Journal of Statistical Physics, Vol. 70, Nos. 1/2, 1993

Periodic Forcing of Ion Channel Gating: An Experimental Approach

D. Petracchi,[1] C. Ascoli,[1] M. Barbi,[1] S. Chillemi,[1] M. Pellegrini,[2] and M. Pellegrino[2]

To get information about the gating process of single ion channels it is important to carry out the periodic modulation of a physical parameter affecting the channels while they are recorded by the patch clamp technique. This paper outlines a possible experimental approach in the case that the membrane potential is the modulated parameter.

KEY WORDS: Patch clamp recording; ion channels; sinusoidal stimulation; Markov systems; dynamical systems.

1. INTRODUCTION

Ion permeation across biological membranes is due to discrete water-filled pores embedded in the lipid bilayer. These "ionic channels" structurally consist of transmembrane glycoproteins that continually undergo conformational changes, some of which cause the pore to open. The probabilities of finding a channel open or closed can be specifically affected by electrical, chemical, or mechanical stimuli. On the other hand, diffusion ion fluxes through open channels are determined by electrochemical gradients.

Direct detection of single-channel gating is obtained with the patch clamp technique,[1] which allows ionic currents flowing across a tiny patch of membrane mounted at the tip of a glass pipette to be recorded (see Fig. 1a). The open–shut transitions of each channel are detected as step changes in the current signal, as shown in Fig. 1b for a patch containing only a single ion channel. The lower level of current is mainly due to

[1] Istituto di Biofisica del CNR, 56127 Pisa, Italy.
[2] Dipartimento di Fisiologia e Biochimica dell'Università, 56127 Pisa, Italy.

0022-4715/93/0100-0393$07.00/0

leakage of ions through the sealing resistance (leakage current), while the difference between the two current levels measures the flow of ions through the open channel. The single-channel current is given by $i_{ch} = g_{ch}(V_m - V_{eq})$, where g_{ch} is the single-channel conductance, V_m the membrane potential, and V_{eq} the Nernst equilibrium potential (see Fig. 2b). Accordingly, the potential at which the membrane is held directly affects the single-channel current. For most channels g_{ch} is constant over a wide range of membrane potentials (nonrectifying channels).

The main interest in studying the gating mechanisms of ion channels is to get the sequence of openings and closings that can be obtained by transforming the recorded current into a dichotomic signal, by means of a two-threshold window discriminator (Fig. 1b). Data reduction gives eventually a pair of distributions, that of openings (T_O) and that of closings (T_C).

The gating transitions of a single channel have been described as

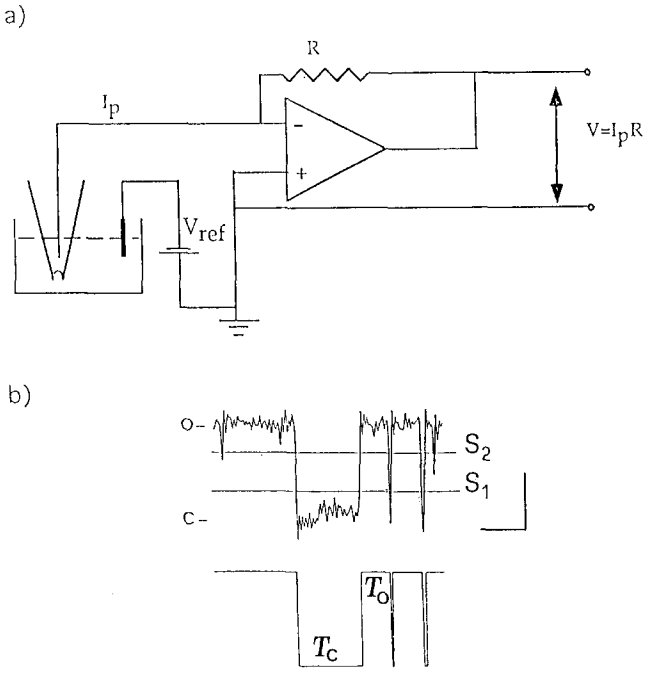

Fig. 1. (a) Schematic diagram of the current-measuring circuit in the patch-clamp technique. I_p: Current flowing through the pipette and the membrane patch on its tip; R: feedback resistance; V: output voltage of the I–V converter; V_{ref}: potential applied to the membrane patch. (b) Upper trace: single-channel current record showing three open–closed transitions (O: open; C: closed); lower trace: the corresponding threshold crossing signal (S_1 and S_2: levels of the window discriminator). Vertical bar: 3 pA; horizontal bar: 2 msec.

