Periodic Forcing of a K^+ Channel at Various Temperatures

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ABSTRACT The random sequence of openings and closings of single ion channels and the channel conductances have been the object of intense study over the past two decades with a view toward illuminating the underlying kinetics of the channel protein molecules. Channels that are sensitive to voltage, such as many K^+-selective channels, have been particularly useful, because the kinetic rates can be manipulated by changing the membrane voltage. Most such studies have been performed under stationary conditions and usually at a single temperature. Here we report the results of experiments with sinusoidal modulation of the membrane potential performed at several temperatures. Dwell time and cycle histograms, objects not normally associated with ion channel experiments, are herein reported. From the last, the transition probability densities for channel opening and closing events are obtained. A new and unusual phase anticipation is observed in the cycle histograms, and its temperature dependence is measured.

INTRODUCTION

The majority of studies dealing with ion channel kinetics have been performed either in stationary conditions or using a variety of stimulation protocols consisting of a set of steps to different membrane potentials, each maintained for a given period of time. The repetition rate of such sets has usually been chosen to allow the system to reach the stationary condition associated with the holding potential. These experiments have provided information on the kinetics of the underlying processes and, thus, on the descriptive accuracy of various stochastic channel models that have been put forth. This approach has been exploited at the macroscopic as well as at the single channel level (Vandenberg and Bezanilla, 1991; Aldrich et al., 1983; Hoshi et al., 1991; Demo and Yellen, 1991) to study the dependence of channel activation and inactivation on both holding and command potentials. Furthermore, double step patterns (Oxford, 1981) have been used to test the details of kinetic models, including the inactivation process. Other nonconstant potentials, including the ramps used in a simulation (DeFelice and Clay, 1983), have been applied to obtain information on the mechanisms and pathways involved in ion channel gating. A useful overview and bibliography have recently been prepared by Ball and Rice (1992).

Here we report the results of applying to single ion channels a sinusoidal modulation of the membrane potential. Periodic modulation of the potential is a largely unexplored way to stimulate ion channels. In a very near field, that of catalyzed chemical reactions in ordered structures, this form of stimulation has instead been more often used. The energy transfer from the applied electric field to chemical reactions and to transport processes through the membranes has been studied, in fact, by experimental and theoretical approaches (Tsong, 1990; Astumian and Robertson, 1989; Woodward and Kell, 1991; Horn, 1993). A comprehensive review of these works has been given by Tsong (1992). It is interesting to note that there seems to be very little interaction between these neighboring fields, for example, the common set of references of the Tsong (1992) and Ball and Rice (1992) reviews are limited to only a few items. Periodic stimulation of single ion channels is suitable to gain insight into the system dynamics, and in some sense it can give more information than when applied to chemical reactions or to the activity of pumps. In fact, in these two last cases what can be measured is the net product of the chemical processes or the net incoming flux inside the cell. Moreover, very often only measurements of the average value of these quantities have been reported (it is not always possible to detect their temporal modulation at the stimulus frequency). Instead, recording from ion channels the single transition from open state(s) to closed one(s) can be measured, so that a more microscopic information is available. The average value of the current flowing through the channel can easily be evaluated by superposing the channel recording during several stimulus cycles; this average corresponds to the macroscopic current that could be measured by recording from a membrane patch containing many identical channels. More subtle quantities can be measured as well by recording single channel transitions. The knowledge of single channel transitions allows the separation of the data into different subclasses according to the state of the channel (open/closed) and to the length of the dwell times, and this makes it possible to measure the conditional probabilities of different events. The use of conditional probabilities has proven to be very useful in the testing of various ion channel models (McManus et al., 1985: McManus and Magleby, 1989; Magleby and Weiss, 1990: Barbi and Petracchi, 1991, 1992; Petracchi and Barbi, 1991; Petracchi et al., 1991), and their use, combined with

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that of periodic stimulation, is explored in this paper. However, there is a price to pay for this microscopic information. In comparison with the macroscopic experiments described in the above cited review, one must carry out single channel experiments over longer periods of time to achieve comparable statistical accuracy.

Another motivation for using sinusoidal or more general periodic forcing in single ion channel experiments is offered by the currently developing discussions on the influence of weak, low frequency electromagnetic fields on living systems (Tsong, 1992; Weaver and Astumian, 1990). This discussion has already included the suggestion that *stochastic resonance* (Moss, 1991) plays a significant role in the amplification of weak electromagnetic signals by thermal noise at the cellular level (Kruglikov and Dertinger, 1993).

Stochastic resonance is a statistical process realized in certain rather general nonlinear dynamical systems, whereby the noise internal to the system actually enhances the transmission of information through the system. At a certain, well defined optimal noise intensity, the signal-to-noise ratio at the system output achieves a maximum. Virtually all previous studies, both experimental and theoretical, have been carried out on *physical* bistable systems stimulated by a weak periodic forcing combined with Gaussian noise (Moss, 1991; Longtin et al., 1993, 1991, Zhou et al., 1990). Recently, however, a *biological* experiment wherein sensory neurons were exposed to weak, noisy, sinusoidal stimuli has been shown to exhibit stochastic resonance (Douglass et al., 1993). In the same spirit as the suggestion (Tsong, 1992) that cells are capable of receiving and processing information and perhaps even decision making, it seemed to us that it might be fruitful to study single ion channels from the point of view of stochastic resonance.

