

Proton Modulation of a Ca^{2+} -activated K^+ Channel from Rat Skeletal Muscle Incorporated into Planar Bilayers

CLAUDIO LAURIDO, SEBASTIAN CANDIA, DANIEL WOLFF, and RAMÓN LATORRE

From the Departamento de Química, Facultad de Ciencia, Universidad de Santiago, Santiago, Chile; Departamento de Biología, Facultad de Ciencias, Universidad de Chile, Santiago, Chile; and Centro de Estudios Científicos de Santiago, Santiago, Chile

ABSTRACT The effect of pH on the activation of a Ca-activated K^+ [K(Ca)] channel from rat skeletal muscle incorporated into planar lipid bilayers was studied. Experiments were done at different intracellular Ca^{2+} and proton concentrations. Changes in pH modified channel kinetics only from the Ca-sensitive face of the channel. At constant Ca^{2+} concentration, intracellular acidification induced a decrease in the open probability (P_o) and a shift of the channel activation curves toward the right along the voltage axis. The displacement was 23.5 mV per pH unit. This displacement was due to a change in the half saturation voltage (V_o) and not to a change in channel voltage dependence. The shifts in V_o induced by protons appeared to be independent of Ca^{2+} concentration. The slope of the Hill plot of the open-closed equilibrium vs. pH was close to one, suggesting that a minimum of one proton is involved in the proton-driven channel closing reaction. The change in P_o with variations in pH was due to both a decrease in the mean open time (T_o) and an increase in the mean closed time (T_c). At constant voltage, the mean open time of the channel was a linear function of $[\text{Ca}^{2+}]$ and the mean closed time was a linear function of $1/[\text{Ca}^{2+}]^2$. Changes in the internal pH modified the slope, but not the intercept of the linear relations T_o vs. $[\text{Ca}^{2+}]$ and T_c vs. $1/[\text{Ca}^{2+}]^2$. On the basis of these results an economical kinetic model of the effect of pH on this channel is proposed. It is concluded that protons do not affect the open-closed reaction, but rather weaken Ca^{2+} binding to all the conformational states of the channel. Moreover, competitive models in which Ca^{2+} and H^+ cannot bind to the same open or closed state are inconsistent with the data.

INTRODUCTION

Biologically important reactions involve the participation of protons, and interest in proton movement through membranes was triggered by the hypothesis that proton gradients were involved in energy transduction (Mitchell, 1961; Williams, 1988). Many membrane processes like carrier- and channel-mediated transport have been

Address reprint requests to Dr. Daniel Wolff, Departamento de Biología, Facultad de Ciencias, Casilla 653, Universidad de Chile, Santiago, Chile.

found to be modulated by pH. For example, changes in pH can produce important changes in the ionization state of chemical groups associated either with binding sites within the ion conduction machinery (Hille, 1968; Begenisich and Danko, 1983) or with those involved in channel gating (Brodwick and Eaton, 1978; Nonner et al., 1980; Hanke and Miller, 1983).

A case of physiological interest is that in muscle, sustained activity can decrease the internal pH by about one unit (Pan et al., 1988; Renaud, 1989), and it is tempting to suggest that the activity of K(Ca) channels in muscle cells is modulated by protons under physiological conditions. In the cell, K(Ca) channels would be upregulated by Ca and downregulated by protons.

This study is concerned with the modification of the gating kinetics of a Ca^{2+} -activated K^+ , K(Ca) channel by changes in the intracellular pH. It has been previously shown, using K(Ca) channels from β cells, epithelia, and smooth muscle cells, that a lowering of the internal pH induced a shift of the channel opening probability vs. voltage curve toward more depolarizing voltages (Cook et al., 1984; Christensen and Zeuthen, 1987; Kume et al., 1990). In other words, intracellular acidification at a fixed Ca^{2+} concentration and voltage lowers the channel opening probability. According to Christensen and Zeuthen (1987), this effect is mainly due to an increase in the channel closed time. On the other hand, Kume et al. (1990) found that the open time distribution was always shorter at higher proton concentrations, suggesting that intracellular pH shortens the open state of the channel. The tentative interpretation of these results was that protons can compete with the Ca^{2+} -binding sites, preventing channel opening. We have studied the effect of intracellular pH on the steady-state and kinetic characteristics of a K(Ca) channel from skeletal muscle membranes incorporated into planar lipid bilayers. Our data are consistent with a kinetic model in which protons exert their action "allosterically" and not by competition with Ca^{2+} at the Ca^{2+} -binding sites. Furthermore, the data also show that protons have a large effect on the binding reactions, but not on the closed-open conformational change.

Part of this work has been presented in abstract form elsewhere (Laurido et al., 1990).

MATERIALS AND METHODS

Membrane Preparation

Plasma membrane vesicles from adult rat skeletal muscle were prepared using a simplified version of the method of Roseblatt et al. (1981) (Moczydlowski and Latorre, 1983a). This preparation has been characterized as transverse tubule membrane with contaminant amounts of surface sarcolemma. Membrane suspensions in 0.3 M sucrose were frozen with dry ice and stored in small samples (~ 50 – $100 \mu\text{l}$) at -80°C .

Planar Bilayers and Channel Incorporation

Bilayers were formed by applying a drop of lipid solution in a hole with a diameter of $\sim 300 \mu\text{m}$ made in a Teflon film of $25 \mu\text{m}$ separating two aqueous solutions. The phospholipid solution was brain phosphatidylethanolamine (Avanti Polar Lipids, Inc., Birmingham, AL) in decane (12.5 mg/ml). Bilayer resistances were always $>50 \text{ G}\Omega$ and had capacitances in the range of

150–300 pF. Planar bilayers were initially formed in aqueous solutions containing 300 mM KCl and 20 mM MOPS-KOH, pH 7. Channel insertion occurs spontaneously after the addition of the plasma membrane vesicles to a final concentration of $\sim 5 \mu\text{g/ml}$. Appearance of single K(Ca) channels was detected as rapid current fluctuations when a constant voltage (30 mV) was applied across the bilayer. As a general rule, channels incorporate into the bilayer with their cytoplasmic side facing the compartment to which the vesicles were added; therefore, the Ca²⁺-binding sites are exposed to this compartment. However, in those few exceptions in which the orientation of the channel was not the usual, this was easily determined by the polarity of their voltage dependence (K(Ca) channels open with depolarizing potentials) or by the absence of activation by Ca²⁺ added to that compartment.

pH Experiments

When pH was the variable to be studied, experiments were started with channel incorporation under symmetrical conditions of salt (300 mM KCl) and Ca²⁺ (usually 20 μM) at pH 7.¹ Current records were taken at different potentials and those lasting at least 60 s were stored on digital tape (see below). This procedure was then repeated at different internal Ca²⁺ concentrations. The internal side was then perfused with a solution containing 300 mM KCl, 30 μM Ca²⁺, and a different pH, and the same protocol described previously was followed. As described by Moczydlowski and Latorre (1983b) and by Oberhauser et al. (1988), there is a variation from channel to channel in the Ca²⁺ concentration dependence. At constant applied voltage, spontaneous shifts in the fraction of time that the channel remains open (P_o) are sometimes observed. Due to these problems, steady-state and gating characteristics of K(Ca) channel at different pH's can only be compared using the same single-channel membrane and channels with stable P_o . This enormously reduced the range of pH that can be studied since most membranes broke after testing two different pH's. In more than 100 trials we were only able to test the effect of three different pH's and several Ca²⁺ concentrations in only two single-channel membranes. In 14 single-channel membranes we were able to test two different pH's and several [Ca²⁺]'s.

