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Periodic forcing of a single ion channel: dynamic aspects of the open-closed switching

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Abstract An unconventional approach to studying ion channel kinetics is exploited. Here we describe the effects of a periodically varying membrane potential on the open-closed transitions of single K^+ channels. The use of cycle histograms allows one to measure the transition probabilities as functions of the stimulus phase. The results show that such probabilities vary with the stimulation frequency and with the stimulus history, thus highlighting a dynamic aspect in the switching of this ion channel.

Key words Ion channels · *Hirudo medicinalis* · Periodic forcing · Kinetics

Introduction

Ion channel switching is normally investigated by measuring it at several values of the holding potential, or by using step-like command potentials to study how channels relax towards the new equilibrium. Two-pulse command potentials were used to limit to a subset the states visited by the channel (Oxford 1981), a method somewhat similar to that of conditional probabilities (McManus and Magleby 1985; Magleby and Weiss 1990; Petracchi et al. 1991; Barbi and Petracchi 1992). However, the use of a long lasting repetitive stimulation allows one to study the channel while it is maintained far from equilibrium. Here, this quite uncommon approach is used by measuring single-channel activity under periodic voltage stimuli. The initial reason for this approach was the issue of the possible existence of dynamic components in the open-closed switching of ion channels. The use of periodic stimuli can be considered as an experimental trick to detect the deterministic nature of noise-like signals: in particular, the presence in the output signal of subharmonics of the forcing frequency can be considered as a signature of nonlinear dynamic systems. However, the results we obtained in the first series of experiments gave no indications in this direction; the output of the experiment was instead a direct evaluation of the rate constants under periodic forcing (Petracchi et al. 1994).

Periodic stimulation was used in another paper (Bezrukov and Vodyanoy 1995) in a study of artificial membranes containing alamethicin channels; in that paper the attention was focused on the enhancement of the current flowing through the membrane due to the noise combined with the periodic stimulus (stochastic resonance), without any evaluation of the rate constants.

According to Petracchi et al. (1994), the investigation of the rate constants as functions of the stimulus phase is made possible by the simultaneous recording of the channel current and the command potential. Thus, non conventional analytical tools, such as cycle histograms, can be used to evaluate the rate constants as functions of the stimulus phase during the stimulation.

The channel studied here, as well as in Petracchi et al. (1994), is responsible for the resting K^+ permeability in central neurons of *Hirudo medicinalis* and was first described as voltage-independent (Pellegrini et al. 1989). In fact this channel is affected only by potentials above +60 mV, which cause the appearance of a class of long closures (LC), lasting for seconds. Step command potentials, used to compare the results obtained with the two different modalities of stimulation, revealed monoexponential relaxations for both the open-closed and the reverse transitions, suggesting a tentative two-state kinetic scheme O↔LC (Petracchi et al. 1994).

The sinusoidal potential synchronizes the LCs and the results of the analysis revealed an unexpected behaviour of the channel: the rate constant for LC-to-open transitions is not a function of the stimulus value, but leads, in phase,

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the imposed potential. In the present paper this unexplained effect is investigated by varying the amplitude, frequency and mean value of the applied periodic potential, aiming to study the relationship between the rate constants and the stimulus parameters.

Materials and methods

Inside-out patch-clamp recordings (Hamill et al. 1981) were performed at 20–22 °C, from single K^+ channels excised from the cell body membrane of identified AP neurones of *Hirudo medicinalis* (Muller et al. 1981). A detailed description of the preparation has been reported in Pellegrini et al. (1989). The experimental bath contained a high K^+ solution: 120 mM KCl; 1 mM $MgCl_2$; 10 mM Hepes/KOH; 10 μM $CaCl_2$; pH 7.2; osmolarity was adjusted with glucose. Patch electrodes with a resistance of 4 MΩ were pulled in two stages from 1.5 mm o.d. glass capillary tubing and filled with a filtered solution, which had the same composition as the bath solution. Channel currents were recorded with a patch amplifier Axopatch 1 D (Axon Instrument, Foster City, CA), low-pass filtered at 0.5 KHz and stored with a modified videotape recorder. Recorded segments were AD converted at 1 KHz with a Labmaster TL1 interface and then analysed with pClamp software (Axon Instrument, Foster City, CA) and with software we developed for this purpose.