In an approach which, to our knowledge, is entirely new to single ion channel work, we draw a direct parallel to the studies on stochastic resonance. In particular, in those studies, time interval histograms were generated, and the profound influence of the *noise intensity* on their shape in the presence of periodic forcing was recorded. We use the patch temperature to control directly the internal noise intensity of the single ion channel, and we record its influence on the interval and cycle histograms. This method of experimentally controlling the internal noise intensity has already been well established for sensory neurons (Stiebler and Narins, 1990). Although not many studies of the temperature sensitivity of ion channel characteristics have been reported, it is evident that the major effect of increasing temperature is to increase all rate constants, thereby increasing the switching rates between open and closed states (Shen et al., 1993, Correa et al., 1992; Nobile et al., 1990). Thus, the internal noise is strongly dependent on the temperature of the channel, so that we were motivated to study the histograms from recordings made at various temperatures. This represents another experimental way to get further information on the dynamic features of a system driven by thermal noise. The occurrence of a selected class of state transitions and their sensitivity to temperature were analyzed.

MATERIALS AND METHODS

Specimens of the medicinal leech *Hirudo medicinalis*, commercially supplied, were maintained at 16°C in artificial pond water (Muller et al., 1981). Segmental ganglia were isolated in leech saline (115 mM NaCl, 4 mM KCl, 7.5 mM $CaCl_2$, 10 mM Tris-maleate buffer, 10 mM glucose, pH 7.4) and maintained in Leibovitz 15 (Gibco, Grand Island, NY) until used. Single ganglia were transferred to the experimental bath containing a high K^+ solution (120 mM KCl, 1 mM $MgSO_4$, 10 mM HEPES KOH, 10 μM $CaCl_2$, pH 7.3, osmolarity adjusted with glucose). A single cell body of the AP neuron, identified according to its size, position, and membrane electrical properties, was exposed by cutting the connective tissue capsule of the ganglion. The membrane was made clear by flushing the bath solution into the cell body with a Pasteur pipette.

The type of channel used in this investigation had been previously identified as a background channel contributing to the membrane resting potassium conductance in the central neurons of the leech (Pellegrini et al., 1989) These channels were identified as potassium channels according to the following criteria: a) the observed shift of the reversal potential for the channel current when the K^+ concentration in the bath was changed was very close to the theoretical potassium equilibrium potential; b) the substitution of acetate ions for chloride ions did not affect the I/V relationship of the single channel current; and c) the substitution of Na^+ for 90% of the K^+ in the bath solution caused the I/V relationship to shift towards the predicted value of E_K and to become asymptotic to the abscissa for values of the depolarization at which the Na^+ was the major current-carrying species.

Patch clamp recordings (Hamill et al., 1981) were performed with electrodes pulled in two stages from 1.5 mm o.d. glass capillary tubing and fire-polished (resistance 2–5 MΩ). The pipette solution had the same composition as the bath solution and, additionally, was filtered with a 0.2 μm Millipore filter (Bedford, MA). Seals of 5–10 GΩ were routinely obtained. Experimental data were recorded with a patch amplifier (Axopatch-1D, Axon Instruments, Foster City, CA) and stored with a modified Sony videotape recorder (Bezanilla, 1985). Single channel currents were recorded from cell-free inside-out patches. The records were filtered at 0.5 kHz and were digitized at 1 kHz. The temperature of the experimental chamber was adjusted and stabilized using Peltier units controlled by a feedback circuit. Arrhenius plots of the single channel conductance versus temperature were used to check that the actual channel molecule temperature changed in accordance with the bath temperature.

Analysis of data obtained as responses to steady-state step voltages was carried out using P-Clamp software (Axon Instruments). Data obtained under sinusoidal modulation of the holding potential were analyzed using software developed for this purpose, as discussed below.

Step stimuli

The typical channel activity recorded at hyperpolarizing values of the membrane potential consists of bursts of openings and two classes of closed dwell times, the shorter ones (intra-bursts) and the longer ones (inter-bursts), can be observed. Apart from details, this pattern does not change when the membrane potential is set at steady values ranging from -100 to 50 mV, and the fraction of total time open maintains a value of about 80% in this range (Fig. 1, *B* and *C*). Accordingly, stepping the potential from hyperpolarizing values up to 50 mV does not affect the channel activity, and the macroscopic current, reconstructed by averaging an ensemble of single channel records, does not exhibit any time or voltage dependence.

When the holding potential is maintained at a steady value greater than 50 mV, very long closures (LC) of the order of seconds in duration appear, and their frequency increases with the strength of depolarization, whence the percent of time open progressively decreases. Step changes of membrane potential from values below 50 mV to values over 50 mV induce single channel closure (Fig. 2 *A*), and the reconstructed macroscopic current displays a relaxation that can be fitted by a single exponential (Fig. 2 *B*). Channel reopening, produced by stepping to hyperpolarizing potentials, follows a faster monoexponential time course (Fig. 2 *B*). The relaxation rate constants obtained by either stepping up or down the potential change as a function of the command potential. We have measured the following values

