J. Membrane Biol. 158, 87–94 (1997)

The Journal of
Membrane
Biology

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Interconverting Gating Modes of a Nonselective Cation Channel from the Tapeworm *Echinococcus granulosus* Reconstituted on Planar Lipid Bilayers

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Received: 30 August 1996/Revised: 31 January 1997

Abstract. A 107-pS (symmetrical 150 mM KCl), nonselective cation channel was reconstituted from a microsomal membrane fraction of the larval stage of the tapeworm *Echinococcus granulosus.* Most of the time, it displayed a high open probability (>0.95) irrespective of either the applied voltage, Ca^{2+}, Ba^{2+}, or tetraethylammonium concentration. Nevertheless, in contrast with this ''leaklike'' behavior, less frequently this ''all-the-time-open'' channel reversibly entered two different kinetic modes. One of them was characterized by lower P_o values and some voltage sensitivity ($V_{1/2} \cong 129$ mV, and an equilibrium constant for channel closing changing *e*-fold per 63-mV change) the kinetic analysis revealing that it resulted from the appearance of voltage-sensitivity in the mean closed times and a sixfold increase in the equilibrium constant for channel closing at 0 mV. The other mode was characterized by a very fast open-close activity leading to poorly resolved current levels and a P_o around 0.6–0.7 which, occasionally and in a voltage-sensitive manner, entered a long-lived nonconducting state. However, the rare nature of these mode-shifting transitions precluded a more detailed analysis of their kinetics. The conductive properties of the channel were not affected by these switches. Model gating alone does not seem to ensure any physiological role of this channel and, instead, some other channel changes must occur if this phenomenon were to be of regulatory importance in vivo. Thus, mode-shifting might constitute an alternative target for channel activity modulation also in tapeworms.

Key words: *Echinococcus granulosus* — Cestode — Nonselective cation channels — Voltage-sensitivity — Modal gating — Planar lipid bilayer reconstitution

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Introduction

We have been concerned with ion transport aspects across the outer surface of the larval form (*protoscolex*) of the tapeworm *Echinococcus granulosus* (Ferreira, Trecu & Reisin, 1992; Ibarra & Reisin, 1994; Grosman & Reisin, 1995), a parasitic invertebrate belonging to the phylum Platyhelminthes which has no alimentary canal and whose outer surface is covered by a syncytial, brush-borderlike epithelium (*tegument*) across which the interchange of substances between the parasite and the host takes place (Morseth, 1967; Chappell, 1980; Brodie & Podesta, 1981). In addition, protoscoleces are tissue-dwelling living-forms affecting both livestock and humans giving thus rise to a condition known as the hydatid disease.

Both the patch-clamp and the planar lipid bilayer reconstitution techniques allowed significant advances in the knowledge of ion channels both as members of the ion transport machinery and as examples of complex macromolecules. Even though these electrophysiological techniques have found a widespread use in many fields of biology, their impact on the understanding of multicellular, helminth parasites at the molecular level has been very scarce thus far (Blair et al., 1991; Day, Bennett & Pax, 1992; Blair & Anderson, 1993, 1994; Day et al., 1993; Grosman & Reisin, 1995).

We have previously reported (Grosman & Reisin, 1995) several general properties of two cation-selective channels from a fractionated microsomal preparation of *E. granulosus* protoscoleces, obtained from naturally infected sheeps, by reconstitution on planar lipid bilayers. One of them displayed a weak-field selectivity sequence (as determined by permeability ratios: $Cs^+ \cong K^+ > Na^+ > Li^+ > Ca^{2+}$), a high open channel conductance (107-pS, symmetrical 150 mM KCl) and, most frequently, a high open probability ($P_o > 0.95$) irrespective of either the

applied voltage, Ca^{2+}, Ba^{2+}, or tetraethylammonium concentration.

