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pH-Modulation of Chloride Channels from the Sarcoplasmic Reticulum of Skeletal Muscle

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Abstract. The understanding of the role of cytoplasmic pH in modulating sarcoplasmic reticulum (SR) ion channels involved in Ca^{2+} regulation is important for the understanding of the function of normal and adversely affected muscles. The dependency of the SR small chloride (SCl) channel from rabbit skeletal muscle on cytoplasmic pH (pH_{cis}) and luminal pH (pH_{trans}) was investigated using the lipid bilayer-vesicle fusion technique. Low pH_{cis} 6.75–4.28 modifies the operational mode of this multiconductance channel (conductance levels between 5 and 75 pS). At pH_{cis} 7.26–7.37 the channel mode is dominated by the conductance and kinetics of the main conductance state (65–75 pS) whereas at low pH_{cis} 6.75–4.28 the channel mode is dominated by the conductance and kinetics of subconductance states (5–40 pS). Similarly, low pH_{trans} 4.07, but not pH_{trans} 6.28, modified the activity of SCl channels. The effects of low pH_{cis} are pronounced at 10^{-3} and 10^{-4} M $[Ca^{2+}]_{cis}$ but are not apparent at 10^{-5} M $[Ca^{2+}]_{cis}$, where the subconductances of the channel are already prominent. Low pH_{cis}-induced mode shift in the SCl channel activity is due to modification of the channel proteins that cause the uncoupling of the subconductance states. The results in this study suggest that low pH_{cis} can modify the functional properties of the skeletal SR ion channels and hence contribute, at least partly, to the malfunction in the contraction-relaxation mechanism in skeletal muscle under low cytoplasmic pH levels.

Key words: pH — Subconductance — Kinetics — Chloride channels — Sarcoplasmic reticulum — Skeletal muscle

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Introduction

The adverse effects of hypoxia on the function of smooth and cardiac muscles [6, 20, 47] are thought to be mediated *via* changes in the regulation of ion channels and/or metabolites intimately connected with muscle contraction [13, 30, 47]. One of the early cytosolic changes occurring in cardiac and smooth muscles under hypoxic conditions and fatigued skeletal muscle is the lowering of $pH_{cytoplasmic}$ which depresses force development [14] *via* modifications in the mechanism(s) involved in Ca^{2+} homeostasis. The understanding of the role of $pH_{cytoplasmic}$ in modulating SR ion channels involved in Ca^{2+} regulation could be important for the understanding of the function of normal and adversely affected muscles. Indeed, lipid bilayer studies reveal that pH_{cis} modulation of single Ca^{2+} release channels of the SR in both cardiac [36] and skeletal muscles [29] has been demonstrated. On the other hand, the pH_{cis} dependency of the regulatory counter-current, which must flow during contraction and relaxation to reduce the SR potential change caused by the transmembrane movement of Ca^{2+}, is not well known.

In addition to the pH-modulated sarcolemmal Cl^--conductance mediated by ClC-1 [39], which is a major Cl^- conductance that plays the important role of stabilizing the membrane potential [7] and underlies myotonia [38], other Cl^- channels found in the SR membrane of skeletal and cardiac muscles have been reviewed recently [23]. The pH modulation of these SR Cl^- channels is not well understood (*see* Discussion). These SR Cl^- channels have been reported to be insensitive to low pH_{cis} [35, 37] and only one study using the lipid bilayer technique has shown that lowering the pH_{trans} increased current amplitude of the cardiac SR Cl^- channel [46]. Using the ''sarcoball'' preparation technique Hals and Palade [18] found that luminal alkalization from 7.0 to

8.0 reduced the unitary conductance of a large SR Cl^- channel from 505 to 257 pS, whereas this channel was unaffected by changes in luminal pH between 7.0 and 4.0. The aim of this study was to examine the direct effects of low pH on the conductance and kinetic properties of the multiconductance SCl channel. The modulation of an SR, or any other intracellular, voltage- and Ca^{2+}-activated Cl^- channel by low pH_{cis} has not been reported previously.

Materials and Methods

Preparation of SR Vesicles

Terminal cisternae or longitudinal SR vesicles from rabbit skeletal muscle [27, 40] were incorporated into lipid bilayers [27, 33]. Muscle was dissected from New Zealand rabbits and either used fresh or stored at −70°C. Cubes of muscle were homogenized in a Waring blender in homogenizing buffer (mM: imidazole, 20; sucrose, 300, adjusted to pH 7.1 with HCl). The homogenate was centrifuged (11,000 × *g*, 15 min) and the pellet resuspended, rehomogenized (4 × 15 sec) and recentrifuged as above. The supernatant was filtered through cotton gauze and centrifuged at 110,000 × *g* for 60 min. The crude SR vesicle pellet was layered onto a discontinuous sucrose gradient containing 45, 38, 34, 32 and 28% sucrose (in 20 mM imidazole, pH 7.1 adjusted with HCl), and centrifuged for 16 hr at 20,000 rpm using a Beckman SW28 rotor. Vesicles were collected from the following density interfaces: Band 1 (B1), 28–32%; Band 2 (B2), 32–34%; Band 3 (B3), 34–38%; Band 4 (B4), 38–45%. Vesicles were diluted 3-fold in 20 mM imidazole (pH, 7.1), pelleted at 125,000 × *g*, resuspended in homogenizing buffer (at 10 to 20 mg/ml protein), frozen and stored in liquid N_2. All procedures were performed at 4°C and all buffers contained the protease inhibitors: leupeptin, 1 μM; pepstatin A, 1 μM; benzamidine, 1 mM and PMSF, 0.7 mM.

Solutions

Cis and *trans* solutions contained choline-Cl (250 mM *cis*/50 mM *trans*) plus 1 mM $CaCl_2$ and 10 mM HEPES (pH_{cis} and *trans* 7.2–7.4, adjusted with Tris). The pH of the *cis* solution was adjusted by either adding aliquots of 100 mM stock HCl or perfusion of the *cis* chamber with buffered solutions. In the perfusion experiments the pH_{cis} was buffered with 10 mM MES (pH range 5.5–6.7). Both methods of lowering the pH_{cis} produced similar effects on the SCl channel activity. The pH of the solutions in the *cis* chamber was measured during pauses (to avoid the large electrical noise pickup and the breaking-up of bilayers) in the ion channel recordings between −40 and −30 mV close to the reversal potential. A final measurement was also taken after termination of the experiment using custom-made pH-probe combination glass Ag/AgCl (Microelectrodes, NH). $[Ca^{2+}]_{cis}$ was adjusted by adding aliquots of 100 mM stock 1,2-bis(2-aminoethyl ether)-N,N′,N′,N′-tetraacetic acid (BAPTA) and the final $[Ca^{2+}]_{cis}$ was calculated as described previously [26].