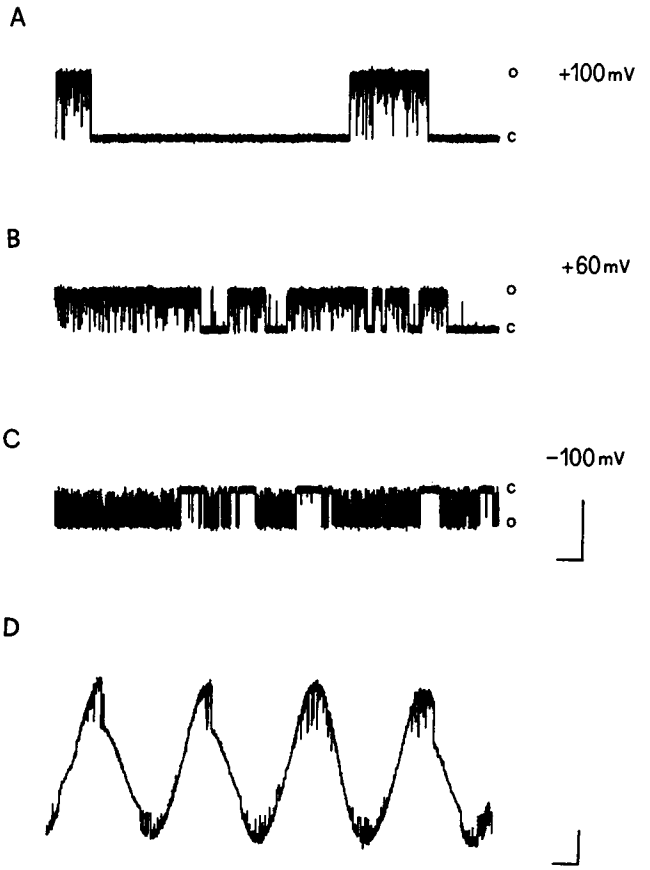

FIGURE 1 General features of potassium channel recordings showing typical steady-state single ion channel activity at the constant membrane potentials: (*A*) 100 mV; (*B*) 60 mV; (*C*) −100 mV showing the changing fraction of closed dwell times; (*D*) an example recording with a sinusoidal applied voltage of +100 to −100 mV peak-to-peak at 0.5 Hz frequency. Note the large probability for the onset of a long closing event near the maximum of each cycle.

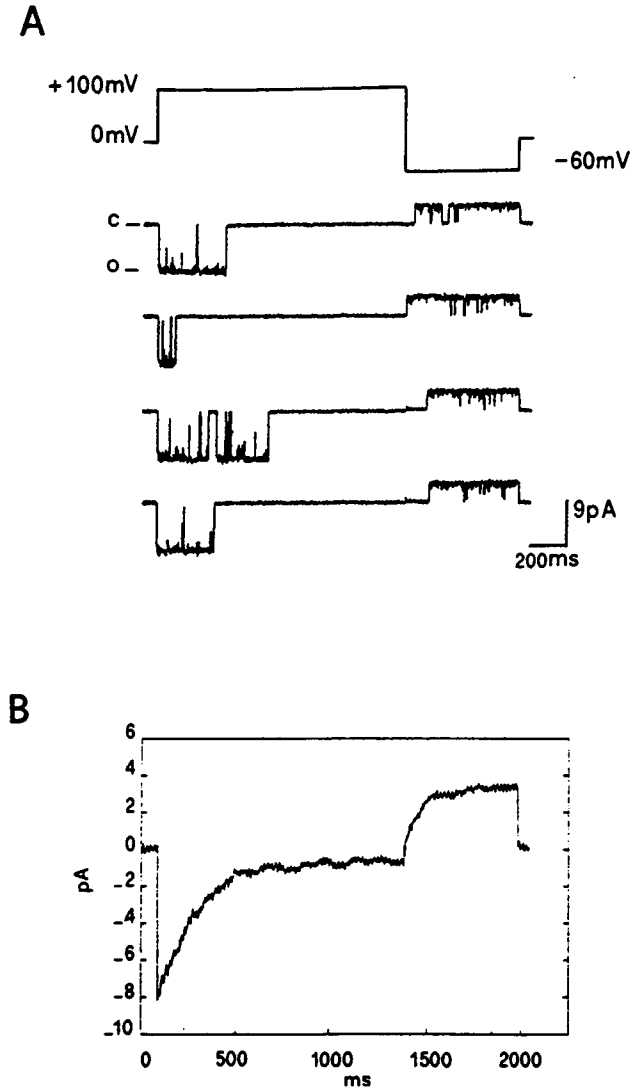

FIGURE 2 (*A*) Several example single channel responses to the stimulus pattern shown on the top, illustrating the occurrence of long closings (LCs) under strong depolarization and reopenings under hyperpolarization. (*B*) Reconstructed macroscopic current obtained by averaging 200 single channel responses like those shown in *A*.

stepping up from zero to 60, 80, 100, and 120 mV, respectively: 1, 2.5, 5, and 12 s^{-1}. Stepping down from depolarizing values to hyperpolarizing ones, the following values were instead obtained, corresponding to −40, −60, and −100 mV, respectively: 8, 12, and 50 s^{-1}. The occurrence of monoexponential relaxations suggests a very simple kinetic scheme with only two states (open and closed), and we discuss briefly in the next paragraph the expectations coming from such a simple scheme when a periodic perturbation is applied to the system.