Electrical Measurements and Data Analysis

The voltage clamp circuit has been described in detail (Alvarez, 1986). The current across the bilayer was measured with a low-noise current-to-voltage converter, filtered at 1 KHz with an eight-pole Bessel low-pass active filter, amplified, and recorded continuously on an FM tape recorder for later analysis. The electrophysiological convention is used, in which the external side of the channel is defined as zero voltage. All experiments were carried out at $22 \pm 2^\circ\text{C}$.

The fraction of time open was measured as a function of voltage and Ca²⁺ concentration at different pH's. For single-channel membranes, the time averaged probability of the open state

¹ Intrinsic to all these experiments is the assumption that the amphoteric lipid PE is neutral at the pH's studied. At pH 7, PE monolayers contain $\sim 3\%$ negative charge (-0.03 e/nm^2). For the present study this amount of charge is negligible. In the pH range from 7 to 5 the PE surface charge becomes slightly positive, but the amount of charge induced by the pH lowering is still of no consequence for the interpretation of the present results ($\sim 3\%$ positive charge at pH 5). However, at pH 8, PE monolayers contain $\sim 17\%$ negative charge or the equivalent to a surface potential of 21 mV at 300 mM KCl. These changes in surface charge density with pH parallel those observed in PE bilayers (McLaughlin et al., 1970). We argue that even this appreciable surface potential does not affect the present study. First, we have shown (Moczydlowski et al., 1985) that Ca²⁺-activation sites sense only a fraction equivalent to the electrostatic potential at a distance of 0.9 nm from the bilayer surface ($< 7 \text{ mV}$). Second, the shift in the voltage activation curve per pH unit is independent of whether the medium is acidified or made alkaline.

was obtained from digitized records (200 μs /point) as the time spent in the open-current level divided by the total time of the record, usually 60 s. Open and closed events were identified by using a discriminator located at 50% of the open channel current. The durations of the open state (above threshold) and on the closed state (below threshold) were corrected taking into account the dead time of the system as described by Colquhoun and Sigworth (1983). The kinetic events of short lifetime were limited to times ≥ 1 ms; thus, events < 1 ms were included as part of the preceding state of the channel. As discussed by Moczydlowski and Latorre (1983b), this procedure has the effect of increasing mean open and closed times. The K(Ca) channel also shows quiescent periods sometimes lasting several seconds. These kinetic events have been interpreted on the basis of a voltage-dependent Ca blockade (Vergara and Latorre, 1983). For this reason, closed events lasting more than 500 ms were also excluded from the kinetic analysis. Dwell-time histograms were constructed using the method described by Sigworth and Sine (1987). Mean open and closed times were obtained by a maximum likelihood method and corrected taking into account the missing events as described by Blatz and Magleby (1986) for a two-state model. For the present purposes more complicated models need not be used, since by eliminating events of short duration, distributions of dwell times can be fitted with single-exponential relaxations.

RESULTS

Fig. 1 shows representative 10-s segments of records of single-channel currents that illustrate the effect of internal pH at fixed voltage and Ca^{2+} concentration. It can be seen that P_o decreases as the internal pH was lowered. A change in pH from 8 to 6 produced a change in P_o from 0.95 to 0.10. This change in P_o is reversible upon returning to the original pH. Conductance is only slightly affected in this pH range. At pH 6 channel conductance was $\sim 30\%$ lower than at pH 8. We often found that raising the pH_i above 9 or decreasing it below 5 produces an irreversible loss of channel activity. This restricts the pH working range to about 4 pH units (9 to 5). All the effects of pH described here can be brought about by changing the pH only on the internal side of the channel, which is the side where the Ca^{2+} -binding sites are located.

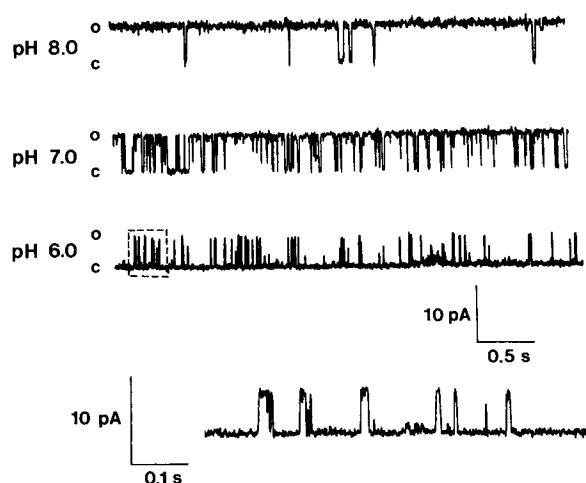


FIGURE 1. Effect of pH on K(Ca) channel activity. Acidification of the intracellular side decreases open-state probability (P_o). At pH 8.0, $P_o = 0.95$; at pH 7.0, $P_o = 0.60$; and at pH 6.0, $P_o = 0.10$. Values of P_o were calculated from records lasting for at least 60 s. Note that the conductance is only slightly modified in this pH range. Symmetrical 300 mM KCl, 20 mM MOPS-KOH. Internal Ca^{2+} concentration was 100 μM and membrane potential was 30 mV.

Equilibrium Behavior

Channel open probability is a sigmoidal function of membrane potential; increasing Ca²⁺ concentration in the internal compartment led to a shift to the left of the P_o vs. voltage curves (Latorre et al., 1982; Methfessel and Boheim, 1982; Wong et al., 1982). Fig. 2 shows P_o as a function of potential at three different pH's and at 100 μ M internal Ca²⁺. Acidification of the internal side shifted the voltage activation curves to the right along the voltage axis. At each pH value the P_o vs. voltage data are well described by a function of the type (Ehrenstein et al., 1970):

$$P_o(V) = \{1 + \exp[-nF(V - V_o)]\}^{-1} \quad (1)$$

where n is a constant that reflects the channel voltage dependence and V_o is the half saturation voltage. In terms of Eq. 1, Fig. 2 shows that lowering the pH_i progressively shifts V_o toward more positive values. On the other hand, the three curves can be fitted to Eq. 1 reasonably well with $n = 1.7$, a result that indicates that pH does not modify the channel voltage dependence. A summary of results at different pH's and

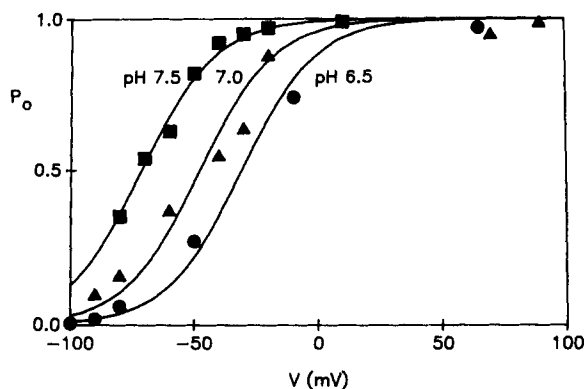


FIGURE 2. P_o as a function of membrane voltage at three different pH's and at 100 μ M Ca²⁺. The solid lines correspond to a fitting of a Boltzmann distribution to the experimental data. V_o values shifted to the right from -72.6 mV at pH 7.5 to -50 mV at pH 7.0 and to -30 mV at pH 6.5. Other experimental conditions as in Fig. 1.