Lipid Bilayer and Vesicle Fusion

The artificial lipid bilayer technique is widely used to record ion channels from internal membranes which otherwise are inaccessible to patch clamp electrodes. Lipid bilayers were formed across a 150 μm hole in the wall of a 1 ml delrin™ cup using a mixture of palmitoyl-oleoyl-phosphatidylethanolamine, palmitoyl-oleoyl-phosphatidylserine and palmitoyl-oleoyl-phosphatidylcholine (5:3:2, by volume), obtained in chloroform from Avanti Polar Lipids (Alabaster, AL). The side of the bilayer to which vesicles were added was defined as *cis*, and the other side as *trans*. The orientation of the cytoplasmic side of the vesicle is thought to face the *cis* chamber [33]. This has been verified by using common ligands which are known to bind to the cytoplasmic domain of the ryanodine Ca^{2+} release channel protein present in the same bilayer [1]. This cytoplasmic orientation is also true for the Cl^- channel proteins [12, 46].

Recording Single Channel Activity

The pClamp program (Axon Instruments, Foster City, CA) was used for voltage command and acquisition of Cl^- current families (*see* Fig. 1) with an Axopatch 200 amplifier (Axon Instruments). The current was monitored on an oscilloscope and stored on videotape using pulse code modulation (PCM-501; Sony) and/or compact disc recorder (ACS Innovations International). Channel recording was made only from bilayers containing an active SCl channel whose incorporation in the bilayer is indicated by using a depolarizing voltage prepulse that removes channel inactivation, induced by sustained hyperpolarizing potentials, and thus aids channel activation at negative voltages. For practical reasons of avoiding the large current noise and bilayer breakage the reference electrode is usually placed in the *cis* chamber rather than the *trans* chamber so the direction of the current is inverted for convention and the voltages are given as *cis* with reference to the *trans* chamber as previously reported [1, 2, 21–27].

Data Analysis

Kinetic analysis was conducted only for optimal bilayers that contained a single active channel [9]. Data were recorded at 5 kHz, filtered at 1 kHz (4-pole Bessel, -3dB) and digitized *via* a TL-1 DMA interface (Axon Instruments) at 2 kHz. The capacitative current was removed by using the pClamp software (Axon Instruments) which allows the subtraction of a family of current traces void of channel activity from families of current traces containing SCl channel activity. An in-house analysis program, CHANNEL 2 (developed by P.W. Gage and M. Smith, JCSMR), was used to measure the following parameters of channel activity (34–250 sec long records): mean open time, T_o (i.e., the average of the open times of all intervals where the current exceeded the baseline noise for 0.5 msec); frequency of opening to all conductance levels F_o; the open probability, P_o (i.e., the sum of all open times as a fraction of the total time); the average mean current, I′ (i.e., the integral of the current passing through the channel divided by the total time). The integral current is determined by computation of the area between a line set on the noise of the closed state and channel opening to various levels. Since measurement of open times and P_o included openings to submaximal conductance levels, the threshold for channel opening or closing was normally set at 1.5 times the maximum baseline noise and approximately at 10–20%, rather than at 50%, of the maximum current amplitude, I. This analysis allows the inclusion of current transitions to subconductance states that are less than 50% of the maximum conductance (e.g., Fig. 1*B* and *C*). The P_{om}, is defined as the fraction of time that the channel is open only at the maximal conductance state (level 6; *see* Fig. 1). The threshold current level in this case was set at 40 pS to include only the maximum conductance, 65–75 pS. The probability of the channel being open to other substates, P_{os}, is obtained by subtracting P_{om} from P_o. Ideally, P_o of each individual subconductance state should be determined. However, it was

not possible to determine the P_o dependence of the different individual subconductance states of the SCl channel as has been done for the double barrel Cl^- channel of the *Torpedo electroplax* [32].

Current amplitude, *I*, was obtained by measuring the distance (in pA) between the peak at 0 pA (representing the closed state) and the extreme peak on the left (representing the open state for the majority of distinct events) in the all-points histogram generated using CHANNEL 2. The value of the current amplitude was also obtained by measuring the distance (in pA) between two lines, one set on the maximum baseline noise of the closed level, where the current amplitude is considered 0 pA, and the other set on the noise of the majority of distinct events, longer than 0.5 msec, which were in the open state. Both methods were used and the results were generally in agreement.

The reversal potential was determined by using minimum polynomial fits of the current-voltage curves. The reversal potential was then corrected for the liquid junction potential and ionic mobility which were calculated by using the JPCalc software [4, 34].

Statistics

Unless stated, each SCl channel was used as its own control and the comparison was between kinetic parameters of the channel before and after changing the pH_{cis}. The data are reported as means ± SEM (n = replicates) and the difference in means was analyzed by Student's *t*-test. Data were considered statistically significant when probability (P) values ≤ 0.01.

Results

Initial experiments were conducted to examine the possibility that low pH_{cis} might indirectly exert its effects on the SCl channel activity by modifying the bilayer properties. These experiments revealed that the biophysical properties of the phospholipids forming the bilayer (n = 3 bilayers) are not affected by pH_{cis} as low as 4. The bilayers maintained a specific bilayer capacitance value of ~0.42 $\mu F/cm^2$ and a chord conductance value for the leak of ~12.5 pS. Under the experimental conditions reported here, these values are indicative of the formation of optimal bilayers.

The effects of low cytoplasmic pH_{cis} on a typical SCl channel activity recorded from an optimal bilayer (250 mM *cis*/50 mM *trans* Cl^-) are shown in Fig. 1. The conductance and kinetic properties of the SCl channel are modified at low pH_{cis} (n = 24 channels in 24 bilayers) (e.g., Fig. 1*B* and *C*). In addition, four runs of low pH_{cis} were repeated on three of these channels. The effect of lowering the pH_{cis} to 6.75 on this multiconductance SCl channel is characterized by a shift in current transitions from those corresponding to the opening of the maximal conductance (level 6) (Fig. 1*A* middle row) to those where the prominent current transitions are between the closed and low conductance states (level 2 and level 1) (middle row in Fig. 1*B* and *C*, respectively). It is important to note that the amplitude of these subconductances was not affected significantly by the pH of the *cis* solution (*see* legend of Fig. 1). They can be seen, though at a different frequency, at different pH_{cis} levels (middle row Fig. 1*A–C*). The prepulses to a positive potential of +40 mV, which remove the inactivation of SCl channels [27], failed to restore the frequency of current transitions of the maximal conductance (level 6). The conventional all-points histograms for the activity of an SCl channel are shown below the expanded current traces in Fig. 1*A–C* (bottom row). The disappearance of almost all transitions to the high conductance states were indicated by the absence of peaks at −5.44 and −7.48 pA (levels 5 and 6) and the prominence of a submaximal conductance (level 2), i.e., −1.61 pA at pH_{cis} 6.75 (Fig. 1*B* middle and bottom rows). The submaximal level of −1.61 pA is one of the six conductance levels (−1.71 pA) observed at pH 7.3–7.4 (Fig. 1*A*) and detailed previously [27]. The changes in the conductance and kinetics of the submaximal state by further reduction in pH_{cis} to 4.28 is characterized by a further shift in the probability of the current peak towards the immediate subconductance −0.66 pA level 1) and the closed state (0 pA level 0) (Fig. 1*C*). This shift in the current peak is despite the appearance of some transitions to the fifth subconductance level −5.44 pA. The inspection of the data traces presented in Figs. 1*B* and *C* (middle row) shows that the current transitions to higher subconductance states (−5.44 pA) increased when the *cis* pH was lowered from pH 6.75 to 4.28. However, because of the low frequency of these current transitions they do not appear as a significant peak on the current amplitude histogram.

