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Bimodal Kinetics of a Chloride Channel from Human Fibroblasts

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Abstract. Excised patches were used to study the kinetics of a Cl channel newly identified in cultured human fibroblasts (L132). The conductance of ca. 70 pS in 150 mM symmetrical Cl, and the marked outward rectification ascribe this channel to the ICOR family. Long single-channel recordings (>30 min) revealed that the channel spontaneously switches from a kinetic mode characterized by high voltage dependence (with activity increasing with depolarization; mode 1), into a second mode (mode 2) insensitive to voltage, and characterized by a high activity in the voltage range ±120 mV. On patch excision the channel always appeared in mode 1, which was maintained for a variable time (5–20 min). In most instances the channels then switched into mode 2, and never were seen to switch back, in spite of the eight patches that cumulatively dwelled in this mode 2.33-fold as compared to mode 1. Stability plots of long recordings showed that the channel was kinetically stable in both modes, allowing standard analysis of steady-state kinetics to be performed. Open and closed time distributions of mode 1 and mode 2 revealed that the apparent number of kinetic states of the channel was the same in the two modes. The transition from mode 1 into mode 2 was not instantaneous, but required a variable time in the range 5–60 sec. During the transition the channel mean open time was intermediate between mode 1 and mode 2. The intermediate duration in the stability plot however is not to be interpreted as if the channel, during the transition, rapidly switches between mode 1 and mode 2, but represents a distinct kinetic feature of the transitional channel.

Key words: Human fibroblasts — Chloride channels — Patch clamp — Channel moding

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Introduction

Several studies have shown that ion channels can interconvert among two or more distinct gating patterns of activity, usually called modes. Interconversion can occur spontaneously, or can be induced by agonists modulating the channel protein. Magleby and coworkers (Blatz & Magleby, 1986; McManus & Magleby, 1988) have shown that fast Cl channels and Ca-activated maxi K channels from rat skeletal muscle can switch among three and four modes of activity clearly distinguishable from the marked difference in the distribution of their open and closed times. In both cases, mode-switching was not induced by agonists or modulators or any other external parameter, but seemed to represent an intrinsic property of the channel proteins that spontaneously interconvert among several kinetic macro, multistate worlds. Single glutamate-activated channels in locust muscle undergo sudden spontaneous changes in their kinetic behavior, evidenced by a major shift in the distribution of their open and closed times (Patlak, Gration & Hushewood, 1979). Mode-switching of channels among several patterns of activity was also postulated by Patlak & Ortiz (1985) and subsequently and more convincingly by Bolhe & Benndorf (1995) to explain the slow component of inactivation of Na channels from rat and mouse myocardial cells.

Other studies have instead shown mode-switching to result from agonist modulation of the channel protein. The phosphorylation state of tyrosine residues on a cation channel determines its gating pattern. In particular, dephosphorylation brought about by membrane-bound phosphatases converts a low-P_{open} burstlike activity of the channel into a high-activity mode, characterized by a continuous high-P_{open} kinetic pattern (Wilson & Kaczmarek, 1993). Mode switching similarly promoted by agonist (dihydropyridines Ca agonist Bay K 8644, and Ca antagonists nimodipine and nifedipine) binding to the channel was proposed to account for the different fre-

quency distribution of the three modes of L-type Ca channel gating in cardiac cells (Hess, Lansman & Tsien, 1984). β-adrenergic stimulation through a cAMP-dependent protein kinase was shown to modulate the intermode transitions on the same L-type Ca channel (Yue, Herzig & Marban, 1990). A generic cellular control was instead proposed to modulate the mode-switching among the three different gating patterns of N-type Ca channel from frog sympathetic neurons (Delcour et al., 1993). These authors complemented previous observations that had originally suggested the existence of "reluctant" and "willing" gating modes modulated by neurotransmitters in N-type Ca channels (Bean, 1989). Regardless of whether occurring spontaneously, or induced by agonists, mode-switching always was shown to be a fast and reversible process.

In this study we report the finding that a Cl channel newly identified in human fibroblasts displays mode-switching. The channel presents two major modes of activity, or gating patterns characterized by markedly different voltage sensitivities, and mean open and closed times. The relevant aspects of this channel moding are that upon patch excision the channel always appeared in the highly voltage-dependent mode, and once it has switched into the voltage-insensitive mode, it was never found to reconvert back into the original mode. Another distinctive aspect of the moding of this channel is that the shift or transition from one mode into the other was not instantaneous, but required several to tens of seconds.