Theoretical background

Let us consider the simplest probabilistic scheme for open-closed transitions:

$$O \leftrightarrow C, \tag{1}$$

and let us assume that α (rate constant for the open closed transition) and β (rate constant for the reverse transition) depend on the applied potential, V, in an exponential way (Stevens, 1978; Tsong, 1990).

$$\alpha(t) = \alpha_0 e^{-rzeV(t)/KT} \tag{1a}$$

$$\beta(t) = \beta_0 e^{(1-r)zeV(t)/KT}, \tag{1b}$$

where e is the electron charge, K is the Boltzman constant, T is the absolute temperature, α_0 and β_0 are the values of the rate constants at zero potential, and rze and $(1 - r)ze$ are the charges that move from the open (closed) state to the activated one on the top of the barrier. In the experiment analyzed here, $V(t)$ is given either by a step potential or by the periodic function

$$V(t) = V_0 \sin(2\pi\nu t). \tag{2}$$

Let us introduce now some quantities that can be measured in an experimental recording. Let $P_o(t)$ be the probability to find the channel open at time t and $P_c(t) = 1 - P_o(t)$ be the probability to find the channel closed at time t. The following equation holds:

$$\frac{dP_o}{dt} = -\alpha P_o + \beta P_c = -(\alpha + \beta)P_o - \beta. \tag{3}$$

At a very low frequency (ν small compared with the smaller of α and β), Eq. 3 can be considered at equilibrium, and in this case (adiabatic approximation) $P_o(t)$ is simply given by

$$P_o(t) = \frac{\beta(t)}{\beta(t) + \alpha(t)}. \tag{4}$$

When, instead, ν is of the same order or greater than α or β, the adiabatic

approximation assumed to obtain Eq. 4 no longer holds, and a numerical integration of Eq. 3 is required.

As shown by Eq. 3, the measurement of the single relaxation time corresponds to $1/(\alpha + \beta)$. In the case analyzed here, for depolarizing potentials, the rate constant for the open-closed transition (α) is much higher than that of the inverse transition (β); thus, the relaxation time constant corresponds approximately to $1/\alpha$. For the same reason, $1/\beta$ is the relaxation time constant measured when stepping down the potential. Using Eqs. 1a and 1b and the data obtained with the step potentials, we obtain $\alpha_0 = 0.083\ s^{-1}$, $\beta_0 = 1.47\ s^{-1}$, $r = 0.53$, and $z = 1.9$.

Let us consider again the particular case in which $\alpha P_o \gg \beta P_c$ (that is, the case of a depolarizing potential in the experiment) or $\beta P_c \gg \alpha P_o$ (hyperpolarizing potential). In these cases, from Eq. 3 we can obtain

$$\alpha = \frac{1}{P_o}\frac{dP_o}{dt} \tag{4a}$$

and

$$\beta = \frac{1}{P_c}\frac{dP_c}{dt}. \tag{4b}$$

Clearly, Eq. 4a holds for depolarizing values of the membrane potential, Eq. 4b holds for hyperpolarizing ones, and both hold only for frequencies far from the adiabatic approximation.

SINUSOIDAL STIMULATION

As shown in Fig. 1 under sinusoidal modulation of the holding potential, between −100 and 100 mV, LCs frequently start near the top of the depolarizing half cycle and continue up to the hyperpolarizing part of the stimulus cycle. Our attention was focused on the transitions to and from the state associated with LCs, so we reduced our data by cutting those closed times with durations shorter than one-tenth of the stimulus period. Therefore, the portion of the kinetic scheme we investigated at different temperatures was reduced to the transitions O ⇔ LC.

The open-closed pattern shows a strong synchronization with the applied stimulus. To study this phenomenon quantitatively, the sequence of open and closed dwell times were extracted from the raw current signal by subtracting the sinusoidal leakage current and correcting for the capacitive phase shift. We previously reported a bias subtraction method, implemented by hardware (Petracchi et al., 1992). This method was not suitable, however, for a current signal with zero crossings, so a software approach was developed for use here. The method is illustrated in Fig. 3. First, it is necessary to determine the membrane current with the channel closed. This is a purely sinusoidal current that flows through the membrane in parallel with the channel current. Fortunately, long segments of the record empty of openings (LCs) were available, eventually at all phases of the stimulus. For example, the LC shown by the top trace in Fig. 3 covers a range of phases between about 240° and 360°. Other LCs were extracted from other cycles of the record at different phases until a complete cycle of the closed channel membrane current was assembled, as shown by the smoother sinusoid in the second trace down from the top. The closed channel membrane current, thus assembled, was then used as a template and subtracted from the overall channel current signal. The resulting channel current is shown in the third trace down. Now we note that harmonic components are