Current amplitude, *I*, is reduced from −6.42 ± 0.18 pA (n = 16) at pH_{cis} 7.26 to −1.34 ± 0.06 pA at pH_{cis} 6.75 ($P < 0.01$, n = 16) and −1.33 ± 0.22 pA at pH_{cis} 4.28 ($P < 0.01$, n = 16). Similarly, the mean current, I', is reduced from −3.57 ± 0.17 pA (n = 16) at pH_{cis} 7.26 to −1.31 ± 0.06 pA at pH_{cis} 6.75 ($P < 0.01$, n = 16) and −1.53 ± 0.10 pA at pH_{cis} 4.28 ($P < 0.01$, n = 16). At low pH_{cis} the decline in I' is largely the result of the disappearance of maximal current transitions (Fig. 1*B* level 6) and changes in the kinetics of current transitions to the maximal current level (level 6) (Fig. 1*C*) rather than a reduction in the amplitude of the current levels (1 to 6).

The effects of low pH_{trans} on the SCl channel activity (250 mM *cis*/50 mM *trans* Cl^-) are shown in Fig. 2. The conductance and kinetic parameters of the channel are not affected significantly ($P > 0.05$, n = 3 channels) when the pH_{trans} is lowered from 7.4 to 6.28 (n = 3 channels in 3 bilayers) (e.g., Fig. 2*B*). The values of I, I', P_o and T_o being −7.18 ± 0.15 pA, −3.52 ± 0.49 pA, 0.65 ± 0.05 and 8.07 ± 0.69 sec, respectively at pH_{trans} 7.4 and −7.13 ± 0.16 pA, −3.37 ± 0.17 pA, 0.63 ± 0.04 and 8.23 ± 0.59 sec, respectively at pH_{trans} 6.28. However, the effect of further lowering the pH_{trans} to 4.07 on the SCl channel, like that of low pH_{cis}, is significant ($P < 0.01$) and characterized by a shift in current transitions towards the closed and low conductance states (level 2 and level 1) (Fig. 2*C*). The values of I, I', P_o and T_o are reduced to −1.79 ± 0.06 pA, −0.64 ± 0.05 pA, 0.21 ± 0.02 and 0.76 ± 0.07 sec, respectively at pH_{trans} 4.07.

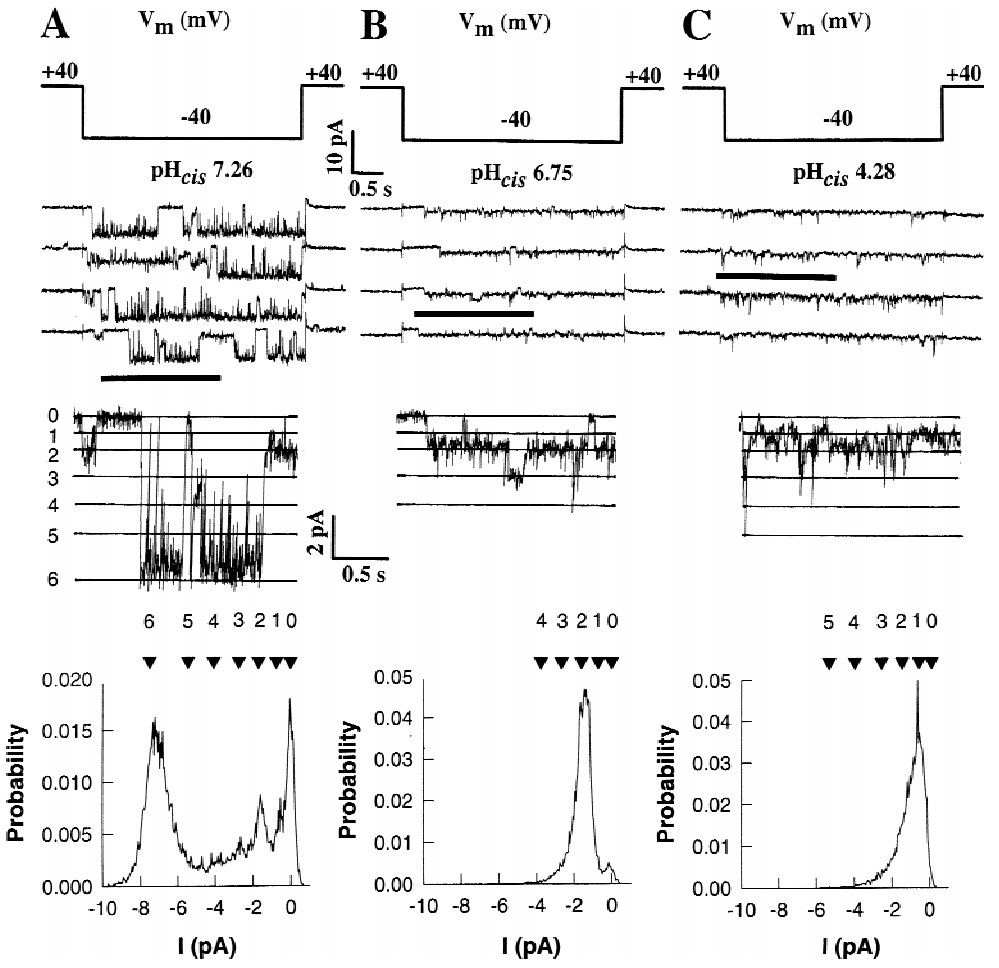


Fig. 1. Representative current traces illustrating the effects of changes in pH_{cis} on single SCl channel activity recorded from an optimal bilayer, i.e., specific bilayer capacitance >42 μF/cm^2, held at −40 mV and asymmetrical choline-Cl (*cis/trans;* 250/40 mM) plus 1 mM $CaCl_2$ and 10 mM HEPES. (*A*) pH_{cis} 7.26. (*B*) pH_{cis} 6.75, and (*C*) pH_{cis} 4.28. Following the convention the downward deflections denote activation of the inward Cl^- current, i.e., chloride ions moving from the *cis* chamber to the *trans* chamber. Solid bars (top row) indicate portions of the current traces presented at a different scale (middle row). For a better display the data are filtered at 1 kHz, digitized at 2 kHz and reduced by a factor of 10, the fast transient capacitative currents are removed and the traces are offset by 10 pA. Filled triangles and Arabic numerals point to current levels in the all-points histograms (bottom row *A–C*) which correspond to current levels shown in the middle rows (*A–C*). The mean of the submaximal current levels at all three pH_{cis} are 1 = −0.71; 2 = −1.62; 3 = −2.68; 4 = −3.96; 5 = −5.43 and 6 = −7.15 pA. The all-points histograms were constructed from longer segments of 57.69 sec (*A*), 57.39 sec (*B*) and 54.89 sec (*C*). The bin width was 0.05 pA. The effects of low pH on the SCl channel were observed in every single bilayer containing SCl channels.