The paucity of knowledge about the subcellular distribution of enzymes in the ovine strain of larval *E. granulosus* severely reduces the usefulness of the determination of marker enzyme activities as a means of estimating a subcellular origin for the isolated membrane vesicles (Grosman & Reisin, 1995). Furthermore, as the few enzymological profile studies reported to date revealed that considerable differences exist between higher animals and tapeworms, between different tapeworms, and even between different strains of the species *E. granulosus* (Kilejian, Schinazi & Schwabe, 1961; McManus & Barrett, 1985; Siddiqui & Podesta, 1985), the extrapolation of a classical marker enzyme distribution pattern to our specific case seems not to be valid. However, the fact that a similar 95-pS (140 mM K^+ 139 mM Na^+, pipette/bath) channel unit was recorded in inside-out patches from the apical membrane of the tegument covering the adult stage of the related parasitic flatworm. *Schistosoma mansoni* (Day et al., 1992) suggests that an outer tegumental origin for the channel recorded by us is likely. Thus, given the single-channel characteristics and its putative localization, it was difficult to propose a physiological role for such an unusual channel in transtegumental ion transport and/or tegumental homeostasis during *E. granulosus'* life cycle (Grosman & Reisin, 1995).

In this paper we report that, in contrast with this ''leaklike'' behavior, less frequently this ''all-the-time-open'' channel reversibly entered different kinetic modes (*modal gating*) exhibiting lower P_o values, voltage-sensitivity, and long-lived, nonconducting sojourns. Thus, modal gating might constitute an important mechanism underlying the physiological regulation of this channel.

Materials and Methods

Membrane Vesicle Preparation

E. granulosus membrane vesicles were prepared as previously described (Grosman & Reisin, 1995). Briefly, *E. granulosus* protoscoleces were obtained from fertile hydatid cysts from livers and lungs of infected sheeps slaughtered at local abattoirs. Vesicles were prepared from a whole-worm homogenate in 10-mM 10-N-[2-Hydroxyethyl]piperazine-N′[2-ethanesulfonic acid] (HEPES)-KOH, pH 7.4 buffer solution containing (mM) 0.2 ethylene glicol-bis (β-amino ethyl ether) N, N, N′, N′-tetraacetic acid (EGTA), 0.4 phenylmethylsulfonyl fluoride (PMSF), and 250 (8.6% w/v) sucrose. The homogenate was allowed to sediment and the supernatant was made 150 mM with solid KI and incubated for 45 min under constant and gentle stirring. The corresponding postmitochondrial supernatant was laid on the top of a discontinuous sucrose gradient (15, 30 and 50% w/v) in a 10-mM HEPES-KOH, pH 7.4 buffer containing 0.2 mM EGTA, and it was centrifuged at 18500 rpm (28,500 × *g*) for 90 min. All steps were carried out at 0–4°C. The vesicles used in the reconstitution experiments came from the 8.6–15% (20% of the experiments) and 15–30% (80% of the experiments) sucrose interfaces.

Channel Reconstitution

Lipid bilayers were formed by spreading a 17-mg/mL neutral phospholipid mixture of synthetic origin (Avanti Polar Lipids, Birmingham, AL.) in *n*-decane (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine:1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, 7:3, by weight) over a 250-μm diameter aperture in a polystyrene cuvette by means of a thin glass or plastic rod. The cuvette was inserted into one of a pair of pits machined on a polyvinyl chloride holder thus defining two aqueous compartments (800 and 1600 μL) separated by a planar lipid film as described by Alvarez, Benos and Latorre (1985). Both sides of the lipid bilayer were bathed by a 10–15 μM Ca^{2+} solution which was H^+-buffered with 10 mM 3-(N-morpholino) propanesulfonic acid (MOPS), adjusted to pH 7.4 with either KOH or N-methyl-D-glucamine. At the beginning of every experiment the *trans* side of the bilayer was bathed only by this solution while that bathing the *cis* side also contained 150 mM KCl so as to generate a transbilayer osmotic gradient that promoted the vesicle-planar bilayer fusion (Cohen & Niles, 1993). Vesicles were added in small aliquots to the *cis* compartment and the resulting suspension was continuously stirred with a small magnetic bar until a channel incorporation was detected. Once a channel insertion took place the composition of the solutions bathing the planar lipid bilayer was modified, as indicated in the text, by adding small volumes of concentrated stock solutions to either compartment.

