much higher Ca^{2+} concentrations at pH 5.8 to attain similar open probabilities as that attained at pH 7.4. The applied potential was 0 mV. (*B*) The effect of pH on single-channel conductance of the Ca^{2+}-activated K^- channel. Solutions contained in mM, 135 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$ and 15 HEPES adjusted with NaOH in the pipette and in the bath 135 KCl, 5 NaCl, 10 EGTA with Ca^{2+} adjusted to 5 μM, and 15 HEPES adjusted to pH 5.8 or pH 7.4

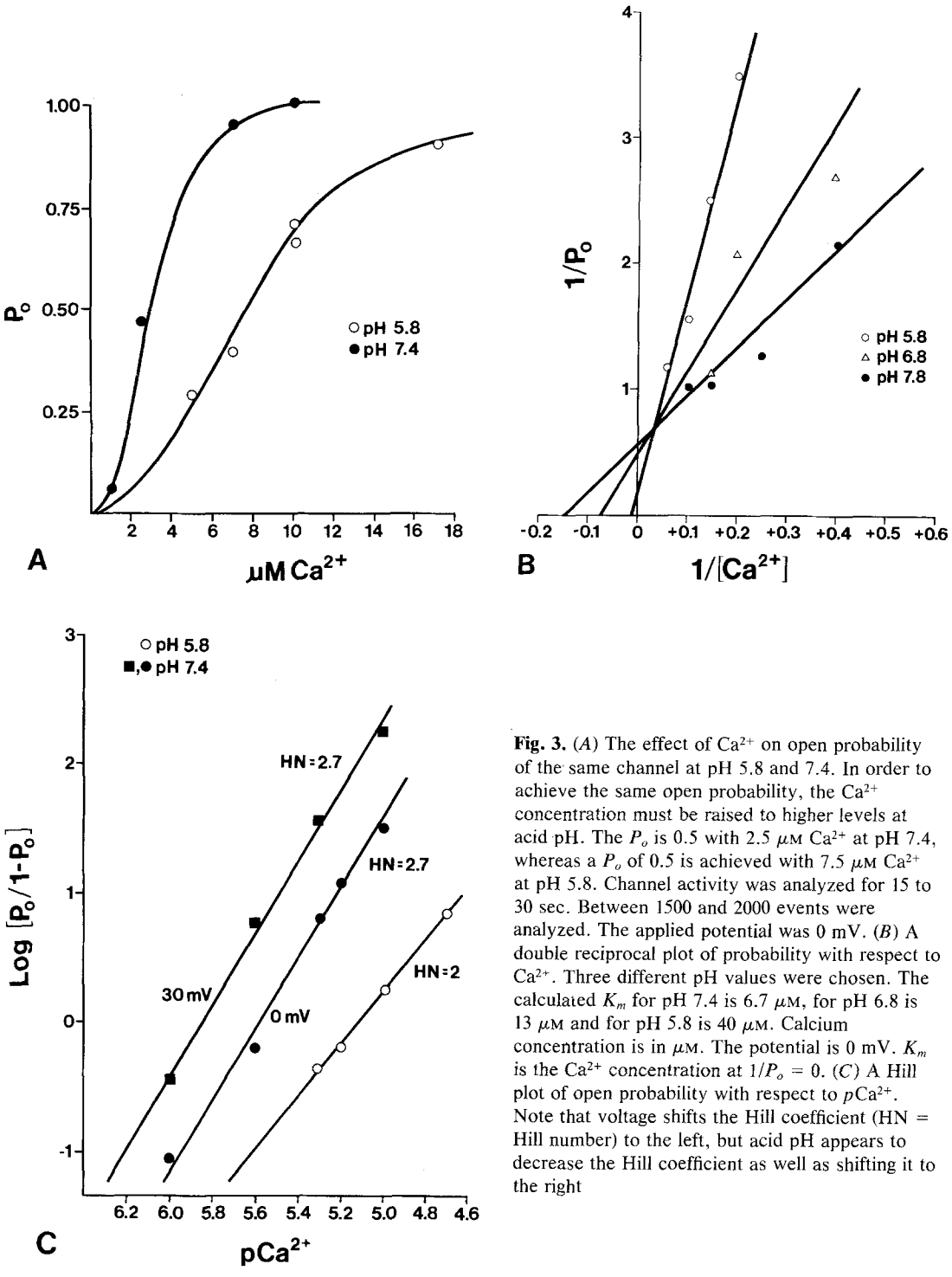

Fig. 3. (*A*) The effect of Ca^{2+} on open probability of the same channel at pH 5.8 and 7.4. In order to achieve the same open probability, the Ca^{2+} concentration must be raised to higher levels at acid pH. The P_o is 0.5 with 2.5 μM Ca^{2+} at pH 7.4, whereas a P_o of 0.5 is achieved with 7.5 μM Ca^{2+} at pH 5.8. Channel activity was analyzed for 15 to 30 sec. Between 1500 and 2000 events were analyzed. The applied potential was 0 mV. (*B*) A double reciprocal plot of probability with respect to Ca^{2+}. Three different pH values were chosen. The calculated K_m for pH 7.4 is 6.7 μM, for pH 6.8 is 13 μM and for pH 5.8 is 40 μM. Calcium concentration is in μM. The potential is 0 mV. K_m is the Ca^{2+} concentration at $1/P_o = 0$. (*C*) A Hill plot of open probability with respect to pCa^{2+}. Note that voltage shifts the Hill coefficient (HN = Hill number) to the left, but acid pH appears to decrease the Hill coefficient as well as shifting it to the right