Ca²⁺ concentrations is given in Table I. It shows that proton inhibition of channel activity resides exclusively in the shift in V_o , which amounts to 23.5 mV per pH unit (range, 14.3–42.6 mV). The shift in V_o induced by protons appears not to be dependent on Ca²⁺ concentration. Thus, for the series of experiments labeled I in Table I, about the same shift in V_o was obtained when the pH was changed from 7 to 5 whether this change was done in the presence of 300 or 100 μ M Ca²⁺. The finding that changes in $[H^+]$ do not promote changes in the Hill coefficient for Ca²⁺ activation curve, but increase the apparent dissociation constant for the divalent cation, allow us to discard a pure competitive inhibition in which protons and Ca²⁺ compete exclusively for the same binding sites. For this case, Segel (1975) has pointed out that in the case of a system displaying cooperative substrate binding in the absence of an inhibitor, the latter should in general increase the sigmoidicity of the activation curve.

Fig. 3 shows the effect of pH on the Ca²⁺ activation curves at a constant voltage of 30 mV. It can be observed that intracellular acidification shifts the curves to the right. The solid lines in Fig. 3 represent a fit to the data of Eq. 14. This expression gives the

TABLE I
Effect of pH on the Equilibrium Parameters V_o and n

Group	pH	[Ca] _i μM	V_o mV	n
I	7	100	-2.4	2.8
I	7	300	-24.0	2.5
I	5	100	43.5	1.9
I	5	300	23.0	1.8
II	7.5	25	-1.2	2.2
II	6	25	34.0	2.0
III	7.5	100	-7.7	2.5
III	6	100	14.0	2.5
IV	7.5	50	28.4	2.3
IV	6	50	32.4	2.3
V	7.5	20	6.6	2.1
V	5.5	20	22.0	2.1
VI	7.5	100	-72.6	1.7
VI	7.0	100	-50.0	1.7
VI	6.5	100	-30.0	1.7

Each group represents data obtained in the same channel.

expected $[Ca^{2+}]$ dependence of the P_o obtained from the kinetic scheme R1 described below.

The apparent number of Ca ions involved in the channel activation can be obtained by fitting the usual Hill expression for P_o to the experimental points.

$$P_o = [Ca^{2+}]^N / (K + [Ca^{2+}]^N) \quad (2)$$

where N is the Hill coefficient, which represents the lower limit of the number of Ca^{2+} sites involved in channel activation.

The best fit to the experimental points was obtained at $N = 2.02$, $K = 30 \mu M$ at pH 8.0, $N = 2.11$, $K = 49 \mu M$ at pH 7.0, and $N = 2.10$, $K = 54 \mu M$ at pH 6.0. Hill coefficients ranging from 1.2 to 2.4 have been found when Ca^{2+} is used as a channel activator (Moczydlowski and Latorre, 1983b; Golowash et al., 1986; Oberhauser et al., 1988). Our results show that N is not significantly modified by pH in the range from

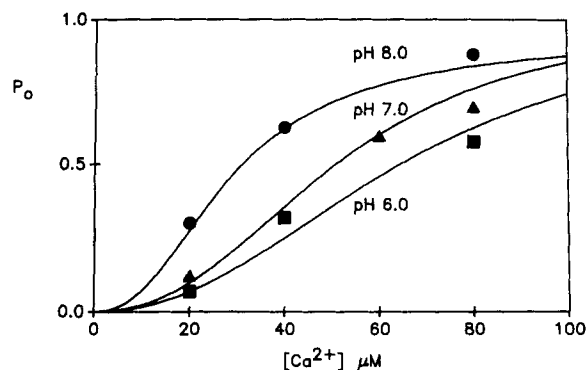


FIGURE 3. P_o as a function of Ca concentration at three different pH's (6.0, 7.0, and 8.0) and at a constant voltage of 30 mV. Symbols represent the experimental data. The solid lines represent the theoretical values obtained from Eq. 14. The values for K_{obs}^o , K_{obs}^c , α , and β were taken from Table II, channel 1.

6 to 8. Therefore, changes in the proton concentration do not promote changes in the number of Ca ions participating in the channel opening.

More information about the mechanisms of interaction of protons with the K(Ca) channel can be obtained by examining the pH dependence of the apparent equilibrium constant for proton dissociation. Fig. 4 shows Hill plots of the open-closed equilibrium vs. pH at different potentials in four different channels. The average slope is 0.90 ± 0.18 . This result suggests that a minimum of one proton is involved in the channel opening reaction.

Effect of pH, Voltage, and Ca on Gating Kinetics

Fig. 5, A and B, shows single-channel dwell time histograms for the closed and open states, respectively, at pH 7. In these histograms the abscissa is logarithmic and the ordinate is the square root of the number of events. Superimposed on the histograms are the fitted probability density functions (pdf's) obtained with a maximum

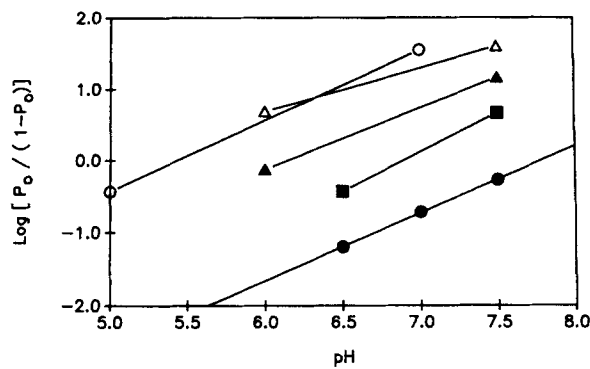


FIGURE 4. Hill plot of open-closed equilibrium vs. proton concentration. Experimental values were obtained from Fig. 2 at -50 mV (filled squares) and -80 mV (filled circles). Points at -80 mV were fitted to a straight line. The remaining points were obtained from the experiments shown in Table 1 at $+30$ mV (open circles: group I, $100 \mu\text{M Ca}^{2+}$; filled triangles: group II, $25 \mu\text{M Ca}^{2+}$; open

triangles: group III, $100 \mu\text{M Ca}^{2+}$). The average slope of the five curves was 0.90 ± 0.18 (mean \pm SD).

likelihood fitting (Sigworth and Sine, 1987). In Fig. 5, C and D, are dwell time histograms and fitted pdf's for the closed and open states, respectively, shown at pH 5. We find that by excluding < 1 -ms events the populations of closed and open dwell times are well described by single exponential functions (see Moczydlowski and Latorre, 1983b, for a discussion on this point). This type of representation of the dwell times was used to calculate closed and open mean times under the different experimental conditions used in the present work. Fig. 5 shows that a decrease in internal pH induces a decrease in the mean channel open time and an increase in the mean closed time. In Fig. 6 we see that both the mean open and closed times are pH and voltage dependent, noting that at the pH's studied mean closed and mean open times vary exponentially with voltage. In this particular experiment the most marked effect of pH is the influence on the mean open time, which decreases by a factor of ~ 10 . On the other hand, mean closed time increases about threefold. Fig. 6 shows that pH does not significantly affect the voltage dependence of the apparent opening

and closing rate constants, which is an additional confirmation that pH does not affect the voltage dependence of the channel. Note also that the changes in the kinetic parameters promoted by pH are essentially independent of the Ca concentration (cf. Fig. 6, *A* and *B*).