Electrical Recordings

Voltage was applied to the *trans* solution with either a DC voltage source or a function generator, the opposite side, *cis*, having been defined as virtual ground. Nevertheless, throughout this paper, the voltage is referred to as the *cis* minus *trans* voltage. Electrical contacts were made through Ag/AgCl electrodes and 200 mM KCl, 2% (w/v) agar bridges. The current across the bilayer was recorded using a current-to-voltage converter with a 10-Gohm feedback resistor. The output voltage was low-pass filtered at 1 kHz (−3 dB) with an eight-pole, Bessel-type filter (Frequency Devices, Haverhill, MA) and displayed on an oscilloscope. Channel recordings were simultaneously digitized with a pulse code modulator (VR-10, Instrutech, Great Neck, N.Y.), stored on videotapes with a VCR and, later, acquired at 4 kHz with an AT computer for subsequent analysis. Some current traces shown in the Results section were further filtered for display purposes. In all cases overall cutoff frequency values (fc) were calculated (Colquhoun & Sigworth, 1983) and are indicated as the –3dB values. Bilayer formation was monitored by applying a 2.5 mV peak-to-peak, 20 Hz, triangular wave. Typically, 100–200 pF bilayers were obtained. All the experiments were performed at room temperature (20–25°C).

Data Acquisition and Analysis

pClamp Version 5.5 (Axon Instruments, Foster City, CA) and Sigmaplot Version 4.02 (Jandel Scientific, Corte Madera, CA) softwares were used.

Results

A 107-pS (symmetrical 150 mM KCl) nonselective cation channel, presumably located at the apical surface of

the tegument of protoscoleces, has been studied by means of the reconstitution technique (Grosman & Reisin 1995). Some single-channel current traces are shown in Fig. 1 where three types of interconvertible kinetics can be observed. In all the examined single-channel bilayers the channel followed the gating mode illustrated in part A during either the total length or, at least, a part of the record. This dominant mode was characterized by a P_o greater than 0.95 and by the occurrence of long open intervals interrupted only by very brief closures at every applied voltage (Fig. 1*A* and Fig. 2).

Figure 1*B* illustrates an alternative mode during which the P_o was lower, and voltage-sensitive (Fig. 2) at the expense of an increase in the mean closed times as applied voltage was made more positive (Fig. 3*B*). Besides, as voltage was made more negative, channel behavior became increasingly more similar to that displayed, at *every* tested potential, by the channel while in the dominant mode. This voltage-sensitive mode was observed in 3 out of 17 single-channel bilayers, alternating with the most usual mode within a given record, an example of which is illustrated in the continuous traces of Fig. 1*C*. We further examined this voltage-sensitivity by plotting P_o *vs.* voltage for both modes. Experimental data points were fitted by the following equation (Boltzmann equation):

$$P_o = \frac{1}{1 + K_o \exp(z\delta FV/RT)} \qquad (1)$$

where K_o is the equilibrium constant for channel closing at 0 mV, z and δ are, respectively, the charge of, and the fractional voltage drop sensed by the gating particle, V is the applied voltage, and F, R and T have their usual meanings. On one hand, for the dominant, voltage-insensitive mode, K_o and $z\delta$ turned out to be 0.02 and 0.08, respectively. On the other hand, for the voltage-sensitive gating mode, these values were 0.13 and 0.40, respectively. Hence, the slightness of the voltage-sensitivity recorded during this different kinetic mode is primarily due to the very low value of the equilibrium constant for channel closing at 0 mV which is reflected by a P_o *vs.* voltage curve shifted towards very positive potentials [$V_{1/2} = (-RT/z\delta F) \ln K_o \cong 129$ mV]. Since we do not know the sidedness of the isolated vesicles, we are not able, thus far, to make any physiological interpretation of this voltage-sensitivity. If vesicles were inside-out oriented (and then the cytoplasmic side of the reconstituted vesicles would face the *cis* compartment) such P_o *vs.* voltage relation would imply that this channel is hyperpolarization-activated, as is the case of a renal dihydropyridine-sensitive Ca^{2+} channel from mouse distal convoluted tubule cells (Matsunaga et al., 1994), and a nonselective high conductance channel from bovine pigmented ciliary epithelial cells (Mitchell & Jacob, 1996). On the contrary, if vesicles were right side-out, such P_o *vs.* voltage relation would correspond to the most common depolarization-induced activation.