stochastic events and modeled mainly in terms of discrete Markov systems.[2–5] The Markov hypothesis leads to the fitting of the dwell-time distributions by sums of decreasing exponentials. However, as the number of exponentials required in the fit increases, the method becomes quite arbitrary, as recently debated, and alternative models have been proposed.[6–8] Nevertheless, in spite of the high number of exponential components often required, the exponential functions appear to have a physical meaning in fitting dwell-time histograms. In fact, the use of conditional distributions, i.e., those of a subset of dwell-time intervals satisfying given conditions, makes it possible to extract from a complex histogram a single exponential component.[8–10]

From a different point of view, ion channels are complex systems (macromolecules of hundreds of kilodaltons), with a high number of degrees of freedom, and a continuous or almost continuous description could be more realistic.[11] So one can consider the Markov scheme as a description of the system, and in the meantime look for what is behind the kinetic scheme.

The use of a periodic external forcing of ion channels could give information about the continuous system that is supposed to be responsible for ion channel gating. In particular, one can test whether there are resonant frequencies.

Alternatively, a system exhibiting stochastic resonance might be a good candidate to account for the behavior of ion channels. The output of an overdamped bistable system actually looks very similar to ion channel currents, also considering its statistical properties. In fact, when the thresholds for transitions are placed on the potential minima, the distributions of dwell times in each potential well are exponential.

Even in the frame of Markov systems the use of an external periodic forcing can give information on the kinetic scheme; for instance, it has been shown that irreversible schemes can have complex eigenvalues[12] and by applying periodic forcing it should be possible to enhance the oscillatory part of the response.

The simplest way to apply a periodic forcing is to periodically modulate the holding potential itself. However, some problems must be solved in order to record channel current under such a stimulation and to get the distributions of the relevant dwell times. In this paper we will deal with these problems, suggesting a solution.

2. THE SCHEME OF THE EXPERIMENT

Sinusoidal modulation of the holding potential can be achieved by using the setup schematically shown in Fig. 2. Herein the ground reference

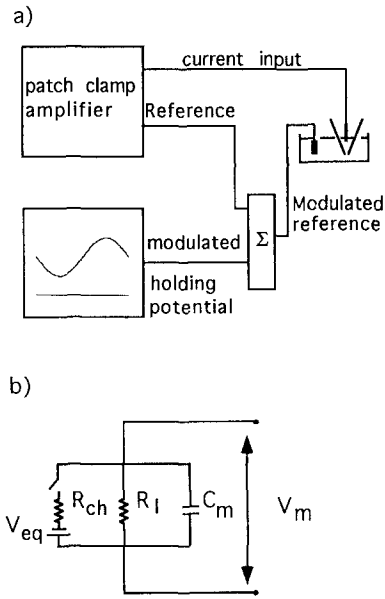

Fig. 2. (a) Block scheme of the experimental setup; (b) equivalent circuit of the membrane patch. C_m: Membrane capacitance; R_l and R_{ch}: leakage and channel resistance, respectively; V_{eq}: Nernst equilibrium potential.

of the patch clamp amplifier, which is usually connected with the bath in which the pipette is immersed, is summed up with the modulated signal. This modulated voltage is directly applied to the membrane on the tip of the glass pipette. Under these conditions the recorded current appears as in Fig. 3: its baseline, i.e., the leakage current, is modulated and, as expected, the amplitude of its step changes at the openings depends on the potential. To perform the statistical analysis of such a signal, the sequence of dwell

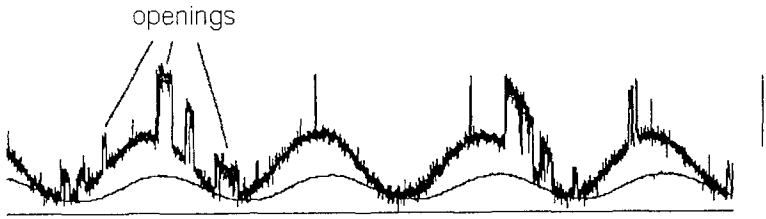

Fig. 3. Patch amplifier output under sinusoidal modulation of the reference potential; lower line indicates zero current level. Upward deflections indicate current from the bath to the pipette. Vertical bar: 15 pA; modulation frequency: 1 Hz; cutoff frequency of signal filtering: 3 kHz.

times at the open and closed levels must be extracted from the raw current signal; in fact, only the modulation of the dwell times carries information on the relationship between the gating mechanism and the applied voltage.