Fig. 7, *A* and *B*, shows the Ca^{2+} concentration dependence of the mean open and closed times, respectively, for a single channel studied at three different pH's and an

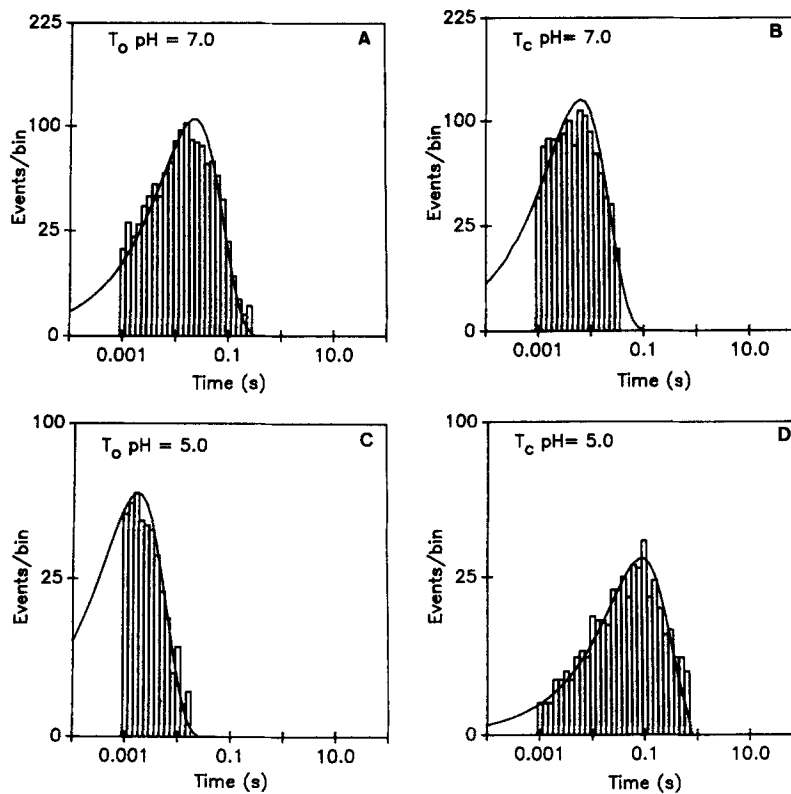


FIGURE 5. Dwell time distribution histograms. Histograms and fitted probability density functions for open and closed dwell times at two different pH's. (*A*) Open time distribution at pH 7.0 from 1,211 events. Data are well fitted by a single component with a time constant (T_o) of 35 ms. (*B*) Closed time histogram at pH 7.0 from 1,155 events. Time constant (T_c) was 8 ms. (*C*) Open time distribution at pH 5.0 from 384 events. T_o was 2 ms. (*D*) Closed time histogram at pH 5.0 from 364 events. T_c was 98 ms.

applied voltage of 30 mV. In Fig. 7 *A* the mean open times (T_o) are plotted vs. Ca^{2+} concentration as in Moczydlowski and Latorre (1983*b*), but the mean closed times (T_c) are plotted vs. reciprocal squared Ca^{2+} concentration instead of $1/[\text{Ca}^{2+}]$. The results show that at any given pH at fixed voltage, T_o is a linear function of $[\text{Ca}^{2+}]$ and T_c is a linear function of $1/[\text{Ca}^{2+}]^2$. If one does not correct for missing events, as shown presently, plots of T_c vs. $1/[\text{Ca}^{2+}]$ very similar to those reported by Moczydlowski and

Latorre (1983b) are obtained (for more detail, see Discussion). Fig. 7, A and B, also shows that both mean open and closed times are strongly pH dependent. However, it is important to notice that protons affect the slope of the straight lines obtained with this particular manner of plotting the data, but at very low (mean open times) or very high (mean closed times) $[\text{Ca}^{2+}]$, T_o and T_c converged to a unique value independent of pH. This was an important result since it allowed us to identify an economical kinetic scheme compatible with the data. Fig. 7, C and D, shows the kinetic behavior of a different single channel at pH 7 and pH 5. It is clear that as in the results shown in Fig. 7, A and B, only the slopes of the straight line that fit the T_o vs. $[\text{Ca}^{2+}]$ and T_c vs. $1/[\text{Ca}^{2+}]^2$ data are pH sensitive. However, this particular channel showed a large probability of opening at much lower $[\text{Ca}^{2+}]$. These results show the usual spread in Ca^{2+} activation for different channels which can be as much as 0.8 P_o units at the same voltage and $[\text{Ca}^{2+}]$ (Moczydlowski and Latorre, 1983b).

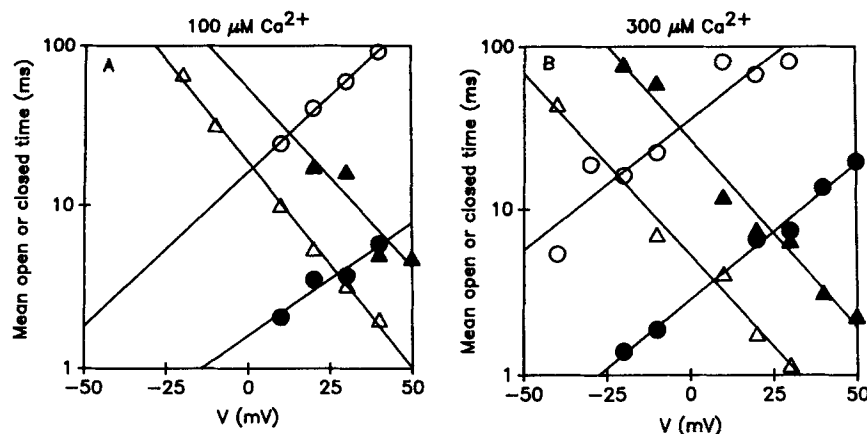
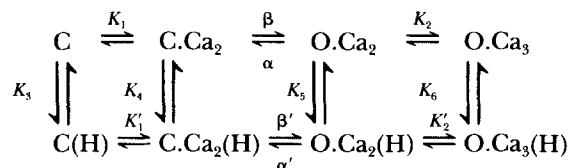


FIGURE 6. Effect of voltage at two different pH's on the mean open and closed times. Filled circles and triangles represent the mean open and closed times at pH 5.0, respectively. Open circles and triangles represent the mean open and closed times at pH 7.0, respectively. Solid lines correspond to a linear regression of the experimental data. (A) Data obtained at $100 \mu\text{M Ca}^{2+}$. (B) Data obtained at $300 \mu\text{M Ca}^{2+}$. Other experimental conditions as in Fig. 1.