A third mode, (Fig. 1*D*, and *E*) was characterized by a very fast open-close activity (*flickery* mode) which led to poorly resolved current levels and a P_o around 0.6–0.7. This mode was observed in 5 out of 17 single-channel bilayers, also alternating with the dominant behavior within a given experiment. In part *D*, some continuous traces recorded during an experiment with 150 mM KCl in the *cis* side and 145 mM LiCl in the *trans* side are shown. In addition to the flickery activity, the voltage-sensitive entry into a long-lived nonconducting state was also evident in this single-channel bilayer. Neglecting these long shut intervals, the voltage-insensitive P_o averaged over the +40 − +80 voltage range was 0.72 while the average mean open and closed lifetimes were 3.41 msec and 1.29 msec, respectively. In part *E*, the reversible dominant ↔ flickery mode interconversion is shown for a different bilayer in symmetrical 150 mM KCl. In this particular case, the sojourn in the flickery mode was much briefer.

To get a better understanding of the voltage-sensitive mode, the individual kinetic constants were investigated and compared with those for the voltage-insensitive one. Dwell time histograms for both kinetic modes were best fitted (plotting number of events *vs.* time, both axes being in linear scales, and not correcting for missed events) by monoexponential probability density functions (Fig. 3*A*). This way, the mean closed time reduces to β^{-1}, where β is the rate constant leading away from the closed state, and the mean open time reduces to α^{-1}, where α is the rate constant leading away from the open state of the channel (*see* scheme in Fig. 3*B*). By assuming that the voltage-sensitivity arose from the displacement of charged or dipolar channel-residues between two positions located at different electrical potentials, kinetic constants were plotted *vs.* voltage and fitted by exponential functions of the form (Fig. 3*B*):

$$\tau = \tau_0 \exp(z\delta FV/RT) \qquad (2)$$

where τ is the mean open or closed time as a function of the applied voltage V, τ_o is the corresponding value at 0 mV, z is the charge of the gating particle, δ is the distance from the energy barrier maximum to either position expressed in terms of the fractional voltage drop, and F, R and T have their usual meanings. The sign of the $z\delta$ parameter indicates whether τ is an increasing (positive sign) or decreasing (negative sign) function of the applied voltage. The τ_o and $z\delta$ values in the voltage-sensitive mode turned out to be, respectively, 15.27 msec and −0.18 for the open state, and 1.82 msec and 0.32 for the closed state. In turn, these values in the usual kinetic mode were 30.44 msec and −0.14 for the open state, and 0.64 msec and a negligible $z\delta$ value for the closed state.