Analysis of channel activity at −40 mV reveals pH_{cis}-modification of channel gating, as deduced from kinetic parameters. There was no significant difference ($P > 0.05$) in P_o at pH_{cis} 7.26 and pH_{cis} 6.75. The values of P_o being 0.73 ± 0.08 [P_o (level 6) is 0.57 and P_{os} levels 1–5) is 0.16] and 0.68 ± 0.06 (entirely due to P_{os} of the subconductance levels 1–5), respectively. At pH_{cis} 4.28 P_o decreased significantly to 0.34 ± 0.03 ($P < 0.01$) [P_{os} (levels 1–5) is 0.33]. This is despite the appearance of a current transition to the maximal conductance state [P_{om} (level 6) is 0.01].

The frequency (F_o) of current transitions to all conductance states decreased from 56 ± 4 events/s, ($n = 16$) at pH_{cis} 7.26 to 19 ± 2 events/sec ($P < 0.01$, $n = 16$) at pH_{cis} 6.75 and returned to 62 ± 5 events/sec ($P > 0.05$, $n = 16$) at pH_{cis} 4.28. It is apparent that the value of F_o at low pH_{cis} is, almost entirely, for transitions to the subconductance states (levels 1–5). The mean open time, T_o, increased from 22.47 ± 2.02 msec at pH_{cis} 7.26 to 33.78 ± 1.21 msec at pH_{cis} 6.75 ($P < 0.01$, $n = 16$) and decreased to 9.28 ± 1.32 msec at pH_{cis} 4.28 ($P < 0.01$, $n = 16$). These changes in the kinetic parameters, like the changes in the conductance parameters, are indicative of a mode shift in the activity of the SCl channels.

The effects of low pH_{cis} on the conductance and kinetics of the SCl channel at different voltages are shown in Fig. 3. Families of single current traces recorded at voltages between −60 and +60 mV in control

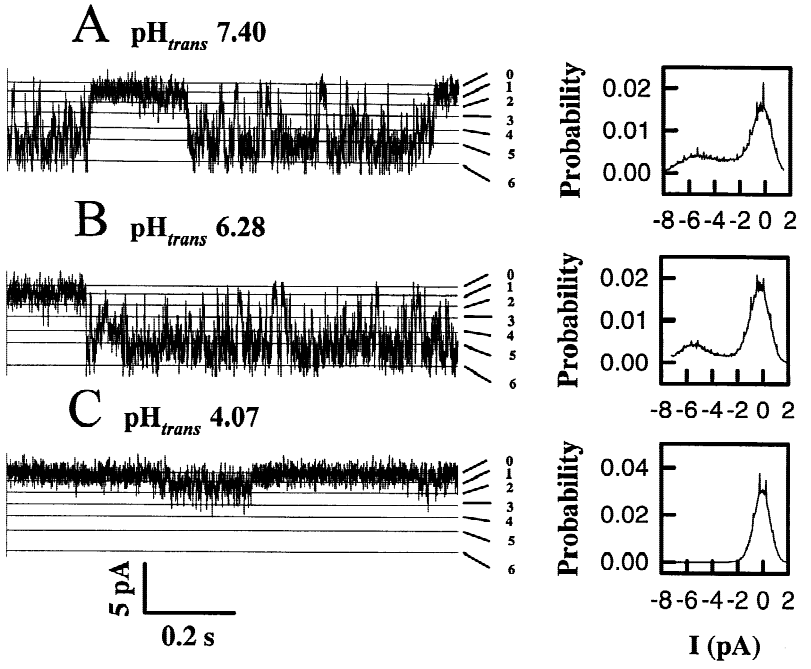


Fig. 2. Effects of pH$_{trans}$ on the SCl channel activity. Current traces recorded at −40 mV in asymmetrical choline-Cl (250 mM/50 mM; *cis/trans*) (*A*) pH$_{trans}$ 7.40, (*B*) pH$_{trans}$ 6.28 and (*C*) pH$_{trans}$ 4.07. The all-points histograms were constructed from longer segments of 34.69 sec (*A*), 63.50 sec (*B*) and 61.74 sec (*C*). The bin width was 0.05 pA.

solution, pH$_{cis}$ 7.37 are shown in Fig. 4*A*. Typically the sensitivity of the SCl channel to low pH$_{cis}$ 4.48 is characterized by the domination of the transitions to submaximal open states and the absence of transitions to the maximal open state at all voltages between −60 and +60 mV (Fig. 3*B*). The effects of low pH$_{cis}$ on the time course of single channel current activity and transition to the maximal conductance state are reversible after perfusion with control solution pH$_{cis}$ 7.37, e.g., current traces at −60, −40, and −20 (Fig. 3*C*). In addition, the transitions to submaximal levels, which are present under the control condition, are also clearly apparent (e.g., see arrows).

Current-voltage relationships constructed to examine the voltage-dependence of pH$_{cis}$-induced changes in channel conductance are shown in Fig. 4. The current-voltage relationship shows that at low pH$_{cis}$ 4.48 current amplitude is reduced from −4.12 pA to −2.56 pA at −40 mV. This reduction in current amplitude is in agreement with a conductance shift to subconductance states (levels 2 and 3). However, the reversal potential, determined from the fitted curves and then corrected for the liquid junction potential and ionic activity [4, 34], shifts non-significantly ($P > 0.05$; $n = 3$ *I-V* relations at each condition from the same channel) from +36.36 ± 2.58 mV at pH$_{cis}$ 7.37 to +34.28 ± 1.64 mV at pH$_{cis}$ 4.48 and recovers to +36.17 ± 1.72 mV after perfusion with control solution pH$_{cis}$ 7.37. This insignificant shift in the reversal potential confirms the nature of this submaximal current as being a Cl⁻ current. According to the Goldman, Hodgkin and Katz equation the $P_{\text{choline}}/P_{\text{Cl}^-}$ is between ~0.03 and ~0.07 indicative of a high selectivity of these subconductance states to Cl⁻.

Analysis of the SCl channel activity, at all current levels 0 to 6, reveals that low pH$_{cis}$ depresses and reduces the steepness of the bell-shaped voltage dependency of the probability of the channel being open P_o and mean open time T_o (Fig. 5*A* and *C*). The frequency, F_o (Fig. 5*B*) and the mean closed time, T_c were not affected at low pH$_{cis}$ (Fig. 5*D*). These findings also suggest that the low pH$_{cis}$-induced decrease in P_o is due to a reduction in T_o and not to changes in T_c. However, at low pH$_{cis}$ the calculated values of P_o, T_o and F_o are mainly for the submaximal conductance states (levels 1 and 2). This analysis together with the channel activity shown in Fig. 1*C* (and also in Fig. 3*B*) suggest that low pH$_{cis}$ enhances channel fluctuations at the submaximal conductance states (levels 1 and 2). Low pH$_{cis}$ induced a nonsignificant voltage shift of −6.86 and −9.60 mV in the voltage-dependency of the P_o and F_o peaks, respectively.