Identification of an Economical Kinetic Scheme Able to Explain Proton Inhibition

There are several lines of evidence indicating that the gating kinetics of K(Ca) channels are complex and involve transitions between several closed and open states (e.g., McManus et al., 1985; McManus and Magleby, 1988). In what follows we have tried to identify the most simple model able to explain the data as a way to understand the pH effect on channel activity. The model is based on the following observations: (a) The slope of Hill plots of the open-closed equilibrium vs. $[\text{Ca}^{2+}]$ ranges from 1.2 to 2.4, corroborating the data of Moczydlowski and Latorre (1983b), Golowash et al. (1986), and Oberhauser et al. (1988) obtained in the same preparation. Values larger than 2 are often found and this result can only be explained if we

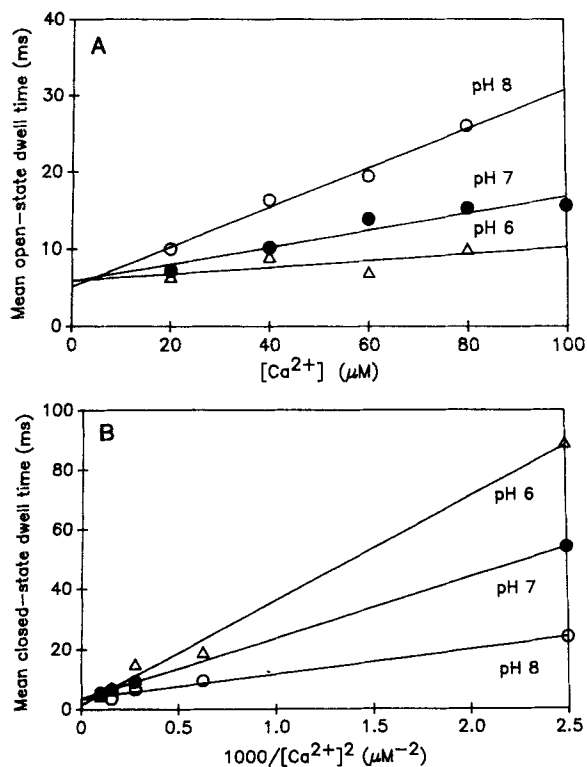
assume the binding of a minimum of three Ca ions for complete activation. (b) The T_o vs. $[Ca^{2+}]$ and T_c vs. $1/[Ca^{2+}]^2$ plots are linear. (c) The slope of Hill plots of the open-closed equilibrium vs. pH is very close to unity, suggesting that only one proton is involved in the proton-induced channel closing. The most economical scheme with only a single protonation follows:



Scheme R1

where C and CH refer to unprotonated and protonated states of the closed channel, and O and OH are the unprotonated and protonated forms of the open channel state. In Scheme R1 all closed states have zero conductance and all open states have the same conductance.

This model therefore assumes a single site able to bind protons independent of the amount of Ca^{2+} bound to the channel. Equilibrium constants, K_i and K'_i ($i = 1, 2$) describe Ca^{2+} binding reactions. The equilibria between protonated and unprotonated



nated open states are described by dissociation constants K_j ($j = 3, 4, 5, 6$). Assuming that the closed–open reaction is slow compared with the Ca²⁺ binding reactions, the kinetic scheme (Scheme R1) predicts the following relations for the mean open and closed dwell times:

$$1/T_o = \alpha P_{OCa_2} + \alpha' P_{HOCa_2} \quad (3)$$

$$1/T_c = \beta P_{CCa_2} + \beta' P_{HCCa_2} \quad (4)$$

where the fractional probabilities, P , are the probabilities of finding the channel in the indicated closed or open state conditioned of being in some of the closed or open states shown in Scheme R1. The problem is now reduced to solve the P 's as a function of $[H^+]$ and $[Ca^{2+}]$. Focusing first on open times, it is easy to show that

$$P_{OCa_2} = 1 / \{1 + [H]/K_5 + ([Ca]/K_2)(1 + [H]/K_6)\} \quad (5)$$

and

$$P_{HOCa_2} = ([H]/K_5) / \{1 + [H]/K_5 + ([Ca]/K_2)(1 + [H]/K_6)\} \quad (6)$$

From Eqs. 3, 5, and 6 we have

$$1/T_o = (\alpha + \alpha'[H]/K_5) / \{1 + [H]/K_5 + ([Ca]/K_2)(1 + [H]/K_6)\} \quad (7)$$

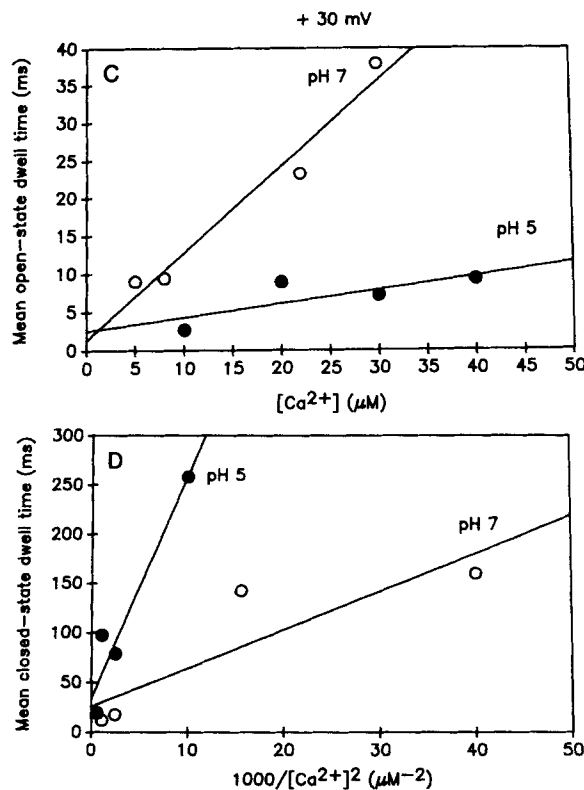


FIGURE 7. Calcium concentration dependence of mean open and closed times at three different pH's. (A, C) Relationship between the mean open-state dwell time and $[Ca^{2+}]$. (B, D) Relationship between the mean closed-state dwell time and $1/[Ca^{2+}]^2$. In A and B, open circles represent data at pH 8.0, filled circles at pH 7.0, and triangles at pH 6.0. In C and D, filled circles are data taken at pH 5 and open circles at pH 7. Applied voltage: 30 mV. Mean open and closed time values were obtained from the dwell time histograms shown in Fig. 4 corrected for missing events.

Since the intercepts of the straight lines described by the T_o vs. $[Ca^{2+}]$ data (Fig. 7, *A* and *B*) do not depend on pH, we have either (*a*) $\alpha = \alpha'$ or (*b*) $\alpha \gg \alpha'[H]/K_5$. From both alternatives, only possibility *a* satisfies the experimental results, since alternative *b* demands that T_o should increase with $[H^+]$ (Eq. 7). Therefore,

$$T_o = (1/\alpha)\{1 + ([Ca]/K_2)(1 + [H]/K_6)/(1 + [H]/K_5)\} \quad (8)$$

Defining

$$K_{obs}^o \equiv K_2(1 + [H]/K_3)/(1 + [H]/K_6) \quad (9)$$

Eq. 8 becomes

$$T_o = (1/\alpha)(1 + [Ca]/K_{obs}^o) \quad (10)$$

Eq. 10, as found experimentally (Fig. 7, *A* and *C*), describes a straight line with a proton concentration-independent intercept $1/\alpha$, and a slope $1/\alpha K_{obs}^o$, which is a function of $[H^+]$ as predicted by Eq. 9. In other words the *observed* dissociation constant for Ca^{2+} varies with $[H^+]$. Table I shows that α remains constant and K_{obs}^o increases as the pH becomes smaller. According to Eq. 9 these results imply that $K_6 > K_5$ and therefore the state $O Ca_2$ binds protons more tightly than $O Ca_3$. On the other hand, microscopic reversibility demands that $K_2 K_6 = K'_2 K_5$, which implies that $K'_2 > K_2$; i.e., Ca^{2+} binds more tightly to unprotonated open states. Unfortunately, the range of pH we have been able to study is not extensive enough to actually calculate the different K 's in Scheme R1. However, an order of magnitude estimation of the dissociation constants can be obtained by a fit to the K_{obs}^o data using Eq. 9 (Fig. 8*A*). The best fit is obtained with $K_5 = 3.0 \times 10^{-8}$ M, $K_6 = 2.6 \times 10^{-7}$ M, and $K_2 = 1.5 \times 10^{-5}$ M. Using this value of K_2 and the microscopic reversibility relationship, we have $K'_2 = 1.28 \times 10^{-4}$ M.