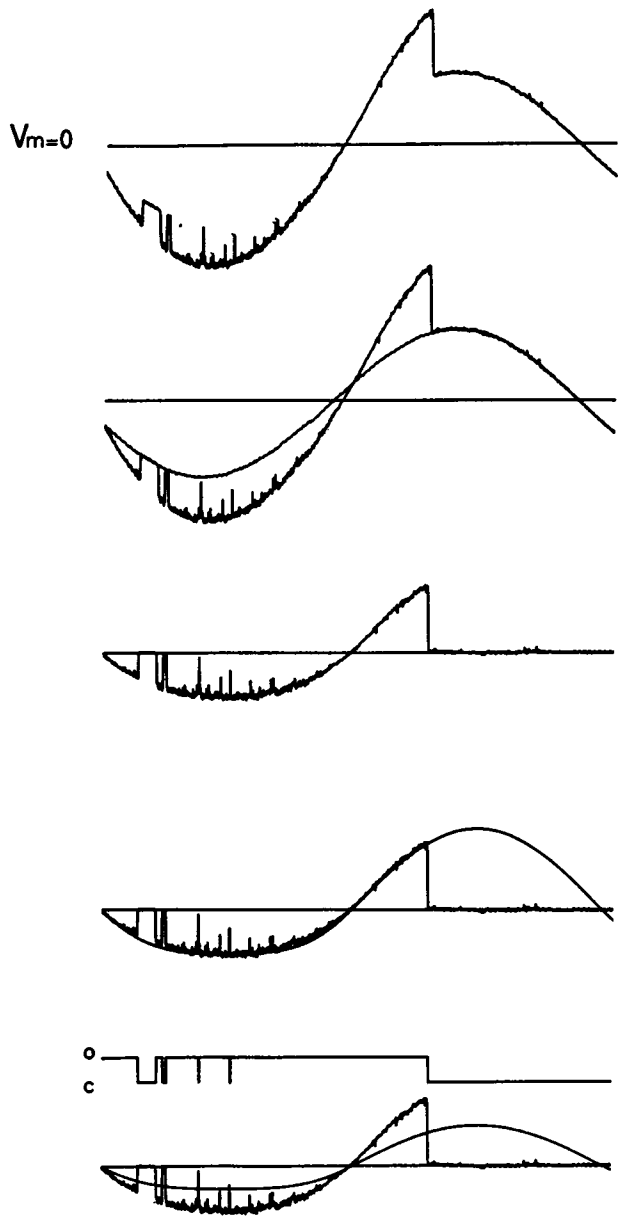

FIGURE 3 The method for extracting the single channel current transitions from the overall membrane current when the applied potential is sinusoidal and the construction of the dichotomic time series (see text).

present in this signal that slightly flatten the current over the negative half cycle. At this point, the sum of a sinusoidal function plus a small second harmonic component in the negative half cycle only, is fit to the channel current. This is shown by the smooth curve in the fourth trace down. Fifty percent of this function is assumed as the threshold and compared with the channel current in the bottom trace. At every crossing of the threshold by the channel current, a single pulse was written into a time series. In this way an idealized dichotomic signal, as shown by the trace marked O-C in Fig. 3, was produced for analysis. This protocol was necessary to catch correctly the transitions occurring near the points of current inversion.

The dichotomic signals, together with a trigger at the start of every cycle, were used to calculate some statistical indicators of the channel activity. The cycle histograms obtained from data of a typical experiment, with modulation from −100 to +100 mV, at 0.5 Hz and a temperature of 25°C, are shown in Fig. 4. The probability of finding the channel open at the phase ϕ of the stimulus cycle, $P_o(\phi)$ (Fig. 4 *A*), was computed by counting the number of times, $N_o(\phi)$, the channel is open at that phase divided by the total number of cycles in the record. The peak-to-peak amplitude in this histogram gives a measure of the degree of synchronization between channel activity and forcing stimulation. The fraction of cycles displaying LCs consistently changes with both frequency and amplitude of the stimulus. According to Eq. 4a, the probability of the transition O → LC, $P_{oc}(\phi)$, was evaluated as the ratio of the number of transitions, $N_{oc}(\phi)$ (plotted in Fig. 4 *B*) divided by $N_o\Delta t$, where Δt is the time span that corresponds to the phase bin in all the cycle histograms. P_{oc}, expressed in s^{-1} is reported in Fig. 4 *C*.

The quantities concerning the transition LC → O are plotted in Fig. 4 *D–F*. A consistent feature, observed in all the experiments, is that *the P_{co} peak leads in phase that of the hyperpolarizing potential.* Fig. 4 *F* shows this feature, where the maximum of P_{oc} occurs at a phase of about 0.3π relative to the stimulus. Such a phase anticipation cannot be explained by the two states model suggested by the responses to step stimuli. In fact, as discussed above, the analysis carried out allows one to obtain an evaluation of the rate constants of the assumed scheme, and these must be in phase with the applied potential (Eqs. 1 and 4 above). The only question that can be raised regarding the results shown in Fig. 4 *F* concerns the statistical accuracy of the data. In particular, one can wonder whether the observed phase anticipation could come from an erroneous counting of the number of transitions from LC to O. Transitions from the closed state with shorter durations than one-tenth of the period were not counted, and some of them might actually belong to the LC population. Thus, omitting the transitions from short duration closings might under estimate the number of transitions from LC, and it makes sense to consider whether this underestimation could be more pronounced at the maximum hyperpolarization, where the rate constant α becomes larger. We have addressed the problem of the statistical accuracy, and that of the possible bias in cutting short closings, by generating a set of numerical data using a simple simulation of a two-state Markov model and by analyzing these data with a procedure *exactly identical* to that used in analyzing the experimental data. The simulation of the two-state Markovian dynamics with sinusoidally varying rate constants is described in the Appendix, and it was done with the same statistical accuracy (that is, the same number of transitions was used in the simulation as were recorded in the experimental data). The results, shown in Fig. 5, are directly comparable, also with regard to statistical accuracy, to the experimental data shown in Fig. 4. Despite the variability of both the simulated and experimental data sets, it is clear that there is a phase anticipation evident in Fig. 4 *F* compared with Fig. 5 *F*. Moreover, the observed anticipation is temperature-dependent, becoming more pronounced at higher temperatures (31°C), and becoming nearly imperceptible at the lowest temperature (10°C). We have reported representative data in Fig. 4, obtained at room temperature (25°C). We have observed this phase anticipation in five independent experiments, and, hence, we believe that this is a new result for which no definitive explanation presently exists. We conclude that the experimentally observed phase anticipation of the probability of channel opening is a biological effect not accounted for by the purely statistical two-state model.