After excision, the channels progressively increased the percent of time open, reaching a typical stationary value around 70%. When steady membrane potentials above +60 mV were applied, the LCs appeared, their frequency increasing with the potential value. The application of sinusoidal command potentials of suitable amplitude across the amplifier virtual ground elicited the onset of the LCs on the depolarizing portion of the stimulus, whereas reopening occurred in the hyperpolarizing portion (Fig. 1 A). In order to analyse the channel current recorded during periodic modulation, a method, fully described in Petracchi et al. (1994), was implemented to subtract leakage and capacitive currents and to extract from the overall current the conductance transitions due to channel switching. Briefly, segments of the record free of openings, spanning at least one cycle, were used as a template and subtracted from the overall channel current signal. The resulting signal (Fig. 1 B) was reduced to a dichotomic signal (Fig. 1 C), using a half-amplitude threshold criterion.

The identification of the LCs at a steady potential was easy: closings shorter than a certain value C were considered part of the normal bursting switching of this channel, while those longer than C were included in the LCs class. Since the two classes were well separated, it was trivial to find suitable values for C. During sinusoidal stimulation the use of such a criterion was more critical: the periodic hyperpolarization forced the channel to reopen, thus shortening the LCs. In order to choose the C value suitable to discriminate LCs, we examined the distribution of the closed-open transitions as a function of the stimulus phase.

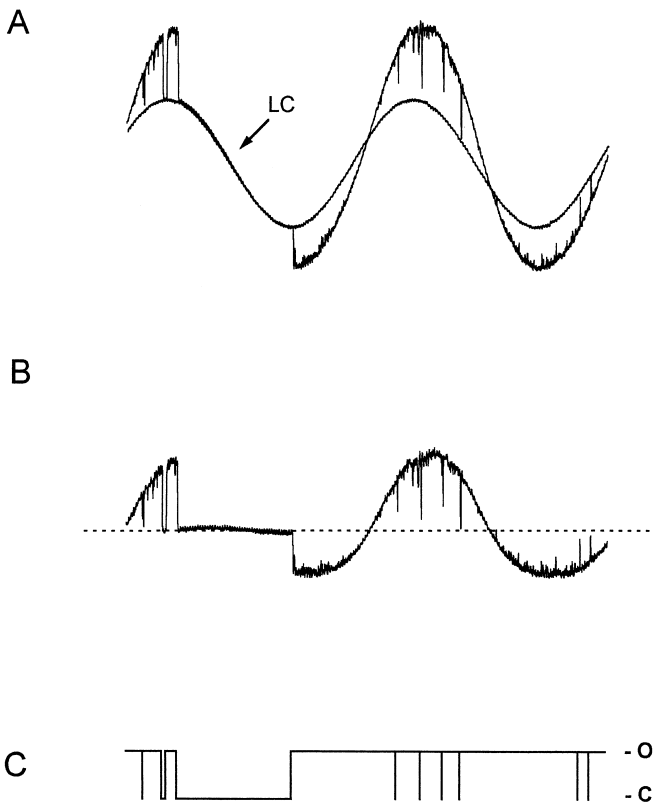

Fig. 1 A–C The reduction of the overall membrane current to construct a dichotomic time series. **A** Superimposed traces of overall membrane current and template, outward currents are displayed upward; **B** channel current afer subtraction; **C** idealized dichotomic signal

For each frequency we tried several values of C and plotted the distribution of the transitions occurring after closings shorter than C, and that of the transitions occurring after closings longer than C. The C values to be chosen for detecting the LCs must give a uniform distribution in the former case, while a phase preference exists in the distribution of closings longer than C. However, by increasing the frequency of stimulation the separation between the two classes of closings was less sharp and for this reason the results obtained at 5 Hz were more noisy than those obtained at 0.5 Hz.

Results

Macroscopic current

The reconstructed macroscopic currents of a single K^+ channel obtained at three different frequencies of stimulation are illustrated in Fig. 2. The average value of the applied sinusoidal potential was zero, while its frequency ranged between 0.5 and 5 Hz. The histograms were constructed by counting the number of times the channel was open at each phase. In all figures the zero value of the phase