The SCl channel is modulated by physiological [Ca^{2+}] [26] on the cytoplasmic side of the channel, hence the effects of lowering pH$_{cis}$ on the activity of SCl channels were also examined at different physiological [Ca^{2+}]$_{cis}$. It was found that, like the low pH$_{cis}$ 6.75-induced reversible effects on the SCl channels at 10^{-3} M [Ca^{2+}]$_{cis}$ (Fig. 1), exposure of SCl channels to low pH$_{cis}$ 6.65 modified the channel at 10^{-4} M [Ca^{2+}]$_{cis}$ (Fig. 6*B*). On the other hand at 10^{-5} M [Ca^{2+}]$_{cis}$, where the subconductances of the channel are already uncoupled [26], the effect of low pH$_{cis}$ 6.57 on the same SCl is not apparent (Fig. 6*E*). The effect of lowering the pH$_{cis}$ to 6.64 at 10^{-4} M [Ca^{2+}]$_{cis}$ on the SCl channel is typically characterized by a shift in current transitions from the maximal conductance (level 6) (Fig. 6*A*) to those transitions between the closed and low conductance states (Fig. 6*B*).

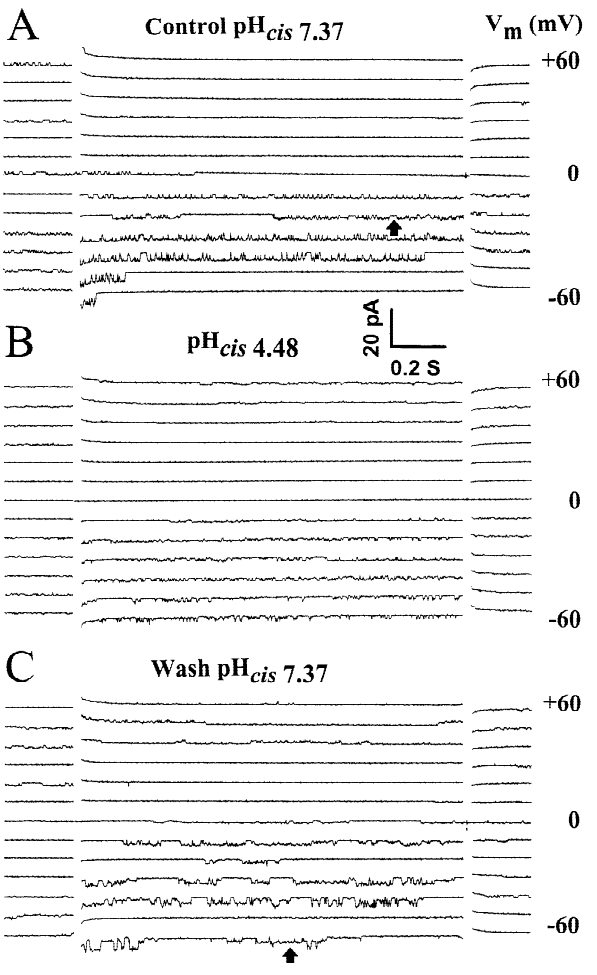


Fig. 3. Effects of pH_{cis} on the voltage-dependence of SCl channel activity. A voltage protocol is used to activate the voltage- and Ca^{2+}-dependent SCl channels currents. From an initial holding potential of 0 mV lasting 250 msec the bilayer potential (V_m) is stepped to voltages between −60 and +60 mV, in steps of +10 mV, for periods lasting 1500 msec. The steps are also separated by 250 msec interval where the holding potential is also kept at 0 mV. (*A*) Control current traces recorded in asymmetrical choline-Cl (250 mM/50 mM; *cis/trans*) pH_{cis} 7.37, (*B*) Current traces recorded at pH_{cis} 4.48. and (*C*) current traces recorded after perfusion with control solution pH_{cis} 7.37. At negative voltages downward deflections indicate an inward current. The submaximal current levels (arrows) became more prominent after wash with control solution pH_{cis} 7.37. The current traces are filtered at f_c = 0.2 kHz. Similar effects were obtained for three other channels with slightly different pH_{cis} of 4.67, 4.28 and 4.23.

Prepulses to a positive potential of +40 mV also failed to remove the inactivation of SCl channels and restore the frequency of current transitions of the maximal conductance (level 6). The conventional all-points histograms for the activity of an SCl channel also confirm that the effect of lowering the pH_{cis} at 10^{-3} and 10^{-4} M $[Ca^{2+}]_{cis}$ is to lower the frequency of transitions to the high conductance states as indicated by the absence of peaks at −5.44 at −7.48 pA (levels 5 and 6) and the prominence of

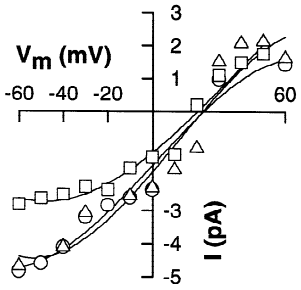


Fig. 4. Effects of pH_{cis} on the voltage-dependence of the SCl channel maximal current amplitude at: (○) control pH_{cis} 7.37, (□) pH_{cis} 4.48 and (▽) wash pH_{cis} 7.37. The solid lines are drawn to a third-order polynomial fits. Family of curves are for the same channel. Four exponentials would have a smoother fit of all data points, however a minimum of third order exponential was sufficient and most suitable since the reversal potential for the maintained 250/50 mM *cis/trans* Cl^- gradient did not change significantly.

a submaximal conductance (levels 1–3) at low pH_{cis} (Fig. 6*B* and *E*).

At $[Ca^{2+}]_{cis}$ of 10^{-4} M the conductance and kinetic parameters of the SCl channel are affected significantly ($P < 0.01$, $n = 3$ channels) when the pH_{cis} is lowered from 7.4 to 6.64 ($n = 3$ channels in 3 bilayers) (e.g., Fig. 6*B*). The values of I, I', P_o and T_o are reduced from −7.21 ± 0.17 pA, −3.10 ± 0.11 pA, 0.68 ± 0.02 and 8.44 ± 0.58 sec, respectively at pH_{cis} 7.4 to −3.31 ± 0.16 pA, −0.64 ± 0.08 pA, 0.21 ± 0.01 and 0.64 ± 0.09 sec, respectively at pH_{cis} 6.64. On the other hand, at $[Ca^{2+}]_{cis}$ of 10^{-5} M the conductance and kinetic parameters of the SCl channel are not affected significantly ($P > 0.01$, $n = 3$ channels) when the pH_{cis} is lowered from 7.38 to 6.57 ($n = 3$ channels in 3 bilayers) (e.g., Fig. 6*E*). The values of I, I', P_o and T_o being −2.86 ± 0.14 pA, −0.60 ± 0.08 pA, 0.21 ± 0.03 and 0.45 ± 0.04 sec, respectively at pH_{cis} 7.38 and −2.81 ± 0.10 pA, −0.52 ± 0.07 pA, 0.21 ± 0.03 and 0.47 ± 0.03 sec, respectively at pH_{cis} 6.57.