In order to better understand the analysis protocol, it is worth recalling how the membrane patch acts in the electric circuit. The equivalent electric scheme of the patch is shown in Fig. 2b. Here C_m represents the membrane capacity and $g_l = 1/R_l$ the leakage conductance, due partly to imperfect sealing between the pipette glass and the membrane and partly to the membrane itself; then $g_{ch}(1/R_{ch})$ is the single-channel conductance and V_{eq} the Nernst equilibrium potential.

The modulated holding potential is given by

$$V_m(t) = V_0(1 + M \sin 2\pi ft) \tag{1}$$

where V_0 is the mean value, M the modulation depth, and f the modulation frequency.

Sinusoidal modulation of the holding potential has been applied as an example to stretch sensitive cation channels of leech central neurons.[13] The cell-free patch was mounted at the tip of the pipette in inside-out configuration and current was recorded in symmetrical 120 mM KCl. Since in this experimental condition the solutions bathing the two sides of the membrane have the same composition, the equilibrium potential for each ion is zero.

The recorded current is the sum of three terms, i.e., capacitive, leakage, and channel currents:

$$I(t) = i_c + i_l + i_{ch} \tag{2}$$

Now the capacitive current leads by 90 deg the applied voltage; so we can express the patch amplifier output as

$$I(t) = A \sin(2\pi ft + \pi/2) + i_l + i_{ch} \tag{3}$$

where A is the amplitude (unknown) of the capacitive current. Then

$$i(t) = I(t) - A \sin(2\pi ft + \pi/2) = i_l + i_{ch} \tag{4}$$

The last two terms (leakage current and channel current), under the conditions described above, are both proportional to the instantaneous value of the voltage. In fact, at least within the range of membrane potential where the channel does not rectify, the current flowing through the open channel is proportional to the driving potential $V_m - V_{eq}$, which in this case is equal to V_m.

Figure 4 shows for simplicity a stretch of record where openings do

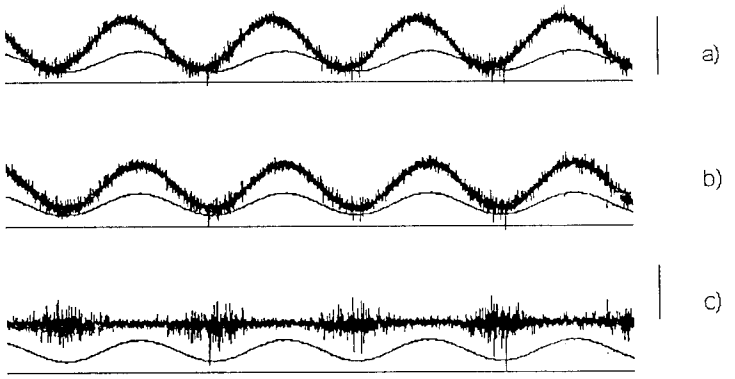

Fig. 4. Application of the method to a record where gating transitions are absent. In each panel the membrane potential is displayed as the middle trace and both the zero-potential and zero-current levels as the lower trace. The upper trace from (a) to (c) shows respectively the total amplifier output, the current signal minus the capacitive current, and the conductance reconstructed as $g = i/V_m$. Vertical bar: 15 pA in (a) and (b), 200 pS in (c). Modulation frequency: 1 Hz; cutoff frequency of signal filtering: 3 kHz.

not occur. In the first panel the amplifier output and the modulated holding potential are displayed. The phase shift between the two signals is due to the capacitive current. The second panel is obtained by subtracting the capacitive current from the recorded current $I(t)$. This was done by software, by computing $i(t)$ of Eq. (4) and varying A until the phases of $i(t)$ and of the potential coincided. Thus the signal in the second panel in Fig. 4 is simply the leakage current, which is proportional to the applied voltage.