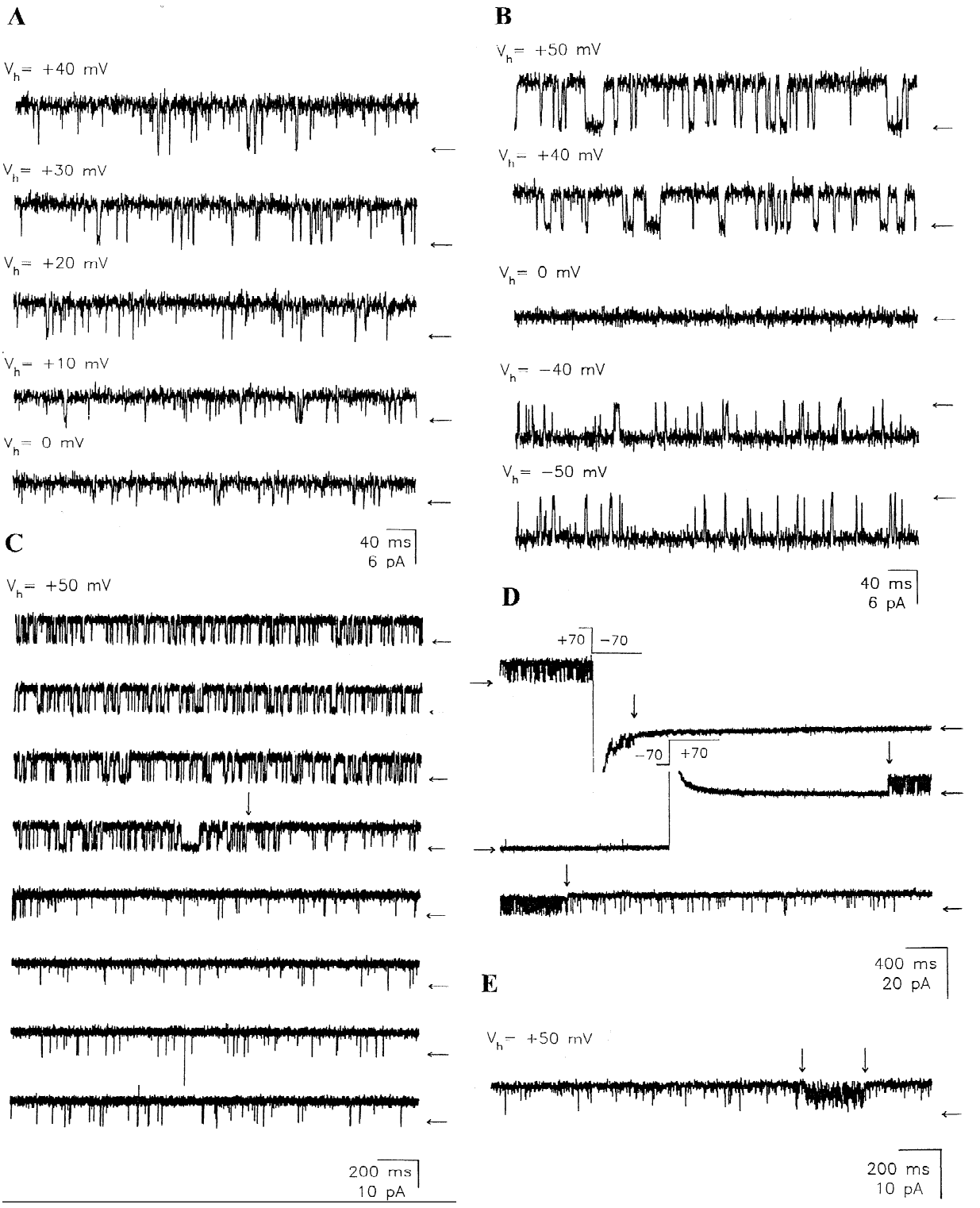

Fig. 1. Single-channel features and kinetic mode shifting. Horizontal arrowheads indicate the closed state of the channel. (*A*) Single-channel traces, representative of the dominant, voltage-insensitive kinetic mode, at different voltages and with 150 mM KCl added to the *cis* side (fc = 1 kHz). (*B*) Single-channel traces, representative of the voltage-sensitive kinetic mode, at different voltages and with 300 mM KCl added to both sides of the bilayer (fc = 1 kHz). (*C*) Continuous single-channel traces recorded at +50 mV with symmetrical 300 mM KCl. Vertical arrowhead indicates a voltage-sensitive → voltage-insensitive mode switch (fc = 1 kHz). (*D*) Continuous single-channel traces in response to ±70 mV voltage steps with 150 mM KCl added to the *cis*, and 145 mM LiCl added to the *trans* side of the bilayer. The first two vertical arrowheads indicate, respectively, the channel entering and the channel leaving an inactivated state. The third one indicates a flickery → dominant mode switch. The capacitive transients were not subtracted (fc = 240 Hz). (*E*) Single-channel trace recorded at +50 mV with 150 mM KCl added to both sides of the bilayer. Vertical arrowheads indicate a dominant → flickery → dominant mode switch (fc = 450 Hz).