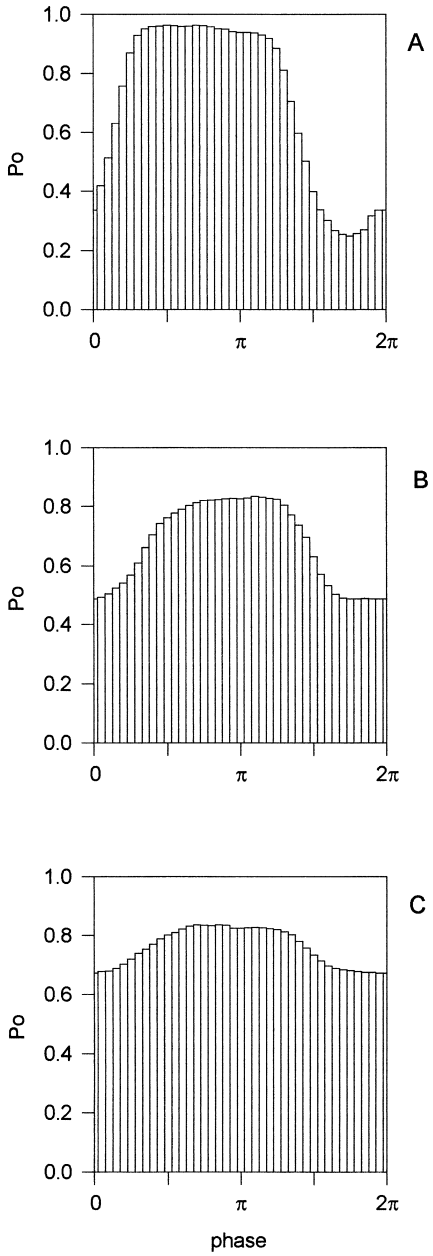

Fig. 2 A–C Reconstructed macroscopic currents. On the *abscissae* the stimulus phase, on the ordinates the fraction of cycles in which the channel is open. **A** 0.5 Hz, 500 cycles; **B** 3 Hz, 790 cycles; **C** 5 Hz, 1800 cycles. Here and in the following figures we assumed that the zero of the phase corresponds to the beginning of the hyperpolarizing portion of the stimulus

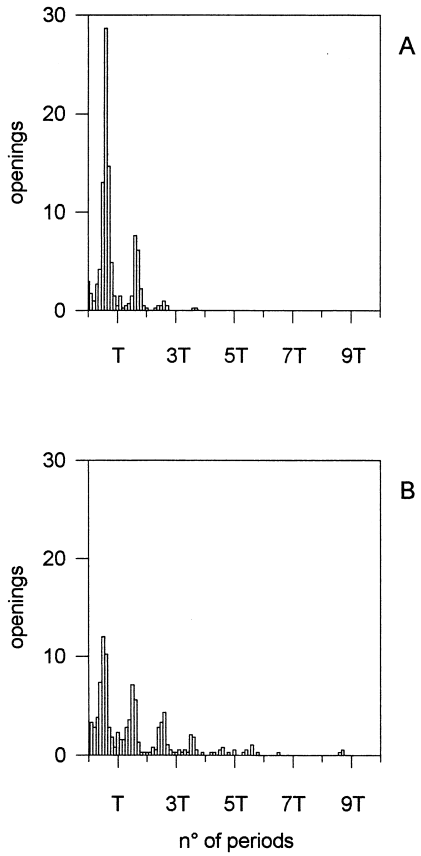

Fig. 3 A, B Histograms of the openings duration at 0.5 Hz and 3 Hz (**A** and **B** respectively). On the *abscissae* the opening duration is reported using the period as time unit; area normalized at 100. Number of events 400

corresponds to the beginning of the hyperpolarizing half of the stimulus and the phase bin is $2\pi/40$.

Clearly the net modulation of this "macroscopic current" declined with the frequency of the stimulus. This did not mean that the stimulus was ineffective. In effect, as shown in the following, the transition probability increased with the frequency either for the open-to-LC or for the opposite transitions. The reason for the lower modulation of the histograms reported in Fig. 2 can be appreciated in Fig. 3, where the histograms of openings are reported. By increasing the frequency many openings persisted for more than one cycle, thus causing a decrease of the overall effect of the stimulus.

Rate constants

The idealized signal, obtained from the pre-analysis, was used to derive some statistical indicators of the channel activity. The evaluation of the rate constants was obtained by counting the number of transitions between the open and closed states (Noc and Nco), as well as the number of times the channel was open or closed (No and Nc) as a function of the stimulus phase. Let Δf be the phase width of each bin in these post-stimulus histograms and Δt its corresponding time duration. The ratio Noc/No divided by $N\Delta t$, where N is the number of cycles, was a direct evaluation of the rate constants for the corresponding transitions in a dynamic condition.