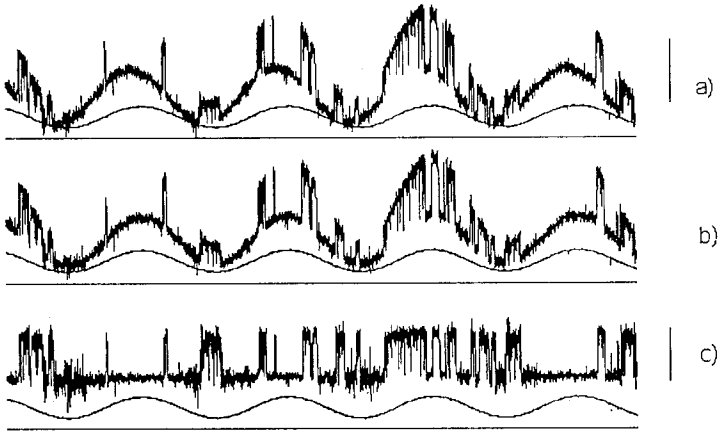

Fig. 5. The traces in each panel have the same meaning as in Fig. 4. The method was applied to a record segment exhibiting gating transitions. Calibrations as in Fig. 4.

Dividing the leakage current by $V_m(t)$ yields the third panel. A flat signal proportional to the membrane conductance is finally obtained; note the expected increase of noise amplitude near the minimum of the input signal (the division by small values causes an amplification).

Figure 5 shows the same procedure applied to a record segment where the channel makes shut–open transitions. The third panel shows the cleaned sequence of openings and closings. The resulting signal can be analyzed with standard procedures, for instance, by selecting openings and closings with thresholds placed as in Fig. 1b.

Fig. 6 shows the superposition of five sweeps; here the original signal is reported in the first panel and the conductance signal obtained from it in the second one. This large-conductance channel, identified as a stretch-activated one, looks insensitive to the membrane potential under our stimulation conditions. This point was not investigated further because at this stage we used this channel only to test the method.

Now, what can be done by software can also be done by hardware. Figure 7 is obtained by using a home-made electronic circuit (the scheme is available from the authors) to process the current signal in the same way as described above.

In Fig. 7 the distributions of all the time intervals T_O and T_C are separately reported on a log-binned time scale[14]; in this representation an exponential distribution appears as a skewed bell-shaped curve. The fitting curves are a single exponential for the openings and a sum of two exponentials for the closings. The time constants are not related to the frequency of modulation.

This work shows that it is possible to apply to the channels recorded in a patch clamp a modulated holding potential *and* to analyze the recorded signal by separating the meaningless baseline and amplitude modulations from the modulation of the sequence of openings and closings.

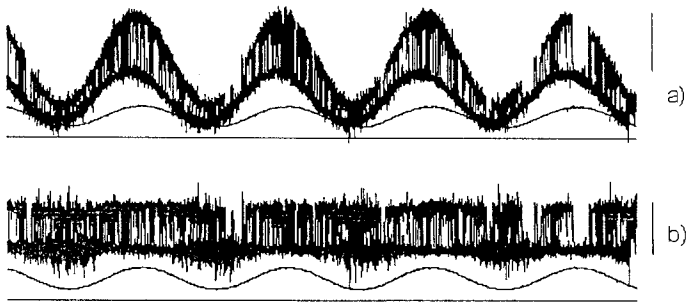

Fig. 6. Five superimposed (a) current and (b) conductance traces showing the absence of modulation in the channel activity. Calibrations as in Fig. 4.