Materials and Methods

Preparation

Cultured human fibroblasts (L132) derived from pulmonary endothelium were plated into Petri dishes at a density of approximately ⅓ with respect to confluency, and used within 72 hr.

Electrophysiology

Single-channel currents were recorded by using the patch-clamp technique in the inside and outside-out configuration (Hamill et al., 1981), with borosilicate pipettes (Hilgenberg GmbH, Malsfeld, Germany), pulled with a programmable puller (PUL-100; WPI, Sarasota, FL), whose resistance ranged between 1 and 2 MΩ when filled with standard 150 mM NaCl pipette solution. Currents were amplified with a List EPC-7 amplifier (List Medical, Darmstadt, Germany), and digitized with a 12 bit A/D converter (TL-1, DMA interface; Axon Instruments, Foster City, CA). The pClamp software package (version 5.6; Axon Instruments) was routinely used on a Compaq Pentium PC (Compaq Computer, Houston, TX) for generating the command voltage pulses, recording and archiving the currents, and preliminary analysis of the data. For online data collection, current signals were usually filtered at 3 kHz and sampled at 60 μsec/point. Experiments were carried out at room temperature (18–22°C).

Solutions

Extracellular solution contained (in mM): NaCl 150, KCl 3, $CaCl_2$ 1, $MgCl_2$ 1, MOPS 5, titrated to pH 7.2 with NaOH. For a good single channel current resolution necessary for a reliable kinetic analysis, intracellular solution contained (in mM): NaCl 500, $CaCl_2$ 1, EGTA 10, MOPS 5, titrated to pH 7.2 with NaOH. NaCl 150 replaced NaCl 500 in the cytoplasmic side of the membrane in the experiments carried out in inside-out configuration to measure channel conductance in symmetric Cl condition.

Data analysis

Open probability of the channel was calculated from amplitude histograms of 60-sec recordings in which only a single channel was present. The duration of open intervals was assessed by using the 50% threshold method (Colquhoun & Sigworth, 1983), and the events were binned according to the logarithm of their durations (McManus, Blatz & Magleby, 1987). The resulting distributions of open events were fitted with a sum of exponential functions and the minimum number of components were estimated by the maximum likelihood method (McManus et al., 1987). An additional exponential component was considered significant when the increase in the logarithm of the likelihood was greater than 3. To reduce the error introduced by the limited time resolution, the detected events with a duration shorter than two times the dead time of our recording system (dead time = 115 μsec) were excluded from the fitting. Stability plots were constructed as described by McManus & Magleby (1988). Briefly, the mean durations of groups of 50 sequential open intervals were measured for a continuous recording of about 5,000 open events and plotted against sequential interval number. Data are expressed as mean ± SEM.

Results

Characterization of the Cl Channel

The bimodal behavior was found on anion-permeant channels newly identified in human fibroblasts. Channels were identified as anion permeant on the basis of their anion *vs.* cation selectivity, as shown by the inward-going current in salt gradient condition (150 mM $NaCl_{ext}$ *vs.* 500 mM $NaCl_{int}$) at zero voltage (Fig. 1*A* and *C*). The zero-current potential in these conditions was 13 mV, a value significantly lower than 30 mV expected for a purely selective Cl channel. This indicates that Na could measurably permeate the channel. Quantitatively, the reversal potential of 13.6 ± 1.8 mV averaged from three experiments could be accounted for by the GHK voltage equation assuming a $P_{Na}/P_{Cl} = 0.37$. The *i/V* plot of Fig. 1*C* also shows the marked outward rectification of the channel. Channel activity was characterized by a high voltage dependence, with P_{open} steeply increasing at positive voltages. This feature is quantitatively illustrated by the P_{open} *vs.* voltage relation shown in Fig. 1*B*. Data points were obtained by measuring the P_{open} of long stretches of single channel recordings at varying voltages, and fitted by a Boltzmann function which gave a valency of the gating charge equal to 3.0 and $V_{1/2} = -29$ mV. In four similar experiments including the one shown in Fig. 1 the gating charge and $V_{1/2}$ were respectively 2.8 ± 0.22, and 31.6 ± 2.4 mV. These features, together with the channel (chord) conductance