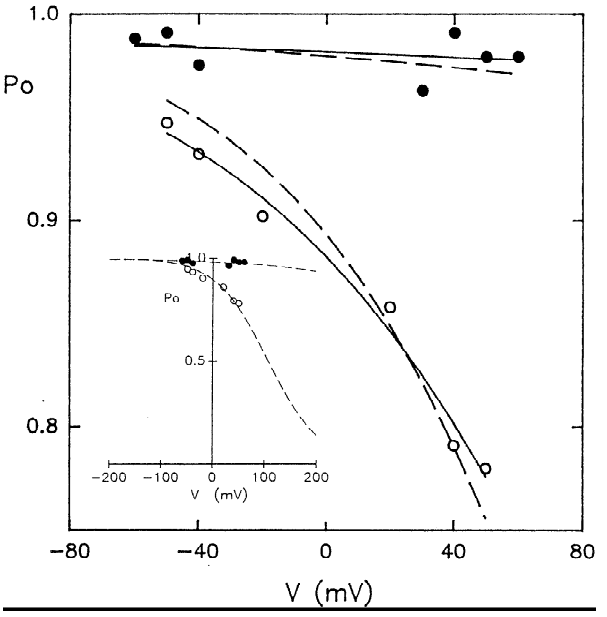

Fig. 2. P_o *vs.* voltage plots for the dominant (filled circles), and for the voltage-sensitive (open circles) kinetic modes. Solid lines are fittings to the Boltzmann equation (Eq. 1). Broken lines are the predicted P_o values on the basis of the estimated rate constants at 0 mV and their respective voltage-sensitivities. Inset: P_o *vs.* voltage plots on an expanded voltage scale. Only the experimental data points and the predicted values are displayed.

These values were combined [$P_o = (1 + \alpha/\beta)^{-1}$] to give the predicted P_o *vs.* voltage parameters (K_o and $z\delta$): 0.12 and 0.50 for the voltage-sensitive, and 0.02 and 0.16 for the voltage-insensitive mode, being consistent with the values obtained from the direct analysis of P_o *vs.* voltage. The dashed lines in Fig. 2 represent the calculated P_o values as a function of voltage. The superposition of the experimental and the expected (on the basis of kinetic constants and voltage-sensitivities) values suggested that channel behavior might be well approximated by a two-state, closed ↔ open kinetic scheme while in either gating mode.

The conductive properties of the channel in the dominating gating mode were described earlier (Grosman & Reisin 1995) and, during the experiments leading to this paper, neither the conductance nor the P_{Cl}/P_K and P_{Li}/P_K ratios were affected by the channel entering the alternative kinetic behaviors. Nevertheless, the paucity of such mode ''excursions'' precluded a more detailed analysis of their kinetics and of the conductive changes (if any) involved in such conversions as well as of the putative factors influencing them.

Discussion

The close approach to ionic channels achieved by their study at the single-entity level has been revealing the occurrence of certain functional heterogeneity among channels displaying a given ion-channel activity. In general, the reasons for such variability are unknown but the proposed mechanisms range from genetically encoded heterogeneity to different activities displayed by a single channel-protein molecule. This phenomenon has been documented to occur between independent experiments and/or during the course of a given experiment under a fixed set of conditions. On one hand, the *inter*-experiments heterogeneity has been attributed, in some cases, to the existence of channel isoforms derived from different genes, different transcripts, splice variants, variable subunit composition and/or irreversible postransla-