Although current amplitude histograms and their response to low pH are qualitatively similar, there were some quantitative variations (Figs. 1, 2 and 6). The amplitude histogram in Fig. 1, lower left panel was constructed from data obtained in response to a 16-episode voltage protocol at pH 7.26 and 10^{-3} M Ca^{2+}. In Fig. 2 the amplitude histogram was constructed from data obtained for a channel that is clamped to −40 mV at pH 7.40 and 10^{-3} M Ca^{2+} for longer periods of recordings than those in Fig. 1. The quantitative differences in amplitude histograms are due to differences in Ca^{2+} levels (Figs. 1 and 6), pH_{cis} (Figs. 1*A* and 2*A*) and the degree of the prominence of the subconductance states (Fig. 1*A*, Fig. 2*A* and Fig. 6*A* and *B*). The data in Fig. 6*A* and *C* indeed highlights the subconductance states of the SCl channel at 10^{-4} Ca^{2+}. The Ca^{2+}-dependence of these subconductances of the SCl channel has been detailed previously [26]. The incomplete reversibility (*see* Fig.

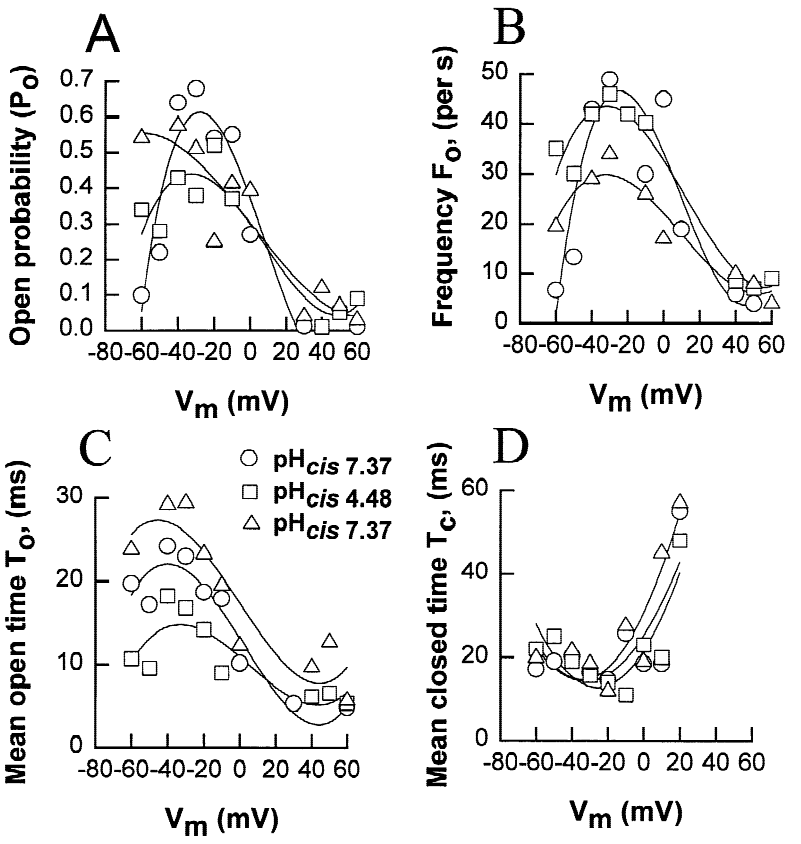


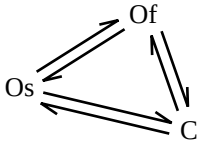
Fig. 5. Effects of pH_{cis} on the voltage-dependence of SCl channel kinetic parameters. (*A*) Open probability (P_o), (*B*) frequency F_o, (*C*) mean open time T_o and (*D*) mean closed time T_c. (○) control pH_{cis} 7.37, (□) pH_{cis} 4.48 and (▽) wash pH_{cis} 7.37. The solid lines are drawn to a third-order polynomial fit in (*A, B* and *C*) and to a second-order polynomial fit in (*D*). Qualitatively similar results were observed for three other SCl channels as mentioned in Fig. 3.

6*A* and *B*) at *cis* pHs 7.40 and 7.38 respectively, could reflect differences in the recovery rates of the main conductance and subconductance states. Any Ca^{2+} role in the reversibility of low pH-induced change in the conductance states of the SCl channel needs further characterization.

Discussion

Model of the pH_{cis} Effects

The low pH_{cis}-induced changes in the biophysical properties of the SCl channel (Fig. 1) could be interpreted in terms of a qualitative three states kinetic scheme reported previously for low O_2 tension-induced modification of the SCl channel [21] and shown here:



The model is consistent with the channel activity that shows transitions between a maximum opening or a fully open state (Of) (level 6), a group of subconductance states (Os) (levels 1–5) and a closed state (C) (level 0) representing the closed conductance state. In such a model current transitions to the maximal conductance state (level 6), i.e., a fully open state 65–75 pS (Fig. 1*A*) dominate at pH_{cis} 7.2–7.4 representing a largely unprotonated gating mechanism. Current transitions to the subconductance states (levels 1–3) (Fig. 1*B*) dominate at pH_{cis} 6.75 representing a partially protonated gating mechanism. Current transitions to the immediate subconductance state (level 1) and the closed state (level 0) (Fig. 1*C*) dominate at low pH_{cis} 4.48 representing a highly though not fully protonated gating mechanism.

The large effect of low pH_{cis} on current amplitude associated with small changes in the reversal potential, reflects a transition to a low Cl^- permeating conductance state (subconductance state), and the otherwise unaltered Cl^- selectivity, of the SCl channel. The low pH_{cis}-induced enhancement of the current fluctuations at the submaximal conductance states (levels 1–3) represents a typical channel behavior that is also observed after the exposure of the channel to IP_3 or low $[Ca^{2+}]_{cis}$ [25, 26]. The findings reported in this study indicate that the channel protein in the main conductance state configuration responds differently to low pH compared with the channel protein when in the subconductance states configuration.