Three characteristics of the open-time distribution are evident at pH 5.8 (Fig. 4). First, two open-time distributions are still present, second, although both distributions are Ca^{2+} dependent, they are less so at the lower pH, and finally, openings cluster into one distribution only when Ca^{2+} is raised to 17 μM compared to 11 μM at pH 7.4. Furthermore, at the 17 μM Ca^{2+} where the two distributions converge at pH 5.8, the mean open time is 11 msec, lower than that (approximately 20 msec) attained at pH 7.4. We conclude, that both at normal and acidic pH, as the open probability is increased, the duration of the

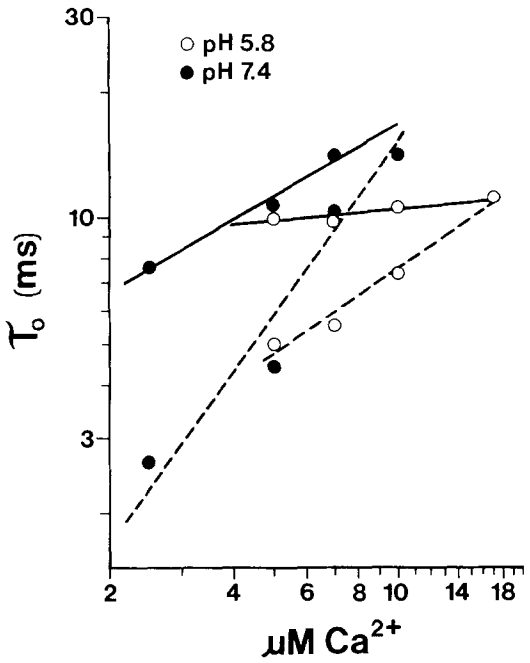

Fig. 4. The effect of Ca^{2+} concentration on mean open time in excised patches. Most of the open events (in msec) fell into two open distributions. The open events at pH 7.4 were somewhat longer than at pH 5.8

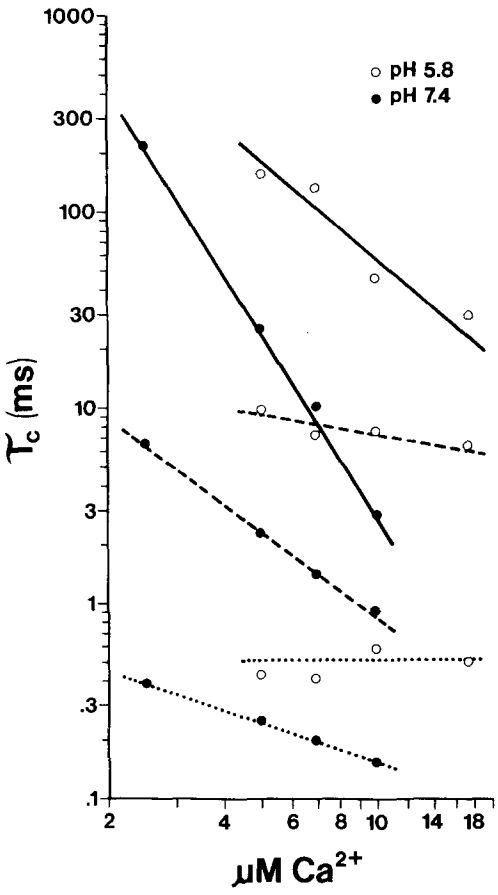

Fig. 5. The effect of calcium concentration on mean closed time in excised patches. At pH 7.4, the closed times were fitted by three distributions. At pH 5.8, there are also three distributions with each of the mean open times of longer duration than the distributions at pH 7.4

open events lengthens and that at more acidic pH a higher Ca^{2+} concentration is needed to shift most of the openings to the longer event distribution.

Closed times can be analyzed into at least three distinct distributions at pH 7.4. As Ca^{2+} is raised at pH 7.4, the closing events in each distribution become shorter so that at 10 μM Ca^{2+}, a concentration at which the probability of opening was nearly 1, most of the closings are brief.