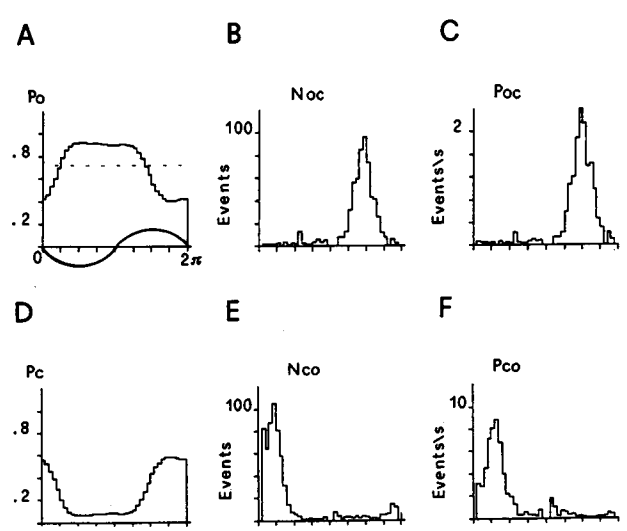

FIGURE 4 Statistical indicators as described in the text: (*A*) probability of finding the channel open versus phase, (*B*) cycle histograms of open-closed transitions, (*C*) probability of open-closed transitions versus phase, and (*D–F*) the corresponding quantities for the closed-open transitions. The histograms were constructed from records of 1000 stimulus cycles.

Another kind of analysis has been done on the data of Fig. 6. The purpose was to test whether the phases at which the transitions occur depend on the previous history of the system, that is, the phase of the previous closing (opening) and/or the length of the closed (open) dwell time. Let us explain the idea behind the analysis. Let ϕ_i be the phase at which a long closing begins (we are here speaking about LCs, but the same holds for openings). The relation between the time interval of the closing, T, the period of the stimulus, T_s, and the phase at which this LC ends, ϕ_e, is simply given by

$$T = T_s(\phi_e - \phi_i)/2\pi. \quad (5)$$

Assuming that there is no history dependence, that is, that the closing phase is independent of the opening phase or of the length of the closing, Eq. 5 is a linear relation. A plot of T versus ϕ_i (mod 2π) should then show a slope of $-T_s/2\pi$ as the signature of such independence. This slope is clearly evident in our experimental data, where Fig. 6 *A* represents the O → LC and Fig. 6 *B* the LC → O transitions. Fig. 6 also reveals typical accumulations: on the vertical axis at intervals spaced by one period of the stimulus (closings rarely persist

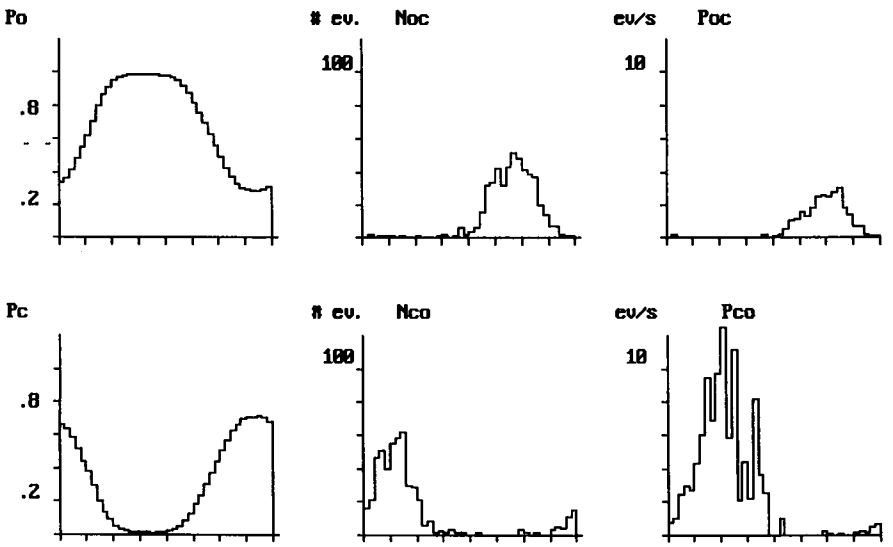

FIGURE 5 The results of the numerical simulation based solely on Markovian statistics and the simple two-state model, which can be compared directly to the experimental results shown in Fig. 4.

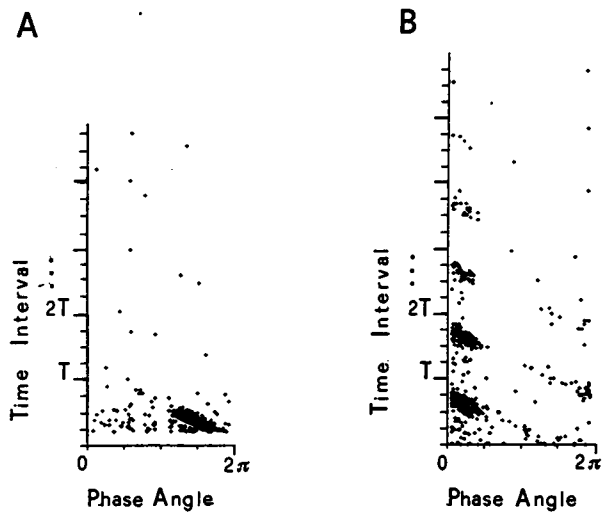

FIGURE 6 Time interval-phase angle return maps assembled from closed states (*A*) and open states (*B*). The abscissas are the phase angles of the onset ϕ_i of the indicated state.

over more than one stimulus cycle) and along the horizontal axis at the preferred initial phase.