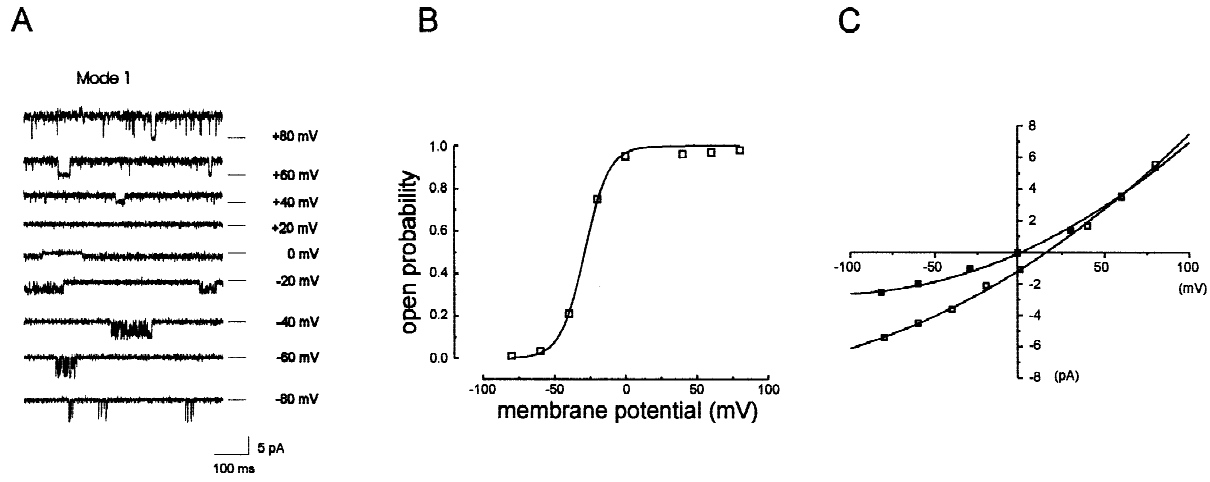

Fig. 1. General features of the ICOR channel from human fibroblasts. (*A*) Single channel records from an excised inside-out patch at varying holding potentials. Internal and external NaCl concentrations were 500 and 150 mM, respectively. Currents were low-pass filtered at 1 kHz. Current levels for open and closed states are indicated in each record. (*B*) Open-state probability *vs.* voltage relationship for the Cl channel shown in *A*. Data points were fitted by a Boltzmann function of the form: $P_{open} = 1/[1 + \exp(zF[V_{1/2} - V]/RT]$, where $V_{1/2}$ is the voltage for half-maximal activation ($P_{open} = 0.5$) and z is the gating charge. The activation function was best fitted with $z = 3.0$, and a $V_{1/2} = -29$ mV. (*C*) Current/voltage relationship in 150 mM symmetrical NaCl (closed squares) and after replacement with 500 mM NaCl in the internal side of the membrane (open squares). Each point in the *i/V* plot was determined via amplitude histograms constructed from single channel records of the type shown in *A*. Each *i/V* relationship is the least-square fit by a polinomial function (solid lines).

of *ca.* 70 pS (at zero mV) in symmetrical 150 mM Cl, assign this channel to the ICOR (Intermediate Conductance Outward Rectifying) family (for review *see* Greger, 1994).

With time, however, these Cl channels often and spontaneously changed the kinetic behavior described above, characterized by a high voltage sensitivity, into a second gating phenotype insensitive to voltage. Typical single channel records taken at varying voltages after the channel had switched into this second gating pattern are shown in Fig. 2. The most dramatic change consists in the loss of the voltage dependence, evidenced by the high activity of the channel at all voltages (Fig. 2), including voltages as negative as −80 mV at which the channel was hardly active before. This aspect is quantitatively illustrated by P_{open} *vs.* voltage plot of Fig. 2*B*. Channel selectivity and permeation were instead unchanged as shown by the constancy of the reversal potential (12 mV) resulting from the *i/V* plot of Fig. 2*C* obtained under same ionic conditions of Fig. 1, and by the outward rectification of the current.