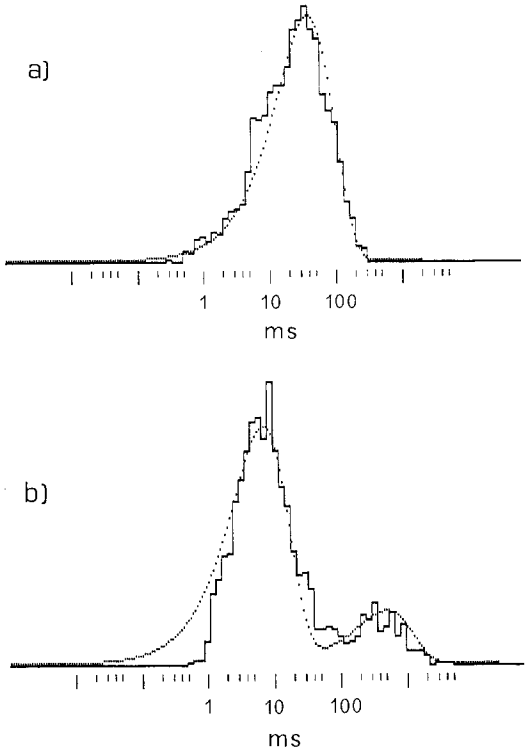

Fig. 7. Log-binned histograms of (a) open and (b) closed time intervals obtained after conversion of the current signal to a conductance signal. Data reduction was performed by hardware. The time constant fitted to openings is 40 msec, those fitted to closings are 7 and 500 msec. A total of 1500 events were used.

ACKNOWLEDGMENTS

The authors are deeply grateful to F. Moss for his enthusiastic encouragement to perform these experiments. Thanks are due to E. Arimondo, A. Fioretti, L. Fronzoni, R. Mannella, and B. Zambon for stimulating discussions. This research was partially supported by NATO grant 1272/90 to F.M. and E.A. and by CNR grant 900122502 to M.P.

REFERENCES

1. O. P. Hamill, A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth, Improved patch clamp techniques for high resolution current recording from cells and cell free membrane patches, *Pfluegers Arch.* **391**:85–100 (1981).
2. D. Colquhoun and A. G. Hawkes, On the stochastic properties of single ion channels, *Proc. R. Soc. Lond. Biol.* **211**:205–235 (1981).

3. D. Colquhoun and A. G. Hawkes, The principles of the stochastic interpretation of ion-channel mechanisms, in *Single Channel Recording*, B. Sakmann and E. Neher, eds. (Plenum Press, New York, 1983), pp. 135–175.
4. D. Colquhoun and A. G. Hawkes, A note on correlations in single ion channel records, *Proc. R. Soc. Lond. Biol.* **230**:15–52 (1987).
5. D. R. Fredkin, M. Montal, and J. A. Rice, Identification of aggregated Markovian models: Application to the nicotinic acetylcholine receptor, in *Proceedings of the Berkeley Conference in Honor of Jerzy Neymann and Jack Kiefer*, L. M. LeCam and R. A. Olshen, eds. (Wadsworth Press, 1985), pp. 269–289.
6. L. S. Liebovitch, Testing fractal and Markov models of ion channel kinetics, *Biophys. J.* **55**:373–377 (1989).
7. G. L. Milhauser, E. E. Salpeter, and R. E. Oswald, Diffusion models of ion-channel gating and the origin of the power-law distributions from single-channel recording, *Proc. Natl. Acad. Sci. USA* **85**:1503–1507 (1988).
8. M. Barbi and D. Petracchi, How to test the Markov nature of ionic channels, in *Proceedings of the 10th European Meeting on Cybernetics and Systems Research*, R. Trappl, ed. (Vienna, 1990), pp. 391–398.
9. D. Petracchi and M. Barbi, Statistical of open and closed dwell times of ion channels, *SIF: Conference Proceedings*, Vol. 31, C. Frediani, ed. (Bologna, pp. 245–252.
10. D. Petracchi, M. Barbi, M. Pellegrini, M. Pellegrino, and A. Simoni, Use of conditional distributions in the analysis of ion channel recordings, *Eur. Biophys. J.* **20**:31–39 (1991).
11. H. Frauenfelder, F. Parak, and R. D. Young, Conformational substates in proteins, *Annu. Rev. Biophys. Biophys. Chem.* **17**:451–479 (1988).
12. M. T. Kirber, J. J. Singer, and J. V. Walsh, Possible forms for dwell-time histograms from single chanel current records, *J. Theor. Biol.* **116**:111–126 (1985).
13. M. Pellegrino, M. Pellegrini, A. Simoni, and C. Gargini, Stretch-activated cation channels with large unitary conductance in leech central neurons, *Brain Res.* **525**:322–326 (1990).
14. F. J. Sigworth and S. M. Sine, Data transformations for improved display and fitting of siongle-channel dwell time histograms, *Biophys. J.* **52**:1047–1054 (1987).