Temperature strongly affects the channel activity under sinusoidal modulation of membrane potential. The results of a typical experiment, in which the temperature assumed three values in the range 10–31°C, are illustrated in Fig. 7. Three main changes can be consistently observed with increasing T: 1) an increase of the peak-to-peak amplitude in the P_o cycle histograms (Fig. 7 *A*); 2) an increase in the mean probability to be open (Fig. 7 *A*); 3) a shift to the left, that is, a phase anticipation of the P_{co} peak (data not shown). The first two changes indicate an increase in the depth of modulation of the channel activity with increasing temperature (however, with constant stimulus strength). This latter effect is consistent with similar results obtained from models with bistable potentials (Longtin et al., 1993), wherein increasing output signal coherence is observed for increasing internal noise intensity (which, in the present experiments, we vary by increasing the temperature). Remarkably, this result is also consistent with what is observed during temperature (noise)-enhanced signal transduction in sensory neurons.

We turn now to another statistical object familiar in the analysis of probabilistic, periodically modulated systems: the time interval histogram. We examine here the distribution of time intervals between identical, successive transitions, that is, the interval from one closed-to-open transition to the next identical transition. Both the open and closed times are affected by the time window used. In consequence of the windowing mentioned above, the open time interval distribution is actually the distribution of burst durations. Histograms of intervals between successive, identical transitions, i.e., between successive O → LC transitions, called here the intertransition intervals (ITIs) are reported in Fig. 7 for three values of the patch temperature and display a sequence of peaks with successively smaller amplitudes, centered on time intervals that are integer multiples of the stimulus period, nT_s (n is an integer) (Douglass et al., 1993; Longtin et al., 1993, 1991, Zhou et al., 1990). Experiments with physical bistable systems have shown that the rate of decay of successive peaks is an indicator either of the stimulus strength or of the noise intensity. For example, the effect of the temperature, which in this experiment establishes the internal noise intensity, can be measured by the changes of this indicator if the stimulus strength is held constant. The decay rate of the amplitude of the peaks of the ITI histograms reported in Fig. 7 *B–D* clearly increases with temperature, and this behavior is completely consistent with results previously obtained for increasing noise intensity in experiments with physical, bistable systems (Longtin et al., 1993, 1991, Zhou et al., 1990) and with sensory neurons (Douglass et al., 1993).

DISCUSSION

Summing up, our results show that: a) periodic stimulation is capable of synchronizing a selected class of dwell times

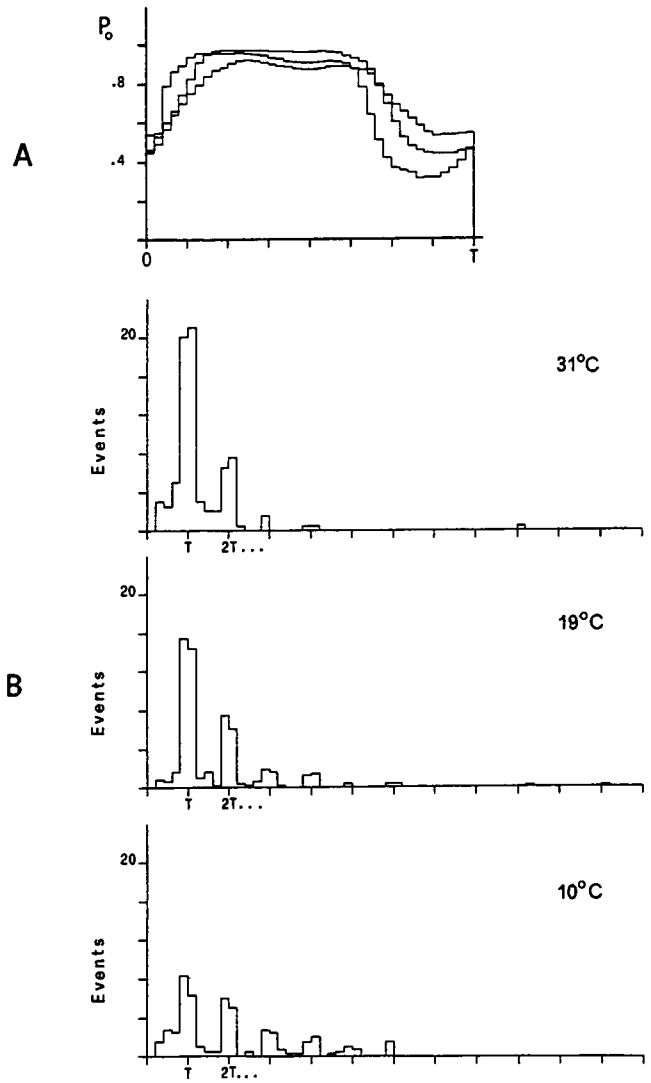

FIGURE 7 Effect of temperature on the cycle histograms of P_o (*A*) and the transition time histograms (*B*). The cycle histograms show a larger modulation depth for the higher temperatures. The transition time histograms show the characteristic peak sequence at integer multiples of the period of the applied potential. The decay rates of the sequential peak amplitudes sharply increase at higher temperatures as the interval probabilities are accumulated in the lower order peaks.