A similar analysis can be done for the closed states. From Eq. 4 and the explicit relations between P_{CCa_2} and P_{HCCa_2} and the dissociation constants, $[H^+]$, and $[Ca^{2+}]$ determined by Scheme R1, the mean closed time is given by

$$T_c = \{1 + [H]/K_4 + (K_1/[Ca]^2)(1 + [H]/K_3)\}/(\beta + \beta'[H]/K_4) \quad (11)$$

Since the intercept of the straight line that defines the relation between the T_c vs. $1/[Ca^{2+}]^2$ (Fig. 7, *B* and *D*) data is pH independent, $\beta = \beta'$ and Eq. 11 becomes

$$T_c = (1/\beta)(1 + K_{obs}^c/[Ca^{2+}]^2) \quad (12)$$

where

$$K_{obs}^c = K_1(1 + [H]/K_3)/(1 + [H]/K_4) \quad (13)$$

The data shown in Table II indicate that $K_4/K_3 \geq 4$ and suggest that the open channel has a larger change in Ca^{2+} binding between protonated and unprotonated states than does the closed channel. An attempt to fit the K_{obs}^c vs. $[H^+]$ data with Eq. 13 is shown in Fig. 8*B*.

From Eqs. 10 and 12, an expression for the Ca^{2+} dependence of the channel open probability can be obtained:

$$P_o = (K_{obs}^o[Ca^{2+}]^2 + [Ca^{2+}]^3)/\{K_{obs}^o[Ca^{2+}] + [Ca^{2+}]^3 + (\alpha/\beta)K_{obs}^o[Ca^{2+}]^2 + (\alpha/\beta)K_{obs}^oK_{obs}^c\} \quad (14)$$

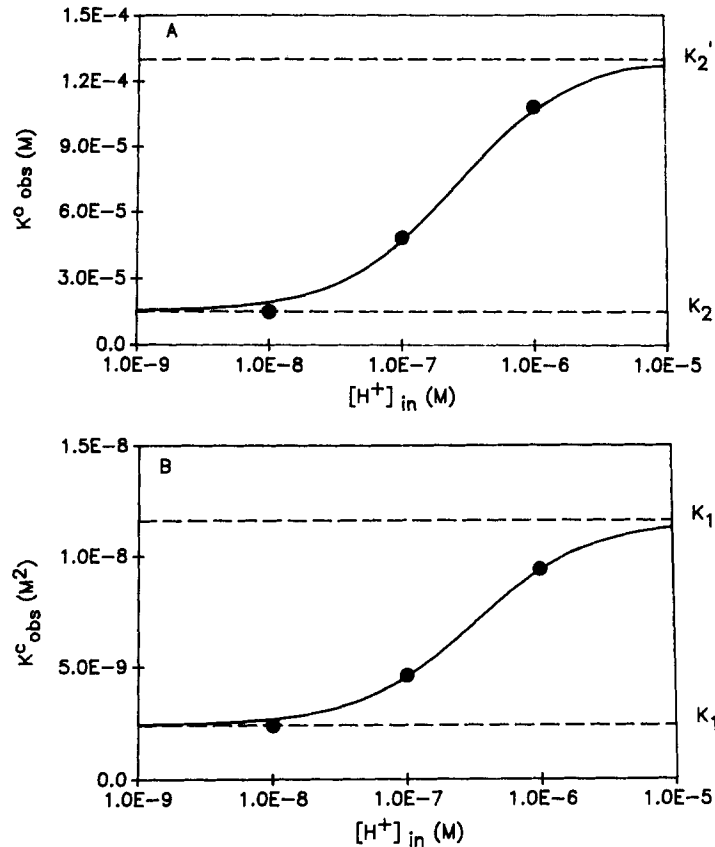


FIGURE 8. "Observed" dissociation constants as a function of proton concentration for the open (A) and closed (B) states of the channel. Points represent the average of the observed dissociation constants, at three pH's, obtained from Fig. 7, A and B. Solid lines represent the fitting of Eqs. 9 and 13 to the observed dissociation constants (Table II). In A, the best fit was obtained with $K_2 = 1.5 \times 10^{-5}$ M, $K_3 = 3.0 \times 10^{-8}$ M, and $K_6 = 2.6 \times 10^{-7}$ M. In B, the best fit was obtained with $K_1 = 2.4 \times 10^{-9}$ M², $K_3 = 6.8 \times 10^{-8}$ M, and $K_4 = 3.3 \times 10^{-7}$ M. For more details, see text.

TABLE II
Values of Dissociation Ca Constants and Open-Closed Transition Constants of the Channel

pH	Open state		Closed state	
	α^{-1}	$K_{\text{obs}}^{\text{o}}$	β^{-1}	$K_{\text{obs}}^{\text{c}}$
Channel 1	ms	μM	ms	nM^2
8	4 ± 1	15.0 ± 2	3.5 ± 0.1	2.4 ± 0.7
7	5 ± 1	48.3 ± 11	3.5 ± 0.1	4.6 ± 1.4
6	5 ± 1	108 ± 24	3.0 ± 1.0	9.4 ± 0.4
Channel 2				
7	1.5 ± 0.5	1.2 ± 0.4	26 ± 4.0	0.10 ± 0.02
5	2.5 ± 0.4	7.3 ± 1.5	34 ± 5.0	0.75 ± 0.10

Data from Fig. 7. Values are the mean \pm SD.

Kinetic Data Allow Us to Reject Simple Competitive Models

It is important to note that a competitive model in which both H^+ and the third Ca^{2+} cannot bind to the open state does not predict the linear plots of Fig. 7, *A* and *C*. In this case it is expected that the intercept and not the slope of the straight lines in Fig. 7, *A* and *C*, should be $[H^+]$ dependent. Obviously the data also violate a competitive model in which H^+ can bind exclusively to the totally Ca-free state (*C*) since this would make H^+ close the channel (as observed), but only by lengthening the closed state; the open state would be pH insensitive, which is not the case (see Figs. 6 *A* and 7 *A*). Furthermore, the plot K_{obs}^c vs. $[H^+]$ for the open state is *sublinear*, which rules out a purely competitive model in which a proton or two Ca^{2+} 's cannot bind simultaneously to the Ca-free closed state. Competitive inhibition predicts that K_{obs}^c should vary linearly with the concentration of the inhibitor. Further, the K_{obs}^o vs. $[H^+]$ curve is also *sublinear*. This is an independent confirmation that a competitive model in which both H^+ and a third Ca^{2+} cannot bind to the open state. In this case, K_{obs}^o should be independent of $[H^+]$ concentration. In summary, the data are incompatible with the most simple competitive models for proton action.