Figure 4 shows the rate constants as a function of the stimulus phase at different stimulation frequencies. It can be noted that Poc increased with frequency, remaining nearly in phase with the stimulus. Pco also increased with

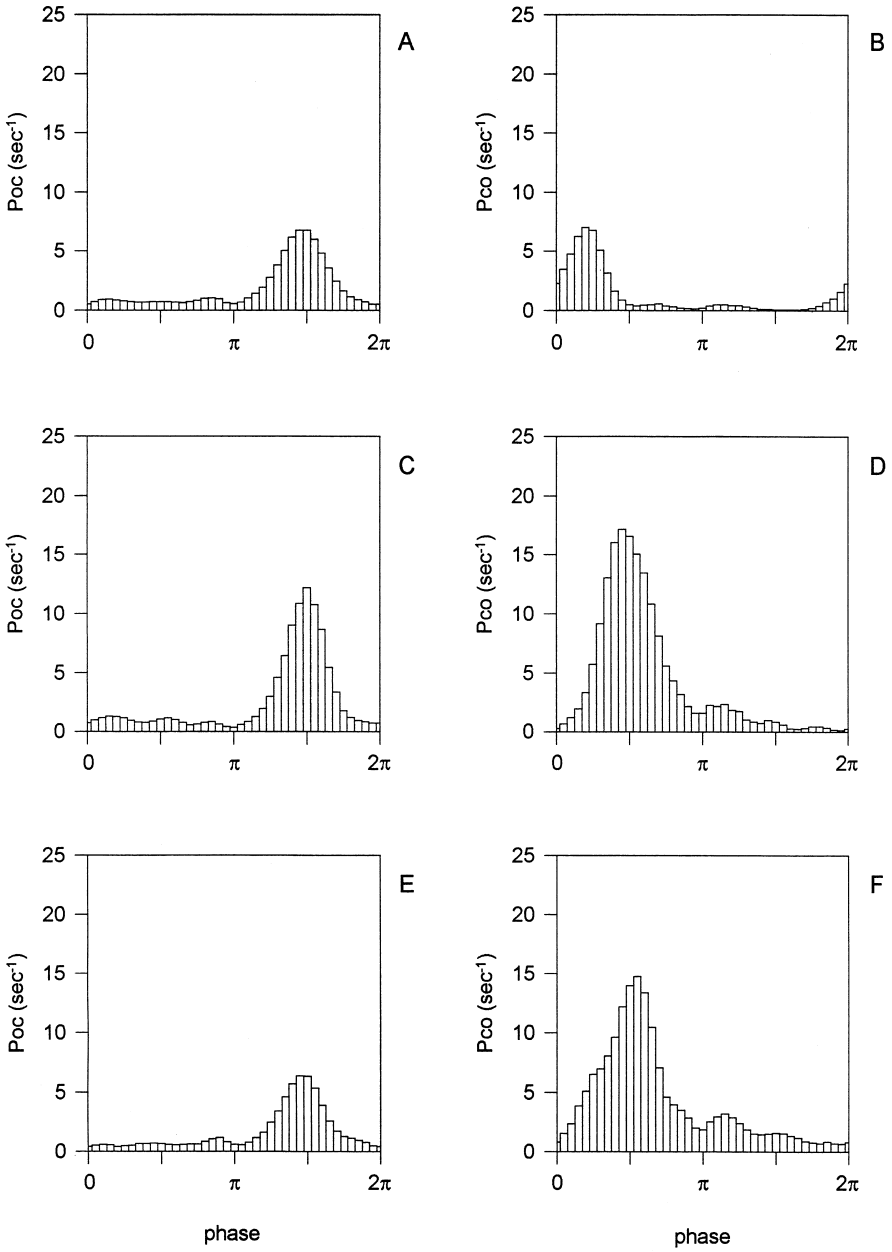

Fig. 4 A–F The rate constants Pco and Poc for the transitions closed-open and the opposite as a function of the stimulus phase are reported. The stimulus frequency was 0.5 Hz (**A** and **B**), 3 Hz (**C** and **D**), 5 Hz (**E** and **F**)

frequency, reaching a maximum at 3 Hz and leading, in phase, the imposed potential at the lowest frequency tested (0.5 Hz). By increasing the frequency, the phase shift declined and Pco coincided in phase with the potential at 5 Hz. These results are shown as a different plot in Fig. 5: the rate constant Pco is here reported as a function of the instantaneous value of the stimulus. This kind of plot highlights how the rate constant does not depend on the stimulus value, but on the stimulus "history". This is still more evident in Fig. 6, where Pco is plotted as a function of the potential for two different amplitudes at the same frequency.