Channel Moding

The change in gating behavior, or moding, is more clearly shown in its temporal development by Fig. 3. Panel *A* presents a continuous 90-sec segment of a long single-channel recording selected to visualize the change in channel behavior (i.e., the spontaneous switch from mode 1 to mode 2). The single-channel recording was taken at −60 mV, a voltage at which the difference in kinetic behavior between the two modes was obvious. The upper trace, typical of the channel in mode 1, is characterized by frequent and extremely short (*ca.* 0.5 msec on average) openings. The lower trace, representative of the channel in mode 2, displays instead very long openings, with the channel open for most of the time. The middle trace presents in its first half a gating pattern intermediate between mode 1 and mode 2, containing both long and very short openings mixed together. This portion of the trace anticipates another major feature of the moding of this channel, that is the moding transition is not instantaneous, but develops over several seconds (*see* later).

The change in the gating pattern, visually detectable in panel *A*, appears clearly in the stability plots of dwell open times and P_{open} (Fig. 3*B* and *C*). The stability plot of dwell open times was constructed by averaging the mean duration of consecutive groups of 50 open time intervals against sequential interval number for the full recording partly shown in Fig. 3*A*, which contained 17,380 intervals. The moving mean of dwell open times fluctuates only negligibly around the overall mean of mode 1 and of mode 2 (indicated by arrows). The stability plot for the closed times is not presented because of the much higher fluctuation of the moving mean around its overall mean value, due to the wider range in the duration of closed events that could be observed in the single channel record. The stability plot of P_{open} for the same recording was constructed by plotting the P_{open} of consecutive segments of the single channel recording containing 50 openings against sequential segment num-

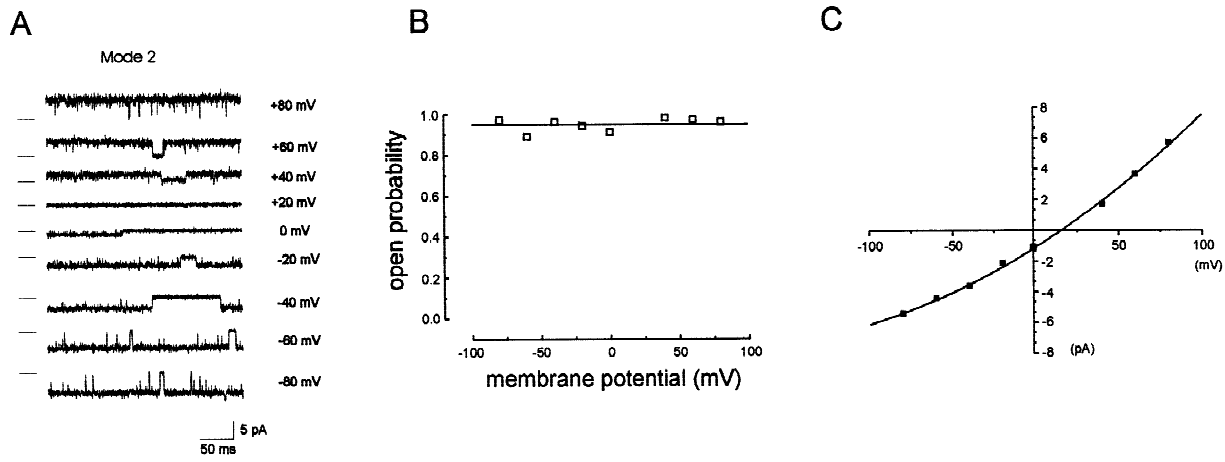

Fig. 2. General features of the ICOR channel in mode 2. (*A*) Single channel records from the same excised inside-out patch of Fig. 1 at varying holding potentials. Internal and external NaCl concentrations were 500 and 150 mM, respectively. Currents were low-pass filtered at 1 kHz. Current levels for open and closed states are indicated in each record. (*B*) Open-state probability *vs.* voltage relationship for the Cl channel shown in *A*. (*C*) *i/V* relationship for the Cl channel shown in *A* (open squares). The individual *i/V* points (symbols) were determined via amplitude histograms constructed from individual segments like those shown in *A*. Each *i/V* relationships is the least-square fit by a polynomial (solid lines).

ber. P_{open} also changed markedly following mode switching (from *ca.* 0.05 to *ca.* 0.85), while fluctuating around the mean value only minor within each mode. The small fluctuation of the moving mean of dwell open times and P_{open} and absence of drifts for both parameters over long recordings indicate kinetic stability of the channel in both modes.