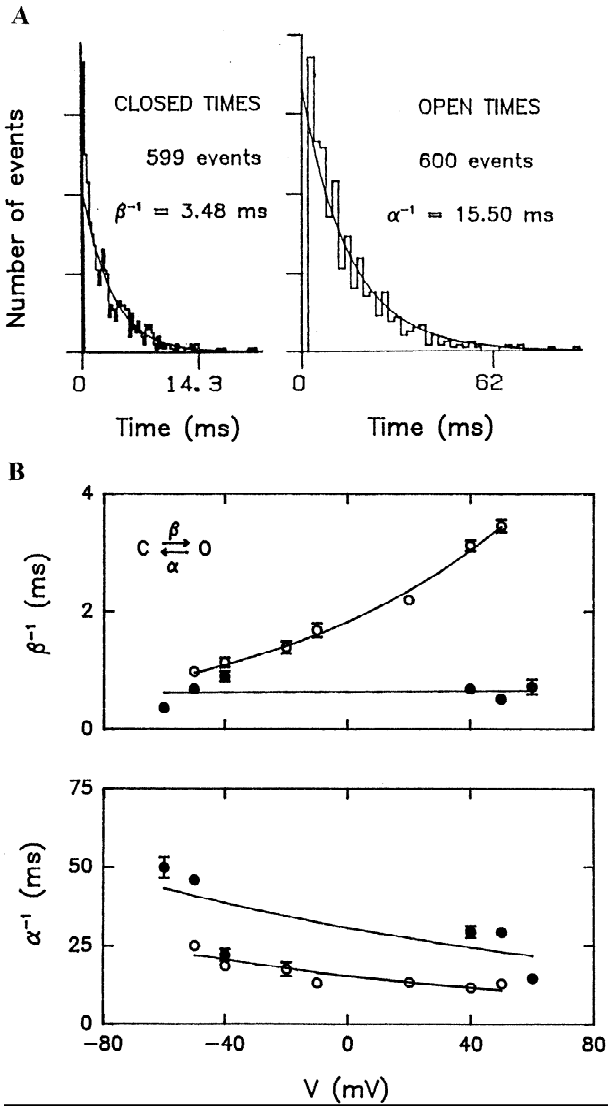

Fig. 3. (*A*) Dwell time histograms for both open and closed intervals at +50 mV during a sojourn in the voltage-sensitive mode. Histograms were fitted by monoexponential functions. (*B*) Time constants *vs.* voltage plots for the dominant (filled circles) and for the voltage-sensitive (open circles) kinetic modes. Continuous lines are fittings to exponential functions. (Eq. 2).

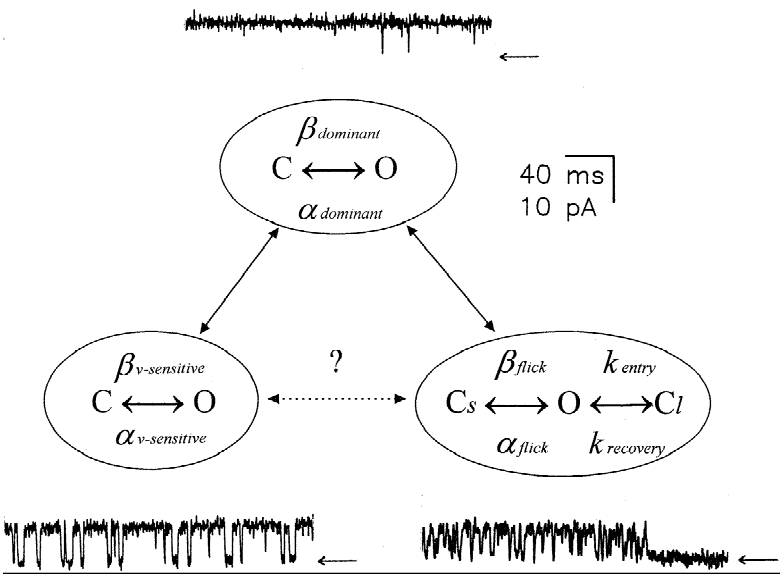

Fig. 4. Working hypothesis for the nonselective cation channel. The C*s* ↔ O ↔ C*l* scheme is meant to illustrate the coupling between the flickery activity and the apparent channel inactivation but it is not intended to be a rigorous model of the kinetics of this mode. C*s* and C*l* denote the short-lived and the long-lived nonconducting states, respectively. The failure to record transitions between this mode and the voltage-sensitive one may simply reflect their very low frequency rather than their impossibility of occurrence.

tional modifications, in such a way that different covalent structures of the channel macromolecule underlie such variable behavior. On the other hand, the occurrence of *intra*-experiment variability needs a dynamic mechanism enabling the channel to adopt, alternatively, different kinetic modes. Though many kinetic studies had success in modelling channel behavior in terms of a few conductive and nonconductive states, in many other cases it was found that a single kinetic scheme does not suffice to account for channel activity but that several such models are necessary, instead (Hess, Lansman and Tsien, 1984; Blatz & Magleby, 1986; McManus & Magleby, 1988; Silberberg et al., 1996). It seems clear that, even though the underlying mechanisms are not yet fully understood, almost every channel type may display such shifting behavior without being restricted to any particular conductive or kinetic characteristic.