Upon inspection of Fig. 5, there are two dominant effects of lowering intracellular pH. First, at pH 5.8, there is an increase in the mean closed times of each distribution compared to pH 7.4. This effect is most dramatic between 10 and 17 μM. A comparison with Fig. 4 shows that even though pH affects the duration of the opening events, the greatest effect is on the closed times. An increase in mean closed times contributes significantly to the decrease in open probability seen at pH 5.8. The second effect of acid pH is a decrease in the dependence of the closed time on intracellular Ca^{2+}.

Effect of pH on Voltage-Gating of the Channel

The effect of pH on voltage-mediated gating of this channel was also investigated. Figure 6 shows the effect of pH on the voltage-dependence of opening probability of a channel in 5 μM Ca^{2+}. Acid pH causes a dramatic decrease in the probability of opening at all voltages between −20 and +40 mV.

Inspection of the open distributions *vs.* voltage in Fig. 7 shows that at constant Ca^{2+}, the mean open time at each voltage between −20 and +40 mV is decreased at acid pH compared to pH 7.4 but that this effect is not large. The closed-time distributions, on the other hand, show a more pronounced pH dependence. For example, at 0 mV the longest closed-time distribution has a closed time of 25 msec at pH 7.4, but is increased to 170 msec at pH 5.8 (Fig. 8). These data suggest that the major kinetic effect of pH is to increase the length of the closed time.

Taken together our data suggest that acid pH has its greatest effect at the Ca^{2+}-binding site influencing the binding of Ca^{2+} to the channel more than changing its disassociation.

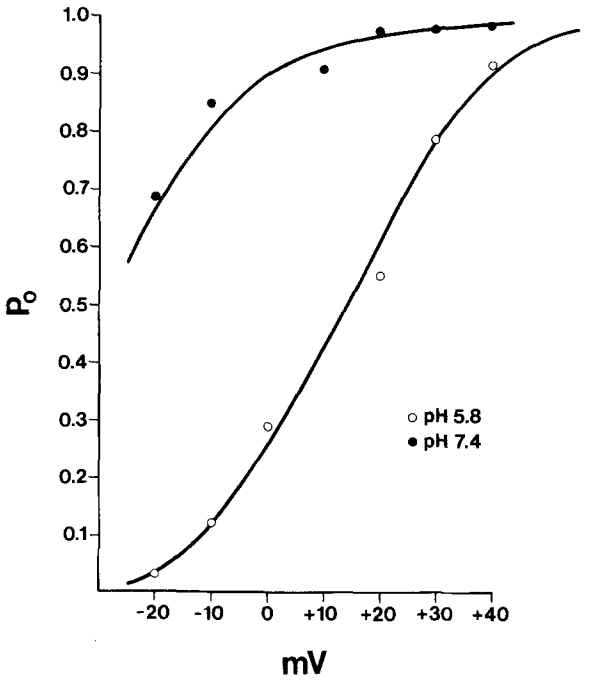

Fig. 6. The effect of pH on voltage-dependent open probability in excised patches. The calcium concentration is 5 μM. A P_o of 0.5 is achieved at pH 7.4 at −30 mV. At pH 5.8, a P_o of 0.5 is achieved at +10 mV. Thus, a pH change of 1.6 units is equivalent to a shift in about 40 mV

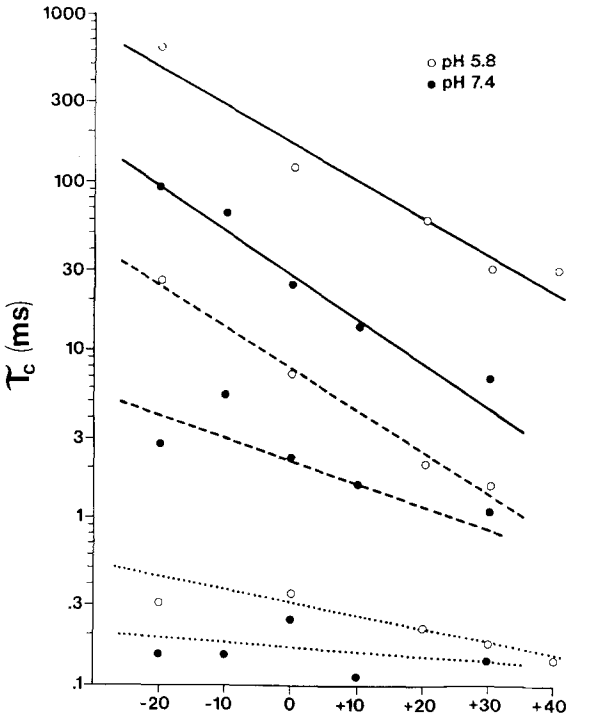

Fig. 8. The effect of voltage on mean closed time in excised patches. Patches were excised into 5 μM Ca^{2+} at pH 7.4 or pH 5.8. The three closed distributions each showed a longer mean closed time at pH 5.8 compared to pH 7.4. The shortest closed distribution was nearly independent of voltage at each pH