Mode switching showed two other relevant features. First, the channel always showed up in the voltage sensitive mode (mode 1), a conclusion drawn from the observation of 22 excised and 3 cell-attached patches where the channel displayed stable and sufficiently long activity. Second, the switch into mode 2 appeared to be a one way process. In eight patches in which we recorded mode switching (from mode 1 into mode 2) we never saw the channel switch back to the initial mode 1, in spite of the eight patches that cumulatively dwelled in this mode 2.33-fold as compared to mode 1. Additionally, mode switching was never observed in the cell-attached configuration of the patch. It need be added that in this configuration this type of Cl channel appeared rarely, and in those few instances ($n = 3$) in which we could record channel activity, the patches did not survive for more than 20 min.

Steady-state Kinetics of Mode 1 and Mode 2

The stability plots presented in Fig. 3 indicating kinetic stability of channels, a prerequisite for steady-state kinetic analysis, has allowed us to perform this type of analysis, which consisted in determining the number of open and closed states in mode 1 and mode 2. Information on the (minimum) number of open and closed states can be obtained from the significant number of exponential components required to describe the distribution of open and closed times (Colquhoun & Hawkes, 1981). To determine the number of significant exponential components, data points were fitted using the maximum likelihood method (McManus et al., 1987). The distribution of open times of mode 1 and mode 2 were best fitted by a single exponential (Fig. 4*A* and *C*), whereas the closed times, again of both modes, were best described by the sum of four exponential components (Fig. 4*B* and *D*). These results show that the two modes of activity are both described by the same minimum number of open and closed states.

Moding Transition

As already shown in Fig. 2, the transition from mode 1 to mode 2 was not instantaneous, but required a variable time in the range of 3–60 sec. This is shown in Fig. 5 by the stability plot of a channel dwell open time during moding transition. For clarity, the channel with the longest moding transition is reported. The stability plot obtained from this recording shows that the mean open time during the transition is intermediate as compared to mode 1 and mode 2. The fluctuation of the running mean (of consecutive groups of 50 openings) during moding transition was instead considerably larger when compared to both mode 1 and mode 2. Because the significant fluctuation of the mean duration of the open events, indication of a lack of kinetic stability, we have not constructed the dwell time histograms for the transition. Figure 5*A* shows three segments of single channel recording typical respectively of mode 1, the moding