Two SR Cl^- channels, a 100 pS (*cis/trans* 100/100

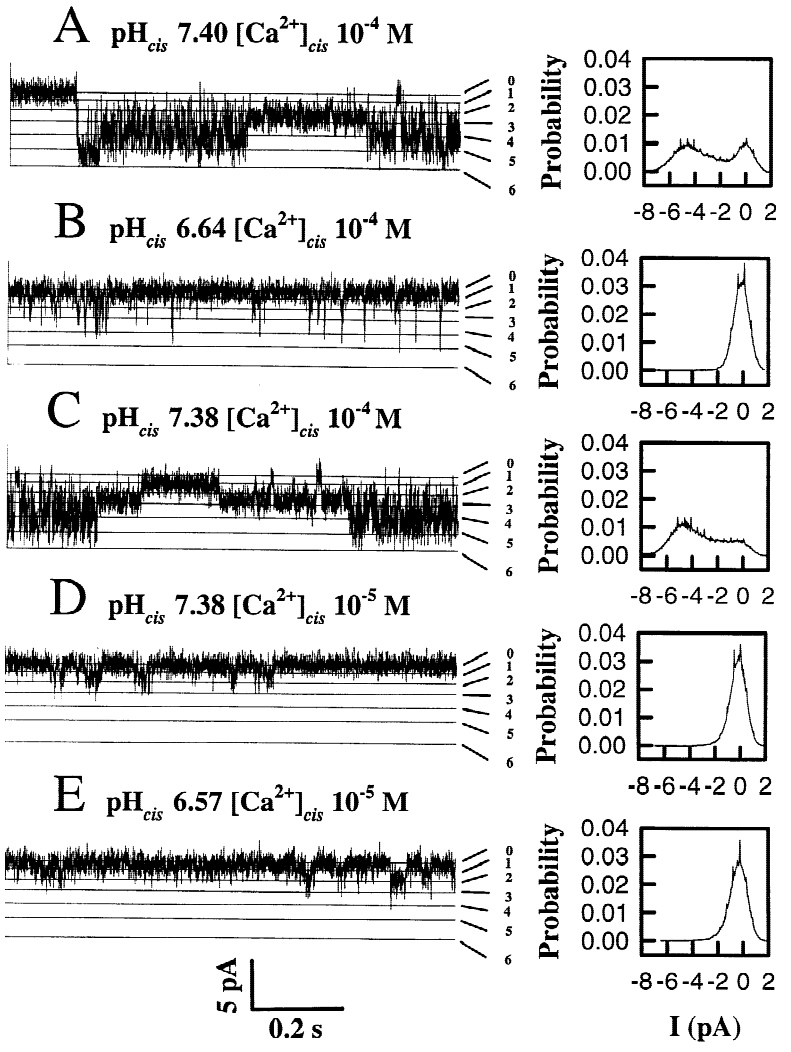


Fig. 6. Effects of pH$_{cis}$ on the SCl channel activity at different [Ca^{2+}]$_{cis}$. Current traces recorded at −40 mV in asymmetrical choline-Cl (250 mM/50 mM; *cis/trans*) (*A*) pH$_{cis}$ 7.40 and 10^{-4} M [Ca^{2+}]$_{cis}$, (*B*) pH$_{cis}$ 6.64 and 10^{-4} M [Ca^{2+}]$_{cis}$, (*C*) pH$_{cis}$ 7.40 and 10^{-4} M [Ca^{2+}]$_{cis}$, (*D*) pH$_{cis}$ 7.38 and 10^{-5} M [Ca^{2+}]$_{cis}$, and (*E*) 6.57 and 10^{-5} M [Ca^{2+}]$_{cis}$. The all-points histograms were constructed from longer segments of 33.74 sec (*A*), 113.59 sec (*B*), 29.94 sec (*C*), 87.54 sec (*D*) and 75.54 sec (*E*). The bin width was 0.05 pA.

mM Cl⁻) and a 130 pS (*cis/trans* 250/50 mM Cl⁻) from skeletal and cardiac muscles respectively, have been reported to be modified by changes in the pH [31, 46]. Miller [31] observed that low pH on both *cis* and *trans* sides of the channel induces a substantial inhibition of the channel conductance in the SR of skeletal muscle. However, no single channel recordings at low pH were presented and the nature of effects on the subconductances and kinetics of the channel were not examined. The multiconductance Cl⁻ channel in the SR of cardiac muscle is modified only by changes in the pH$_{trans}$ in such a way that the conductance changes between ~190 pS at pH$_{trans}$ 5.5 and ~60 pS at pH$_{trans}$ 9.0 [46]. However, it appears that there were no apparent effects on the conductance states 130, 61 and 154 pS and the kinetic parameters, P_o, T_o, and T_c of this channel. Rousseau [35] and Rousseau, Roberson & Meissner [37] reported that the conductance and kinetic properties of the 55 pS (*cis/trans* 250/60 mM Cl⁻) and 95 pS (*cis/trans* 260/50 mM Cl⁻) Cl⁻ channels, in the SR of skeletal and cardiac muscles respectively, were insensitive to low pH$_{cis}$. Perhaps, this is because the pH was not lowered below 6.8. Decrouy, Juteau & Rousseau [12] reported that the open probability of the 90 pS channel (*cis/trans* 250/50 mM Cl⁻) was insensitive to raising the pH$_{cis}$ from 7.4 to 9. However, they did not report the effects of low pH$_{cis}$ on this 90 pS channel. It is not known whether these differences in pH sensitivity are entirely due to variations in the experimental conditions such as tissue types and isolation procedures and/or to intrinsic differences in channel proteins.

In addition to the effects of pH on the SR Cl⁻ channels it has also been found that lowering the intracellular pH (pH$_i$) reduces the amplitude and slows the deactivation of the macroscopic Cl⁻ currents of sarcolemmal skeletal muscle expressed in Sf-9 insect cells and *Xenopus oocyte* expression systems [39]. However, the effects of low pH$_i$ on the conductance and kinetics at a single channel level are not known. pH-induced changes in Cl⁻ channel properties have also been reported for

nonmuscle Cl^- channels. For example, pH_i alkalization from 6 to 9 reduced Cl^- currents in epithelial cells [17] whereas the extracellular acidification decreased the activity of the cystic fibrosis transmembrane regulator (CFTR) Cl^- channels [41] and Cl^- channels in gastric parietal cells [10].

Molecular Mechanism of Low pH_{cis} Action

The response of the SCl channel to low pH_{cis} was not *via* changes in the biophysical properties of the phospholipids forming the bilayer (*see* Results). Hypothetically there are few potential mechanisms to explain the pH_{cis}-induced changes in the conductance and kinetics of the SCl channel. These include: (i) protonation of a site in the pore, (ii) protonation of a site that moves to constrict the pore, (iii) protonation of a site that causes changes in subunit assembly and (iv) combinations of these mechanisms. The protonation-induced reduction in channel conductance, deduced from current amplitude (Figs. 1, 2, 3 and 6), results either from a compression mechanism of the channel subconductance states or from a mechanism that involves uncoupling of these subconductances or both [16]. In the first mechanism, the pH_{cis}-induced modification in the gating of the ion channel proteins could be caused by the acifification-induced flexible carboxyl end that acts as a ''movable particle'' of the channel protein blocking the movement of ions through the channel in analogy with the ''ball and chain'' model proposed for the skeletal muscle Cl^- channels [15] and Shaker K^+ channels [5]. However, the primary structure of the SCl channel has not yet been determined and thus, it is not known whether these channels are regulated by the ''ball and chain'' as described for the Shaker K^+ channels and the skeletal muscle Cl^- channel. In the second mechanism, the effects of low pH_{cis} on the SCl channel are due to the competition between Ca^{2+} and H^+ for binding sites leading to protonation of the channel protein. This causes the uncoupling or loosening of the Ca^{2+}-dependent channel proteins and thus induces current transitions that are dominated by those of the low subconductance states. The data reported in this study regarding a low pH_{cis}-induced shift to subconductance states is in agreement with the second mechanism. The conductance and kinetics parameters of the channel at low pH_{cis} are determined by those of the subconductance states (level 1–3). For example, at low pH_{cis} the value of P_o 0.68 ± 0.08 is equivalent to P_{os} of the submaximal conductance levels.