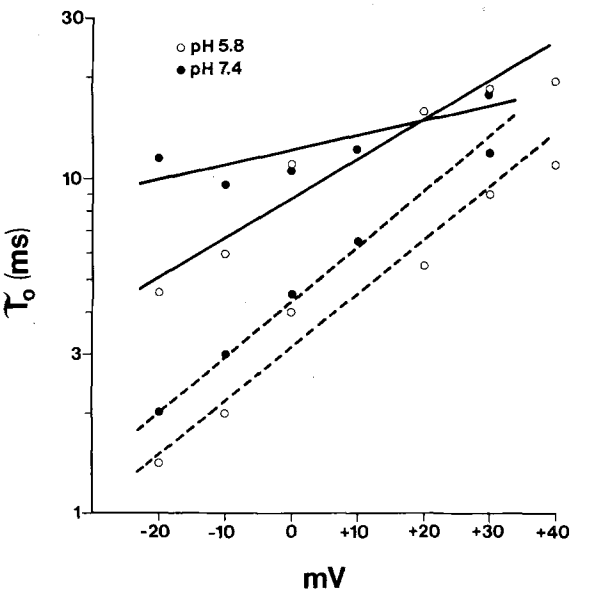

Fig. 7. The effect of voltage on mean open time in excised patches in 5 μM Ca^{2+}. Mean open times at pH 7.4 were fitted by two open distributions. Mean open times at pH 5.8 also displayed two open times both of which increased with positive voltages applied at the intracellular face of the patch

Discussion

The Ca^{2+}-activated K^+ channel is a ligand gated channel, which opens in response to increases in intracellular Ca^{2+} or to depolarizing voltages. Recently, it has been shown that factors, such as intracellular Mg^{2+}, can modulate the Ca^{2+} and voltage gating of these channels. Specifically, Mg^{2+} does not have a direct action on channel gating. Instead, it increases the apparent Hill coefficient for Ca^{2+} in skeletal muscle (Golowasch, Kirkwood & Miller, 1986). The net effect of Mg^{2+} is to increase channel opening probability at a given intracellular Ca^{2+} concentration.

Direct chemical modification of the Ca^{2+} site on the gating mechanism also affects opening of the Ca^{2+}-activated K^+ channels. For example, Pallotta (1985) found that the opening probability of Ca^{2+}-activated K^+ channels is reduced dramatically following treatment with N-bromoacetamide (NBA), a protein modifying agent. This modification of channel gating reduces the Ca^{2+} affinity of channel opening and can be prevented by increasing intracellular Ca^{2+} (Cornejo, Guggino & Guggino, 1987). The Ca^{2+} sensitivity and hence, the activity of the chan-

nel in response to increases in Ca^{2+} concentration would be reduced if similar modifications were to occur in vivo caused perhaps by naturally occurring factors, which directly affect channel gating. Indeed, the literature suggests that the Ca^{2+} affinity of Ca^{2+}-activated K^+ channels may be tissue specific (Blatz & Magleby, 1987) and may also change during development (Blair & Dionne, 1985).

A decrease in affinity for Ca^{2+} induced by acid pH is yet another mechanism by which the opening probability is modified by intracellular factors. In acute acidosis, the peritubular pH of the blood is lowered causing a drop in the intracellular pH. Such a decrease in intracellular pH would then cause a direct decrease in activity of Ca^{2+}-activated K^+ channels. We have found that acid pH causes a reduction of opening probability of the Ca^{2+}-activated K^+ channel, an effect which seems to be at the Ca^{2+}-binding site. H^+ binding to the channel decreases opening probability primarily by increasing mean closed times. The effect of acid pH appears to be competitive for Ca^{2+}, since protons decrease the apparent Ca^{2+} affinity and Hill coefficient. One possibility is that H^+ binds to the closed state, decreasing Ca^{2+} binding and stabilizing the closed state. Such a decrease in apparent binding affinity would significantly decrease the probability of opening of this channel such that even after an increase in intracellular Ca^{2+}, induced for example by Ca^{2+}-mobilizing hormones, Ca^{2+}-activated K^+ channels would not contribute to K^+ conductance.

Another possibility for the mechanisms by which acid pH could affect the activity of this channel is via an allosteric modulatory site such as the Mg^{2+}-binding site. Thus, the effect of protons may not be to interfere with Ca^{2+} binding directly, instead the protons may operate at a site, which regulates the Ca^{2+} affinity of the gating process. However, this possibility remains to be tested in detail.

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