(the LCs), as indicated by our analysis tools—time interval and cycle histograms, time interval versus phase first return maps, and reconstructed macroscopic currents of the idealized dichotomic signal of the channel transitions; b) there is no memory of the opening or closing initial phase or of the time interval evident in the final phase; and c) the maximum of the probability of the closed-open transition leads in phase the peak of the hyperpolarization. The last observation is remarkable, because the simple scheme for the O ↔ LC transitions on the temporal scale of the LCs, as suggested by the results obtained with step command potentials, indicates that both P_{oc} and P_{co} should be in phase with the membrane potential and that the phase relation does not change with temperature (internal noise intensity). This assertion is tested by the Markovian simulation described in the Appendix. Even assuming more complicated schemes, one should expect phase lags, rather than leads, to show up in purely Markovian systems. Because such an effect cannot be explained on statistical grounds alone, we tentatively conclude that it is biological in origin. This nonlinear phenomenon, as well as its dependence on the temperature, still awaits an interpretation.

The increase of P_o in response to increasing temperature might be interpreted as stochastic resonance (Douglass et al., 1993; Moss, 1991). This is a nonlinear effect exhibited by bistable systems, or more generally, by every single barrier system driven by a subthreshold, coherent signal plus noise. Typically, for low noise levels, transitions are synchronized with the forcing signal; higher levels of noise increase the transition probabilities without destroying the synchronization with the stimulus, thus increasing the coherence between the dichotomic output signal (the channel current) and the forcing signal, up to a maximum. As the noise becomes larger yet, the transitions progressively lose their synchronization with the input signal. We can ask whether this effect is present in the actual behavior of ion channels, that is, do the ion channels operate in a range where stochastic resonance might be observed? The increase of synchronization with temperature that we consistently observed seems, at first sight, in keeping with this phenomenon. However, we must take into account that in these first experiments only three or four values of the temperature were imposed on each channel: probably an insufficient number to test convincingly for the existence of the aforementioned maximum in coherence. The number of temperatures was limited, because of the long time required to collect a sufficient number of transitions for satisfactory statistical averages.

Coming back to the main result of this work, namely the phase lead of the closed-open transition probability with respect to the forcing potential, there are two lines to be considered in interpreting this result.

The first hypothesis is to suppose that the gating current associated with this transition actually leads in phase the applied field. This hypothesis has some implications for the energy flow, as already discussed in a recent paper (Markin et al., 1990). By directly measuring the phase of the displacement current, one could clearly test this hypothesis. Experiments of this kind are not easy but can be done (Mika and Palti, 1994).

The other hypothesis to be considered is that other effects not directly related to the gating current can occur that determine the observed phase lead. For example, an enzymatic mechanism, promoted by depolarization with a trigger excited by a threshold crossing of the membrane potential, could also be a candidate to account for these results. The independence of the phase of the C → O transitions on the duration of closed intervals indicates that the O → C transition does not trigger the process that, at a later time, produces the channel reopening. Further experiments designed to understand better the interactions of the channel molecule with the medium are in progress, aiming to define the nature of the physiological mechanisms underlying the onset and the break of LCs. Inactivation and ion block can be tested by

ionic substitution experiments, whereas the role of enzymatic modulation can be studied using the cell-attached configuration.

As a last observation, these results indicate the utility of applying various stimuli, including sinusoidal ones, in experiments designed to characterize ion channel kinetic systems.

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APPENDIX

The simulation was based on the simple two-state Markov model. The steady-state rate constants governing the scheme, O↔LC, are given by

$$\alpha(t) \propto \alpha_0 e^{-zeV(t)/KT} \tag{A1}$$

and

$$\beta(t) \propto \beta_0 e^{-zeV(t)/KT}, \tag{A2}$$

where $V(t)$ is the time-dependent membrane potential, k is Boltzmann's constant, T is the absolute temperature, and ze is the equivalent charge that undergoes the transition (Stevens, 1978). To simulate the experiments with symmetric modulation, we take the potential to be

$$V(t) = V_0 \cos(\omega t). \tag{A3}$$

The transition times were obtained by using a very efficient algorithm (Zambon, 1993) that generalizes the well known one valid for constant rates of transition (De Felice and Clay, 1983). Let us briefly describe it considering, for example, an open-to-closed transition. Suppose at time t_0 the channel just opened, and take the time interval

$$t_1 = (1/\alpha_m)\ln r_1, \tag{A4}$$

where r_1 is a random number uniformly distributed between 0 and 1 and α_m is the maximum value of the closing rate given by

$$\alpha_m \propto \alpha_0 e^{-zeV_0/KT}. \tag{A5}$$

Time $t_0 + t_1$ will be considered to be the transition time with probability

$$p_1 = \alpha(t_0 + t_1)/\alpha_m \tag{A6}$$

(this choice implies the extraction of a new random number). If the transition does not occur, one considers $t_2 = (1/\alpha_m)\ln r_2$ and sums up to $t_0 + t_1$, accepting $t_0 + t_1 + t_2$ as the transition time with probability $p_2 = \alpha(t_0 + t_1 + t_2)/\alpha_m$. Even if the average number of iterates required to compute one transition time increases as α_0 falls lower than α_m, the method is much faster than the obvious step-by-step procedure, and it is exact (Zambon, 1993).

This simulation was used to generate time interval data, which was analyzed in the same way as the experimental data assembled on Fig. 4. The parameter values were chosen to produce cycle histograms for P_o like those of Fig. 4, and the simulated results, shown in Fig. 5, can be directly compared to those of Fig. 4. It is worth noting that the simulation has been carried out with the same number of transitions as in the experimental case, so that the same statistical accuracy is to be expected for both. And despite the statistical variability, Fig. 5 does not show any phase anticipation, thus indicating that the observed effect has a biological origin not predicted by the statistical two-state model.

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