We have previously demonstrated that the SCl channel is Ca^{2+}-dependent and exposure of the channel to low $[Ca^{2+}]_{cis}$ as with low pH_{cis}, uncouples the subconductances of the channel [26]. However, it is difficult to ascertain whether the effects of low pH_{cis} (*see* Fig. 6) are due to a competition between Ca^{2+} and H^+ for (i) Ca^{2+}-activating binding sites and/or (ii) binding sites for the assembly of the subunits of the channel protein. Indeed, protonation-induced separation of subunits of channel protein as an inhibitory mechanism has also been proposed as an explanation for low pH_{cis}-induced changes in the gating properties of the ryanodine receptor Ca^{2+}-release [29, 36] and K^+ channels [16]. The pH_{cis}-induced changes in the SCl channel kinetics and the decline in current amplitude, due to uncoupling of the subunits of the channel protein, might suggest H^+ binding to a site outside the pore. The fact that low pH_{cis} 4.28 induced a significant increase in the frequency of transitions to the subconductance states (Fig. 1*C*) compared with that observed at pH_{cis} 6.75 (Fig. 1*B*) explains the pH-dependent frequency of the subconductance states. Such a pH_{cis}-dependent transition could lead to synchronization in the transitions to subconductance states in such a way that transitions to the high conductance states (levels 5 and 6) will become apparent. However, the small number of transitions to the high conductance states suggests that such synchronization is partial (Fig. 1*C* middle row) and thus indicates incomplete protonation of the channel protein. The effects of low pH_{cis} on the conductance and kinetic properties of the Ca^{2+}-activated SCl channel are consistent with H^+ titration or binding to Ca^{2+} activating binding sites in the cytoplasmic domains of the channel proteins in a manner similar to that suggested for the multiligand gated Ca^{2+} channel [36].

Physiological and Pathological Significance

Low pH_{cis} induced a mode shift in the conductance and kinetics of the SCl channel (Figs. 1, 2, 3 and 6). The low pH_{cis} mediated subconductances (levels 1–3) not only change the amount of the current, indicated from I', that passes through the SCl channel but also could have significantly different functional behavior than the main open state (level 6). For example, subconductances differ in their sensitivity to the metabolic state of the muscle fiber [22] and to second messengers such as cytosolic Ca^{2+} and IP_3 [25, 26]. These facts which point to the presence of multiregulatory mechanisms in vivo together with the variable subconductance states of the SCl channel that indicate a channel substructure fluctuating between opening and closing of nonidentical Cl^- diffusion pathways could provide a mechanism for a tightly regulated response to changes in the intracellular environment. It is concluded that adverse physiological conditions (e.g., hypoxia and fatigue) under which the pH_{cis} becomes acidic could affect contraction *via* modification of the Ca^{2+} counter-current through SCl channels. The fact that both SCl channels (this study) and Ca^{2+}-release channels [29, 36] are similarly modulated by low pH_{cis} and the redox state of these channels [48] supports a

functional linkage between the SCl and Ca^{2+}-release channels acting as components of a molecular mechanism for protecting the muscle from Ca^{2+}-loading under adverse conditions.

Recently, ATP-induced inhibition of the SCl channel was found to be *via* an open channel block mechanism [22]. Furthermore, this channel was modulated by the regulators of the ATP-sensitive K^+-channel [24]. The ATP inhibition of the SCl channel has been confirmed by Ahern & Laver [2] who suggested that the sub mM ATP-induced block of the SCl channel would compromise the role of this channel, particularly in the presence of the ''big'' chloride channel (BCl channel), for providing a counter-current [2]. Firstly, biophysical parameters of ion channel activity recorded from artificial lipid bilayers characterize the minimum requirement for the channel function, and do not necessarily quantitatively reflect the regulatory mechanisms of the channel in vivo. It is too simplistic if these biophysical parameters are used in isolation from other known in vivo regulators, e.g., Ca^{2+}, voltage, pH_{cis} and the reduced environment of the cell. The implication here is that the concentration threshold in vivo for the ATP sensitivity of the SCl channel could be modified by pH_{cis}, ATP/ADP ratio, ADP, lactate, metabolic stress or ATP gradient from the cytoplasm to the ATP-binding sites on the channel protein as has been suggested for ATP-sensitive K^+ channels [3, 11, 44]. Secondly, because ion channel pathways are more efficient than pumps and carriers (*see* [19]), it would require only a small number of briefly open SCl channels to sufficiently provide a counter-current for the Ca^{2+} pump.

Another important point is how can the SCl channel, or any other SR channel including the ryanodine receptor Ca^{2+}-release channel, function in the presence of the large conductance Cl^- channels. This point was raised and addressed by Kourie [23]. Briefly, the high conductance and high P_o for the BCl channels would electrically ''shunt'' any SCl channel contribution to the SR conductance and it would have been difficult to understand the role of the SCl channel if the BCl channels were not regulated in vivo by cellular factors, e.g., luminal ATP inhibits the 150 pS Cl^- channel [22]. Ligand suppression of ion channels in various tissues is well established and is not unique phenomenon confined to large Cl^- channels. The ryanodine receptor Ca^{2+}-release channel in the SR of skeletal muscle is inhibited and activated by physiological concentrations of Mg^{2+} and ATP, respectively [28], while the SR ATP-sensitive SCl channel and the sarcolemmal ATP-sensitive K^+ channels are inhibited by physiological concentrations of ATP [22, 43, 45]. A working hypothesis for the SCl channel operation in vivo based on the in vitro experimental evidence that also considers other cytosolic factors has recently been suggested [21–23]. According to this hypothesis the operation requirements for the SCl channel include an increase in the cytosolic $[Ca^{2+}]_{cyt}$, development of voltage, reduction in the sensitivity of the channel to the redox state and a decline in $[ATP]_{cyt}$.

Ahern and Laver [2] speculated that the ATP-inhibited SCl channel may provide a pathway for ATP transport. This is very unlikely for the following reasons: (i) the ATP-inhibited current seen in the presence of Cl^- gradients remains that of the Cl^- movement through the channel, in a manner similar to that seen in the presence of any other SCl channel blocker, e.g., DIDS and IP_3, and (ii) from an evolutionary stand point, ion channels evolved as efficient transport pathways, allowing the transfer of 10^6 to 10^7 ions per second, for signal transduction mechanisms rather than inefficient transport pathways where ion transfer across the membrane has to be achieved by ''squeezing'' or ''gate crashing'' through these pathways. The large ATP-insensitive Cl^- channels are potential candidates as pathways for the transport of polyanions. Indeed, such a role has already been proposed for the voltage-dependent anion channel [8, 42]. Similarly, it has been suggested that the 150 pS ATP_{cis}-insensitive Cl^- channel may provide a pathway for polyanion transport to the luminal side of the SR [22].

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