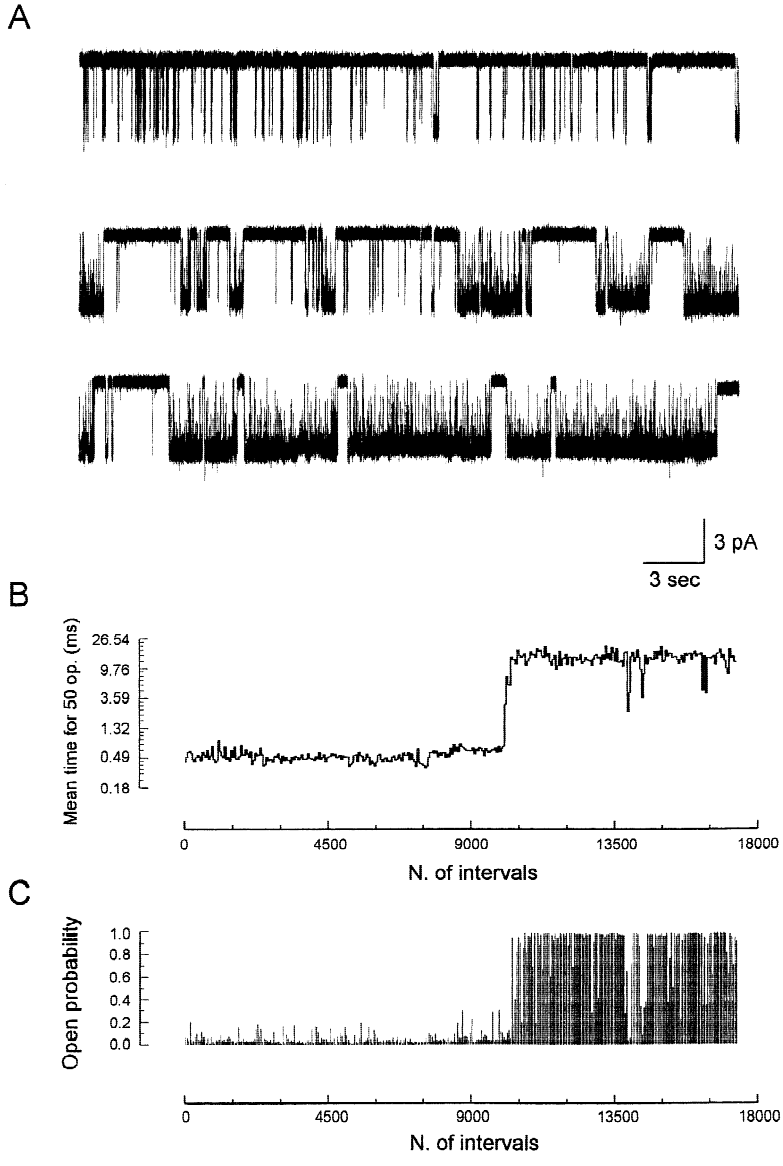

Fig. 3. Switching of the ICOR Cl channel from mode 1 into mode 2. (*A*) Consecutive single-channel records in excised outside-out configuration showing the channel in mode 1 (upper trace), during moding transition (middle trace), and in mode 2 (lower trace). Holding potential is −60 mV, and ionic conditions are $500_{INT}/150_{EXT}$ mM NaCl. Channel openings produce downward currents. (*B*) Stability plot of the moving mean duration of consecutive groups of fifty open intervals showing the transition of the channel between mode 1 and mode 2. Data obtained from the trace partially shown in *A*. (*C*) Open probability plot obtained from the trace partially shown in *A*. Each vertical bar represents the fraction of time spent in the open state in a time segment containing 50 consecutive open and closed events.

transition, and mode 2. It clearly appears that the channel behavior during the transition is different from both modes, presenting open and closed events with intermediate duration when compared to mode 1 and mode 2. The intermediate duration in the stability plot is then not to be interpreted as the channel rapidly switching, during moding transition, between mode 1 and mode 2.

Discussion

In this study we have investigated the gating behavior of a Cl channel from human fibroblasts. The channel displays two major gating patterns that differed markedly for the channel's sensitivity to voltage. In particular, the channel was observed to switch from a highly voltage-sensitive gating phenotype (with apparent gating charge of 2.8), into a voltage-insensitive one (with the channel highly active at all voltages). This feature can be further documented by comparing the P_{open} *vs.* voltage relations of the channel in the two gating modes (Figs. 1*B* and 2*B*), as well as from the stability plot of P_{open} made at −60 mV (Fig. 3*C*).

Apparent loss of voltage sensitivity, with the channel remaining active at all voltages, could result from a shift of channel activation curve towards more negative voltages. In other words, the voltage range within which the channel would previously (mode 1) not be active, now (mode 2) falls in the saturating segment of the Boltzmann curve. To test this possibility, we brought (voltage insensitive) channels in mode 2 at very negative voltages (down to −160 mV), but even at these voltages