When the sinusoidal stimulation was not allowed to cross the zero potential, sweeping only on depolarizing values, a poor modulation of the reconstructed current was observed, even at 0.5 Hz (Fig. 7 A). The inspection of the evaluated rate constants showed that Poc was still modulated by the stimulus (Fig. 7 B) while Pco was constant, and low, at each value of membrane potential (Fig. 7 C).

Discussion

Since step command potentials induced monoexponential relaxations for both the open-closed and the reverse transitions of the channel studied (Petracchi et al. 1994) a two state kinetic scheme O↔LC can be assumed. Now, let us summarize the results reported above concerning the two rate constants. They show: i) a dependence of the phase at which Pco reaches its maximum on the stimulation frequency, ii) a dependence of both Poc and Pco on the fre-

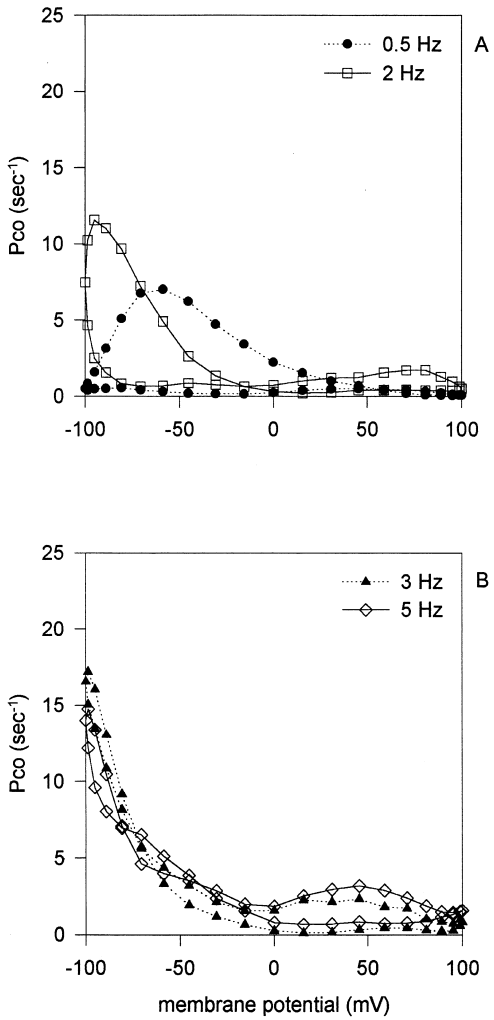

Fig. 5 A, B The rate constant Pco for the closed-open transition is reported as a function of the instantaneous value of the membrane potential. Stimulus frequency 0.5 Hz and 2 Hz in **A**, 3 Hz and 5 Hz in **B**. Same data as in Fig. 4

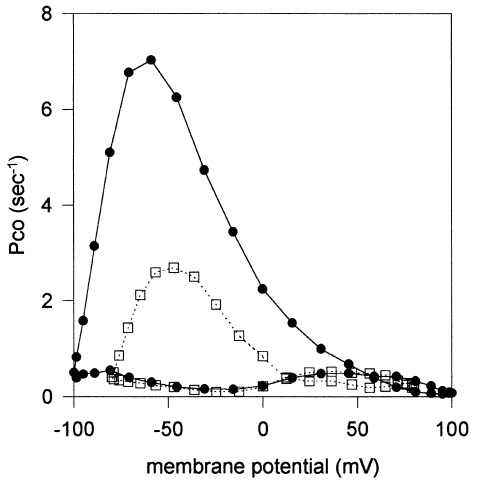

Fig. 6 The rate constant Pco for the closed-open transition is reported as a function of the instantaneous value of the membrane potential. For both curves the stimulus frequency was 0.5 Hz while the amplitude was 100 mV (*continuous line*) and 80 mV (*dotted line*)