DISCUSSION

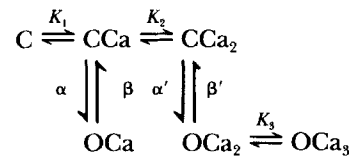
Maxi K⁺ Channels and Proton Inhibition

Cook et al. (1984), working with pancreatic β cells, were the first to observe the effect of internal pH on the activity of Ca^{2+} -activated K^+ channel of large unitary conductance. They reported a much larger shift toward the right in the potential required for half-maximal channel activation (V_o) upon acidification than the one found here (61 mV per pH unit). More in agreement with our results, Christensen and Zeuthen (1987), working in the epithelium of choroid plexus, reported an average shift in V_o of ~ 33 mV per pH unit. Recently, Kume et al. (1990) reported the effect of intracellular pH on a $K(Ca)$ channel from rabbit tracheal smooth muscle. This channel is particularly sensitive to Ca^{2+} and pH, and from their data we have estimated a displacement in V_o of ~ 80 mV when pH is changed from 6.8 to 6 at an internal $[Ca^{2+}]$ of 10 μM . However, to our knowledge no explicit kinetic model for proton action on this channel has been put forward in the literature. At the most, it has been suggested that protons compete with Ca^{2+} for the Ca-binding sites and that " H^+ produces an increase in the closed times by preventing Ca^{2+} from binding to the channel site" (Christensen and Zeuthen, 1987). Kume et al. (1990), on the other hand, concluded that acidification induces a reduction of the channel Ca^{2+} sensitivity and a shortening in the mean open time. Competition between protons and Ca^{2+} for binding sites is also suggested in the work of Cook et al. (1984). Furthermore, previous studies did not explore in detail the effect of $[Ca^{2+}]$ on the inhibitory action of protons in both steady-state and kinetic channel parameters. Our view of the proton effect on channel activity departs from previous ones, and it is our contention that protons exert their effect in a site quite different from that of Ca ions.

Gating Mechanisms for the Ca²⁺-activated Potassium Channel and Proton Inhibition

Observing the durations of open states adjacent to specified closed states, McManus et al. (1985) concluded that brief openings are making connection with long closings,

and that open states of long duration are adjacent to short closings. These findings exclude linear models for the K(Ca) channel in which there is only one pathway that leads to the open state (e.g., a model of the type C-C-O-O). On the other hand, McManus and Magleby (1985, 1988) reported that the distribution of the closed intervals is well described by the sum at least size five exponentials and described the distribution of open dwell times as the sum of three exponentials. A simplified kinetic scheme for the K(Ca) channel is given below (Magleby and Pallota, 1983).



Scheme R2

The mean average time in state OCa is 0.41 ms (Magleby and Pallota, 1983). They correspond to brief apertures, most of which are lost if the cut-off time is set to ≥ 1 ms. The state CCa has a much longer mean duration than CCa₂, and the set of states CCa and CCa₂ would be seen in our measurements as a single closed state. Therefore, assuming that the kinetic mechanism that commands K(Ca) channel activity is similar in muscle cell in culture and in adult muscle, by setting a cut-off time of ≥ 1 ms we have reduced the kinetic model given by Scheme R2 to a linear model similar to that described by Moczydlowski and Latorre (1983*b*), but in which in the first reaction two Ca's are involved (Scheme R1). Therefore, our data are *not* in disagreement with those of Magleby and Pallota (1983) or McManus and Magleby (1985, 1988). Our results simply reflect a primary gating mode of the K(Ca) channel identified with a cut-off time of ≥ 1 ms. Furthermore, the observation of a single exponential for the open- and closed-state probability distributions is predicted by Scheme R1 if the Ca binding reactions are fast compared with the open-closed transition. This observation justifies the use of the simple two-state model for correcting by missing events.

Scheme R1 is proposed as the minimum model for the proton inhibition, based on the following evidence. (a) The dependence of the channel open probability on Ca²⁺ concentration indicates the involvement of more than two Ca²⁺'s in the channel opening reaction. In agreement with this conclusion, Hill coefficients up to six have been found in the presence of Mg²⁺, a divalent cation unable to activate the K(Ca) channel in the absence of Ca²⁺ (Golowash et al., 1986; Oberhauser et al., 1988). Golowash et al. (1986) have proposed that Mg²⁺ "reveals" Ca²⁺ binding sites already present in the K(Ca) channel. Furthermore, the pure reciprocal first-order Ca²⁺ concentration dependence of the mean closed time, as found by Moczydlowski and Latorre (1983*b*), is lost when single-channel current records are corrected by missing events and a 1/[Ca²⁺]² dependence is found instead. This, together with the pure first-order [Ca²⁺] dependence of the mean open time, strongly suggests the participation of at least three Ca ions in the gating process. (b) We have considered that protons and Ca²⁺ are not competing for the same site(s) simply because the kinetic data cannot be predicted from the most obvious competitive kinetic schemes and

because the shift in the activation curves promoted by pH changes appears to be independent of $[Ca^{2+}]$. This assumption has direct support in the experiment labeled I in Table I. The other experiments shown in Table I also corroborated the idea that the shift in V_o induced by pH is rather independent of $[Ca^{2+}]$. On the other hand, the kinetic model described in Scheme R1 nicely predicts the main features of proton inhibition of the K(Ca) channel.

A comparison with other Ca-binding protein is in order here. Fuchs (1974) reported that the Ca binding constant and the number of Ca sites present in troponin C remain essentially constant in the pH range of 5–8. These results suggest that Ca binding sites in this protein contain solely carboxyl groups which are completely dissociated at $pH \geq 5$. This conclusion is supported by the fact that the most common ligand at the Ca-binding sites in the vast majority of Ca-binding proteins are the peptide carbonyl groups and the carboxyl groups from aspartic and glutamic residues, both with pK 's < 4 (Einspahr and Bugg, 1978). If the Ca-binding sites in K(Ca) channels have any resemblance to those found in troponin C, it is not known at present. If this is the case, we would not expect to see competition between protons and Ca^{2+} until the pH is < 4 . (c) Changes in proton concentration affect both open and closed states. Protons only affect the slopes of the straight lines originated when plotting T_o vs. $[Ca^{2+}]$ or T_c vs. $1/[Ca^{2+}]^2$, indicating that the Ca-binding reactions are modified by pH, but that pH does not change the rate constants α and β that define the open–closed equilibrium. (d) The data suggest that only a single proton participates in the reaction and that protons do not compete with Ca^{2+} for the same sites, since the pH effect appears to be independent of $[Ca^{2+}]$. Since the apparent pK_a 's of the protonation reaction in Scheme R1 range from 6.6 to 7.5, this may be consistent with a single histidine group (pK_a 6.8).

According to Scheme R1, inhibition of the K(Ca) channel by protons is a consequence of an increase in the dissociation constants for the Ca-binding reactions with the channel. Our results also indicate that this increase is independent of the channel conformational state: both the open and the closed states can be protonated. These results suggest that proton sites are located outside the ion conduction system of the Ca^{2+} -activated K^+ channel.

Special thanks to Dr. Osvaldo Alvarez for his critical appraisal of the present work and for being there every time we needed help with kinetic models, and to Dr. David Naranjo for many stimulating discussions. Dr. Christopher Miller, with his usual wit, showed us the right approach to the kinetic scheme (Scheme R1) when we were lost.

This work was supported by the Fondo Nacional de Investigación 451-1988, National Institutes of Health grant 35981, and a grant of the Tinker Foundation. R. Latorre is the recipient of a John S. Guggenheim Fellowship. R. Latorre wishes to thank the Dreyfus Bank (Switzerland) for generous support from a private foundation that they made available to him.

Original version received 8 February 1991 and accepted version received 13 June 1991.

REFERENCES

- Alvarez, O. 1986. How to set up a bilayer system. *In* Ion Channel Reconstitution. C. Miller, editor. Plenum Publishing Corp., New York. 115–130.