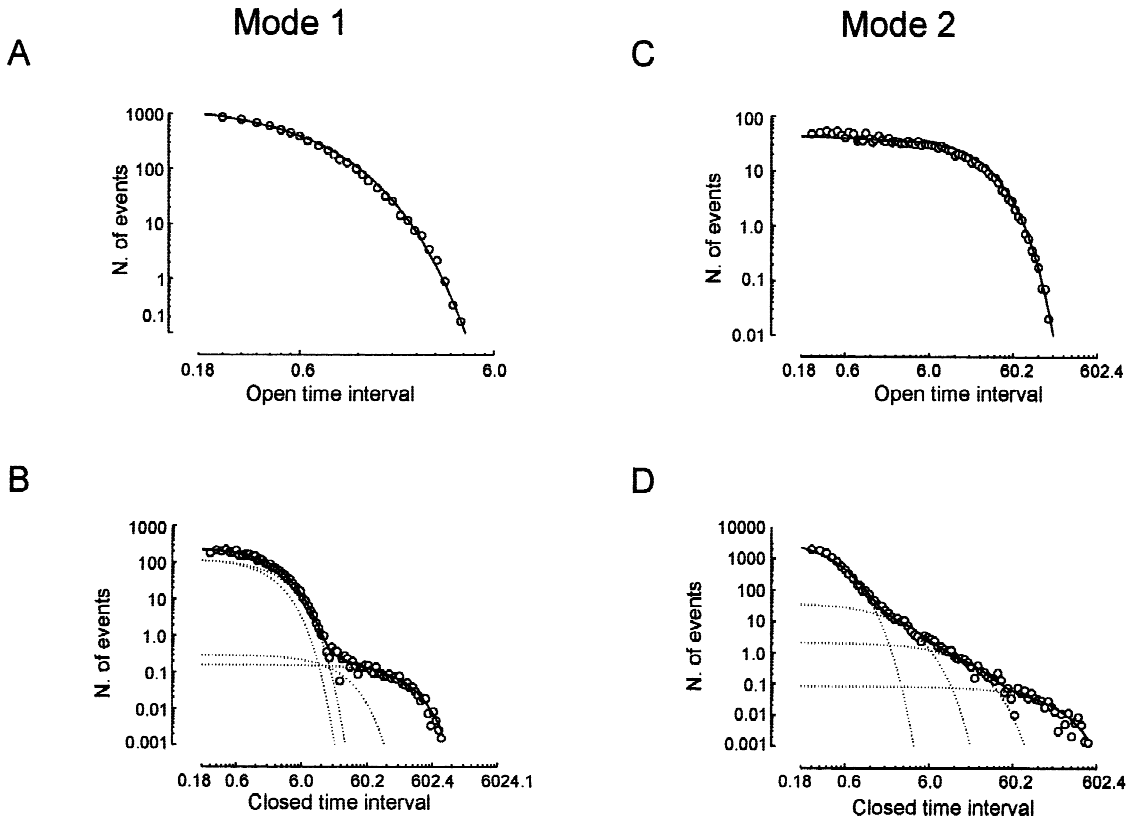

Fig. 4. Histograms of the open and closed times for the ICOR Cl channel in mode 1 and mode 2. (*A* and *B*) Open and closed time distributions in mode 1. The smooth lines are the maximum likelihood fits with the sum of one exponential component to the open intervals, and four exponential components to the closed intervals. The dashed lines in the closed time distribution represent the four individual exponential components. The time constant of the open distribution is 0.44 msec. The time constant of the closed distributions are 200, 19.6, 2.4, and 1.64 msec. (*C* and *D*) Open and closed time distributions in mode 2. The smooth lines are the maximum likelihood fits with the sum of one exponential component to the open intervals, and four exponential components to the closed intervals. The time constant of the open distribution is 21.7 msec. The time constant of the closed distributions are 125, 11.1, 1.79, and 0.26 msec.

the P_{open} was not sensibly lower than at positive voltages, always remaining well above 0.7. This observation may suggest that either the activation curve has shifted further beyond our experimentally testable voltage range, or that the channel has in fact lost its sensitivity to voltage. This event may occur if, upon mode switching, some part(s) of the protein (the voltage sensor, for instance) may get stuck in a "permissive" position (*cf.* Catterall, 1986) so that the voltage-dependent transition between the open and closed state occurring upon its movement back to its "nonpermissive" position is no longer allowed (or allowed with great difficulty), with the result that the channel remains almost constantly open. The marked increase in the duration of open times (Fig. 3*B*) following channel switch into mode 2, expected for a channel that can hardly close, is consistent with this view. The above mentioned test at very negative voltages showing that channel activity was not influenced by this hyperpolarization discards this parameter as a possible determinant of moding. This is a pertinent question given that mode switching has been often reported to be induced by membrane potential (Hoshi & Smith, 1987; Pietrobon & Hess, 1990).