In this paper we reported the occurrence of the latter phenomenon in a nonselective cation channel of parasite origin. Since the 107-pS channel was reconstituted from fragmented membrane vesicles (and thus the cytosolic constituents were greatly diluted) and the bilayer was bathed by solutions containing only salts and a pH buffering system, the role of binding of ligands or of reversible covalent modifications (phosphorylation, thiol-disulfide exchange) of the channel or channel-associated proteins as the molecular basis of the exhibited behavior is unlikely. In addition, as modal gating was reported to occur in many native membranes, as revealed by patch-clamp experiments, the artificial nature of the *n*-decane-containing planar lipid bilayers used in our experiments is unlikely to be responsible for such a kinetic feature though some effects cannot be totally ruled out. Thus, most probably, these kinetic mode interconversions reflect intrinsic properties of the channel or neighboring proteins themselves which, in turn, might be modulated in vivo. This way, for this nonselective cation channel, mode-shifting would arise from slow transitions between three different sets of kinetic states (modes) whose corresponding transition rates are on a much faster timescale than those governing such slow gating mode-connecting processes (Fig. 4). A simple mechanism for the dominant ↔ voltage-sensitive mode switch would invoke the existence of a gating particle having two possible positions, in either gating mode, within the electric field. Most commonly, these positions would be at very similar electrical potentials such that the probability of finding the gating charge in either one (reflected by the P_o) would be unaffected by the applied voltage (dominant, voltage-insensitive mode). Nevertheless, more rarely, a proper reorientation of these positions (occurring on a much slower time scale than the gating activity does) would change their location with respect to the electric field such that they are no longer isopotential. In such a scenario, the equilibrium constant of the charge at either position would, thus, become a function of the applied voltage (voltage-sensitive mode). Moreover, such rearrangement would not only lead to the appearance of voltage-sensitivity but also to an increase in K_o from 0.02 to 0.12–0.13, namely to a relative stabilization of the closed state with respect to the open state by ~1.1 kcal/mol [$\Delta\Delta G = RT\ln(K_{01}/K_{02})$] even in the absence of an applied voltage. The finding that the mean open times during the voltage-sensitive mode were lower, at every examined voltage, than those during the voltage-insensitive one arises, at least partly, from the fact that the longer mean closed times during the former allowed more closing events to be detected (events were detected following a half-amplitude threshold-crossing criterion), thus reducing the number of undetected gaps between openings

as compared with the dominant mode. If this measurement artifact were the only cause of such mean open time shortening then the difference between both modes would reside, only, in the increase of the energy barrier the channel has to surmount before it can get open, leaving the barrier leading to the closed state unaffected. However, modal gating alone does not seem to ensure any physiological role for this channel: the voltage required to attain a P_o of 0.5 ($V_{1/2}$) in the reconstituted bilayer system is as much as 129 mV during the ''voltage-sensitive'' mode, which is an indication that some other channel-changes must also occur if mode-shifting were to be of regulatory importance in vivo.

Modal gating has been extensively reported for example, for the glutamate receptor channel from locust muscle (Patlak, Gration & Usherwood, 1979), for the Ca^{2+}-activated K^+ channel from rat skeletal muscle (Moczydlowski & Latorre, 1983; McManus & Magleby, 1988), for the cardiac dihydropyridine-sensitive Ca^{2+} channel (Hess, Lansman & Tsien, 1984), for the cardiac SR K^+ channel (Rousseau et al., 1992), for the ryanodine-sensitive Ca^{2+}-release channel from rat brain (Vélez et al., 1993) and from canine cardiac muscle (Zahradníková & Zahradník, 1995), for the fast chloride channel from rat skeletal muscle (Blatz & Magleby, 1986), and for the anion channel from plant guard cells (Dietrich & Hedrich, 1994), among others. Yet, its physiological role is still uncertain. Thus, even when the 107-pS nonselective cation channel proved to be insensitive to a variety of agents known to affect other channels' activity, and the interconversion between the different kinetic modes appeared spontaneously, modal gating might constitute an alternative target for channel activity modulation also in the syncytial epithelium covering the body surface of platyhelminth parasites.

This work was partially supported by grants from CONICET (PID 3673/92) and the University of Buenos Aires (FFA 070 and 126). CG is a fellow of the University of Buenos Aires. The skillful technical assistance of Mrs. Marisa Battelli de González Avila is greatly acknowledged.

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