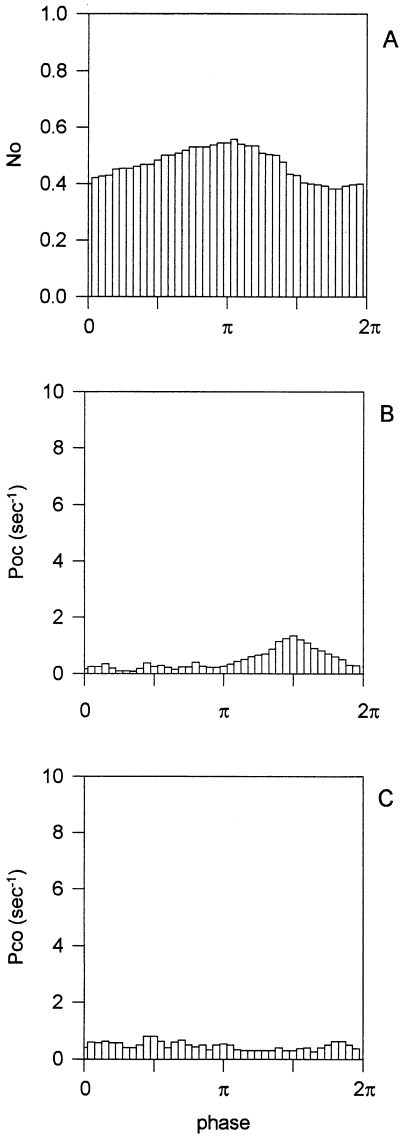

Fig. 7 A–C The membrane potential was sinusoidally modulated around the mean value of +60 mV, with an amplitude of 30 mV at a frequency of 0.5 Hz. In **A** the reconstructed macroscopic current given as fraction of cycles in which the channel is open is reported as a function of the stimulus phase; in **B** and **C** the rate constants Poc and Pco are shown as functions of the stimulus phase

quency, indicating a resonance at a stimulus frequency of a few Hertz.

It is clear that the features of the evaluated rate constants Pco and Poc cannot be explained simply by assuming a two-state kinetic scheme. We considered the question of whether the existence of a hidden state, not revealed by the relaxation experiments, could account for our findings, i. e. the observed phase lead and hysteresis. In principle, the answer is positive, provided that a border state between the open and the closed set becomes empty before the potential reaches its maximum. However, a careful analysis of the first latency distribution confirmed the goodness of monoexponential fitting of relaxation experiments, so that the possibility of other explanations remains

open. Moreover, other ad hoc hypotheses would be required to account for the observed dependence of the two rate constants on the stimulation frequency. Therefore the hypothesis that our findings are a signature of a more complex phenomenon should also be considered.

As far as the underlying mechanism is concerned, we consider two different possibilities: the observed effects might be due to conformational changes of the protein (gating) or to extrinsic factors. According to Stevens (1978), the barrier for a gating transition is assumed to be:

$$U' = U + ze\, f(V) \quad (1)$$

where **e** is the electron charge, **U** is the barrier between these two stable conformations in the absence of any electric field across the membrane and **z** the charge units involved in the gating. If f(V) is a monotonic function this implies that for a periodic V(t) the rate constants have to be always in phase with the applied potential.

The model is essentially a steady-state model and, until it holds, it implies that the rate constants should be independent of the frequency and in general of the "history" of the time varying potential. On the other hand, the validity of such assumptions cannot be unlimited. A range of frequencies should exist at which the motion of the charged groups around the stable positions occurs in a time comparable with that of the stimulus. In this frequency range the motion around the stable positions becomes important. In this context a dependence of the effect of the applied potential on the frequency – also a resonance – could be conceived. Therefore, the kind of method used here could allow one to get some insights into the internal dynamics of the channel.

On the other hand, it is difficult to rule out the possibility that factors different from the protein conformational changes contribute in determining the open-LC switching. Let us consider the case in which accessory subunits are involved in the switching behaviour; in this case the interaction of the channel molecule with cytoplasmic components could exhibit an hysteresis-like behaviour.

Actually, for the channel we studied, we cannot be sure that what we observe is exclusively related to conformational changes of the protein. This is also supported by the observation that the LCs were irreversibly abolished when the bath solution rapidly flushed the cytoplasmic side of the patch, suggesting that a quite labile cytoplasmatic structure of the channel might be involved in LC generation.

To discriminate between these different interpretations further experimental work is required. At the present stage of the work the conclusion that can be drawn is that the use of periodic stimulation represents a valuable experimental procedure to test a kinetic scheme and to extend the information achieved by stationary and relaxation experiments.

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