Moding Transition

Relevant observations regarding moding transition were the following. (i) the channel always showed up in the voltage sensitive mode (mode 1); (ii) moding transition into mode 2 occurred after a long residence (five to twenty minutes) in mode 1; (iii) switch into mode 2 appeared to be a one-way process, as the channel was never found to switch back to mode 1; (iv) the transition from mode 1 into mode 2 was not instantaneous, but took tens of seconds to complete. Although the mechanism underlying the channel moding described here is not known, the features described above are relevant to this question excluding several interpretative options, while favoring others. The channel always appearing with mode 1, and switching upon excision, although with delay, into the second mode of activity without ever revert-

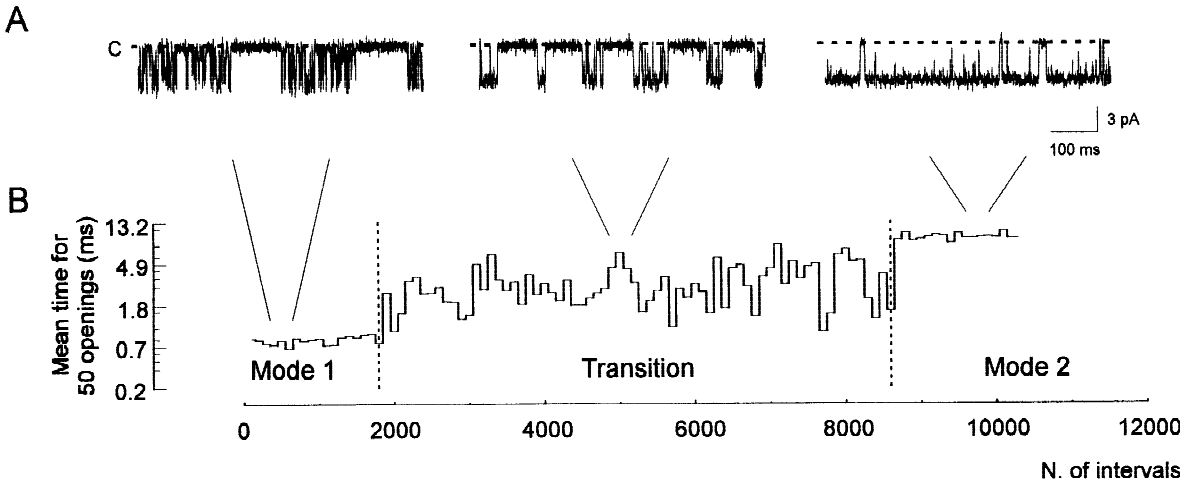

Fig. 5. Kinetic features of the transition mode. (*A*) Single-channel records from excised outside-out configuration in mode 1 (left trace), during the transition mode (middle trace), and in mode 2 (right trace). Holding voltage is −60 mV. Ionic conditions are $500_{INT}/150_{EXT}$ mM NaCl, and channel opening is downward. (*B*) Stability plot of the moving mean durations of consecutive groups of fifty open intervals against sequential interval number.

ing to the original mode may indicate that some cytoplasmic modulator that is lost with patch excision keeps the channel in mode 1. The observation that the phosphorylation state of the channel by cytoplasmic protein kinases is commonly reported as a determinant of mode switching (Wilson & Kaczmarek, 1993; Neumann, Catterall & Scheuer, 1991; Yeu et al., 1990), in conjunction with the dephosphorylation action of membrane-embedded phosphatases may suggest the following hypothetical mechanism. In the native cell the channel is phosphorylated and kept in mode 1. In this mode it is found upon patch excision, and in this mode it remains until membrane-intrinsic phosphatases dephosphorylate the channel, once cytoplasmic protein kinases have diffused away. Dephosphorylation by phosphatases induces the channel to switch into mode 2. The observation that the channel remains phosphorylated for a significant time (up to 20 min) could be explained by considering that in excised patches a considerable amount of cytoplasm is often sucked into the pipette (Sakmann & Neher, 1983; Ruknudin, Song & Sachs, 1991), forming a microenvironment where cytoplasmic macromolecules as protein kinases can be trapped for a significant time. During this time the channel would remain phosphorylated and in mode 1. The observation that the mode transition is not instantaneous, but may take tens of seconds to complete can be explained, within this picture, by postulating that dephosphorylation does not itself induce channel moding, but only makes the channel prone to further changes such as protein oligomerization, the actual determinant of mode switching.

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