- Begenisich, T., and M. Danko. 1983. Hydrogen ion block of the sodium pore in squid axons. *Journal of General Physiology*. 82:599–618.
- Blatz, A. L., and K. L. Magleby. 1986. Correcting single channel data for missed events. *Biophysical Journal*. 49:967–980.
- Brodwick, M., and D. Eaton. 1978. Sodium channel inactivation in squid axon is removed by high internal pH or tyrosine-specific reagents. *Science*. 200:1494–1495.
- Christensen, O., and T. Zeuthen. 1987. Maxi K^+ channels in leaky epithelia are regulated by intracellular Ca^{2+} , pH and membrane potential. *Plügers Archiv*. 408:249–259.
- Colquhoun, D., and F. J. Sigworth. 1983. Fitting and statistical analysis of single channel records. In *Single Channel Recording*. B. Sackmann, editor. Plenum Publishing Corp., New York. 135–175.
- Cook, D. L., M. Ikeuchi, and W. Y. Fujimoto. 1984. Lowering of pH inhibits Ca^{2+} -activated K^{2+} channels in pancreatic β cells. *Plügers Archiv*. 391:85–100.
- Ehrenstein, G., H. Lecar, and R. Nossal. 1970. The nature of the negative resistance in bimolecular lipid membranes containing excitability-inducing material. *Journal of General Physiology*. 55:119–133.
- Einspahr, H., and C. E. Bugg. 1978. Crystal structures of calcium complexes of amino acids, peptides and related models. *Proceedings of the International Symposium on Calcium Binding Proteins and Calcium Function in Health and Disease*. R. H. Wasserman, editor. Elsevier/North Holland, New York. 13–20.
- Fuch, F. 1974. Chemical properties of the calcium receptor site of troponin as determined from binding studies. In *Calcium Binding Proteins*. W. Drabikowski, H. Strzelecka-Golaszewska, and E. Carafoli, editors. Elsevier Scientific Publishing Co., Amsterdam. 1–37.
- Golowash, J., A. Kirwood, and C. Miller. 1986. Allosteric effects of Mg^{2+} on the gating of Ca^{2+} -activated K^+ channels from mammalian skeletal muscle. *Journal of Experimental Biology*. 124:5–13.
- Hanke, W., and C. Miller. 1983. Single chloride channels from *Torpedo* electroplax. *Journal of General Physiology*. 82:25–45.
- Hille, B. 1968. Pharmacological modifications of the sodium channels of frog nerve. *Journal of General Physiology*. 51:199–219.
- Kume, H., K. Takagi, T. Satake, H. Tokuno, and T. Tomita. 1990. Effects of intracellular pH on calcium-activated potassium channels in rabbit tracheal smooth muscle. *Journal of Physiology*. 424:445–457.
- Latorre, R., C. Vergara, and C. Hidalgo. 1982. Reconstitution in planar lipid bilayers of a Ca^{2+} -dependent K^+ channel from transverse tubule membranes isolated from rabbit skeletal muscle. *Proceedings of the National Academy of Sciences, USA*. 79:805–809.
- Laurido, C., D. Wolff, and R. Latorre. 1990. Effect of pH in a Ca^{2+} -activated K^+ channel [K(Ca)] from rat skeletal muscle. *Biophysical Journal*. 57:507a. (Abstr.)
- Magleby, K. L., and B. S. Pallota. 1983. Ca^{2+} dependence of open and shut intervals distributions from Ca^{2+} -activated K^+ channels in cultured rat muscle. *Journal of Physiology*. 344:585–604.
- McLaughlin, S. G. A., G. Szabo, G. Eisenman, and S. M. Ciani. 1970. Surface charge and the conductance of phospholipid membranes. *Proceedings of the National Academy of Sciences, USA*. 67:1268–1275.
- McManus, O. B., A. L. Blatz, and K. L. Magleby. 1985. Inverse relationships of the durations of adjacent open and shut intervals for Cl and K channels. *Nature*. 317:625–627.
- McManus, O. B., and K. L. Magleby. 1985. The large conductance calcium activated potassium channel in cultured rat muscle has at least three open states of similar conductance and six shut states. *Biophysical Journal*. 47:137a. (Abstr.)

- McManus, O. B., and K. L. Magleby. 1988. Kinetic states and modes of single large-conductance calcium-activated potassium channels in cultured rat skeletal muscle. *Journal of Physiology*. 402:79–120.
- Methfessel, C., and C. Boheim. 1982. The gating of single calcium-dependent potassium channels by an activation-blockage mechanism. *Biophysics of Structure and Mechanism*. 9:35–60.
- Mitchell, P. 1961. Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. *Nature*. 191:144–148.
- Moczydlowski, E., O. Alvarez, C. Vergara, and R. Latorre. 1985. Effect of phospholipid surface charge on the conductance and gating of a Ca^{2+} -activated K^+ channel in planar bilayers. *Journal of Membrane Biology*. 83:273–282.
- Moczydlowski, E., and R. Latorre. 1983a. Saxitoxin and ouabain binding activity of isolated skeletal muscle membrane as indicators of surface origin and purity. *Biochimica et Biophysica Acta*. 732:412–420.
- Moczydlowski, E., and R. Latorre. 1983b. Kinetics of Ca^{2+} -activated K^+ channels from rat skeletal muscle incorporated into planar lipid bilayers. Evidence for two voltage-dependent Ca^{2+} binding reactions. *Journal of General Physiology*. 82:511–542.
- Nonner, W., B.C. Spalding, and B. Hille. 1980. Low intracellular pH and chemical agents slow inactivation gating in sodium channels of muscle. *Nature*. 284:360–363.
- Oberhauser, A., O. Alvarez, and R. Latorre. 1988. Activation by divalent cations of a Ca^{2+} -activated K^+ channel from skeletal muscle membranes. *Journal of General Physiology*. 92:67–86.
- Pan, J. W., J. R. Hamm, D. L. Rothman, and R. G. Shulman. 1988. Intracellular pH in human skeletal muscle by ^1H NMR. *Proceedings of the National Academy of Sciences, USA*. 85:7836–7839.
- Renaud, J. M. 1989. The effect of lactate on intracellular pH and force recovery of fatigued sartorius muscles of the frog, *Rana pipiens*. *Journal of Physiology*. 416:31–47.
- Roseblatt, M., C. Hidalgo, C. Vergara, and N. Ikemoto. 1981. Immunological and biochemical properties of transverse tubule membranes isolated from rabbit skeletal muscle. *Journal of Biological Chemistry*. 256:8140–8148.
- Segel, I. H. 1975. *In Enzyme Kinetics*. John Wiley & Sons, Inc., New York. 957 pp.
- Sigworth, F. J., and S. M. Sine. 1987. Data transformations for improved display and fitting of single channel dwell time histograms. *Biophysical Journal*. 52:1047–1054.
- Vergara, C., and R. Latorre. 1983. Kinetics of Ca^{2+} -activated K^+ channels from rabbit skeletal muscle incorporated into planar bilayers: evidence for a Ca^{2+} and Ba^{2+} blockade. *Journal of General Physiology*. 82:543–568.
- Williams, R. J. P. 1988. Proton circuits in biological energy interconnections. *Annual Review of Biophysics and Biophysical Chemistry*. 17:71–97.
- Wong, B. S., H. Lecar, and M. Adler. 1982. Single calcium-dependent potassium channels in clonal anterior pituitary cells. *Biophysical Journal*. 39:313–317.