5. M. D. Desch and M. L. Kaiser, *Geophys. Res. Lett.* **8** 253 (1981).
6. B. A. Smith and G. E. Hunt, in *Jupiter*, T. Gehrels, Ed. (Univ. of Arizona Press, Tucson, 1976), pp. 564–585.
7. I thank the National Space Science Data Center for supplying the data used in this study, which comes from the Voyager imaging experiments (team leader, B. A. Smith). I thank M. Belton and J. Christou for reading a draft version of this paper. The National Optical Astronomy Observatories are operated by the Association of Universities for Research in Astronomy, Inc., under contract to the National Science Foundation.

28 July 1989; accepted 16 January 1990

Steady-State Coupling of Ion-Channel Conformations to a Transmembrane Ion Gradient

EDWIN A. RICHARD AND CHRISTOPHER MILLER

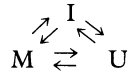
Under stationary conditions, opening and closing of single *Torpedo* electroplax chloride channels show that the number of transitions per unit time between inactivated and conducting states are unequal in opposite directions. This asymmetry, which increases with transmembrane electrochemical gradient for the chloride ion, violates the principle of microscopic reversibility and thus demonstrates that the channel-gating process is not at thermodynamic equilibrium. The results imply that the channel's conformational states are coupled to the transmembrane electrochemical gradient of the chloride ion.

ION CHANNELS ARE A UBIQUITOUS class of integral membrane proteins that catalyze the passive diffusion of specific ions across biological membranes by forming hydrophilic pores. Channels can exist in nonconducting ("closed") and in ion-conducting ("open") conformations, whose interconversions may be driven by ligand binding, covalent modification, or electric fields. It is usually assumed that the opening and closing of ion channels represent conformational equilibria that are separate and distinct from the kinetic process of ions diffusing through the open pore. This study provides an example of an ion channel, the Cl^- channel from the electric organ of the electric ray *Torpedo californica*, whose individual transitions among conducting and nonconducting states display a strong asymmetry in time at the single-channel level and hence are not at equilibrium. Under our experimental conditions, the only free energy sources available for keeping the system away from equilibrium are transmembrane electrochemical gradients of aqueous solutes.

The Cl^- channel of *Torpedo* electroplax is unusual in that it operates by a "double-barreled shotgun" mechanism (*1, 2*). The channel complex is made up of two Cl^- diffusion pathways or "protochannels," which open and close independently of one another on the millisecond time scale. This behavior is apparent in the single-channel record shown in Fig. 1A; the channel displays three short-lived conductance levels, labeled U, M, and D, which represent states in which 2, 1, or 0 protochannels are open, respectively. An "inactivated" state, labeled I, is also apparent as long-lived nonconducting intervals (hundreds of milliseconds) separating bursts of rapid transitions between U, M, and D. This inactivated state is the result of a conformational change that shuts both protochannels simultaneously; bursts of channel activity occur when the channel complex leaves this inactivated state (*2*).

The traces in Fig. 1B show at a higher time resolution the seven inactivated periods displayed in the top trace. These events reveal a remarkable property of the transitions into and out of the I state: a high degree of asymmetry. In six of these seven cases, the channel entered the I state from M but left the I state into U. In 63 inactivated episodes, 31 were M→I→U and 1 was U→I→M. The remaining inactivated intervals were symmetrical: 29 M→I→M and 2 U→I→U.

To clarify the fundamental meaning of these observations, we should consider the directly observable transitions among U, M, and I. Records as in Fig. 1 show that this channel gates according to the following cyclic scheme, in which each state directly communicates with the other two:



The excess of M→I→U over U→I→M transitions means that the forward and backward rates of interconversions between the pairs of adjacent states U/I and M/I are unequal. This time asymmetry shows that detailed balance does not hold here and that therefore the three conformational states are not at thermodynamic equilibrium. Instead, they are maintained in a cyclic steady state in which net "clockwise" movement occurs around the state diagram. The maintenance of any reaction in a steady state always requires the input of external energy to keep the system away from equilibrium (*3*).

In these single-channel experiments, the individual conformational histories of single-channel molecules are observed directly. Therefore, the fluxes around the cycle in each direction, J_+ and J_-, could be measured directly from the channel record as the number of M→I→U→M and U→I→M→U cycles per unit time. It can be readily shown (*3*) on general thermodynamic grounds that the "asymmetry ratio," defined as the ratio of these unidirectional

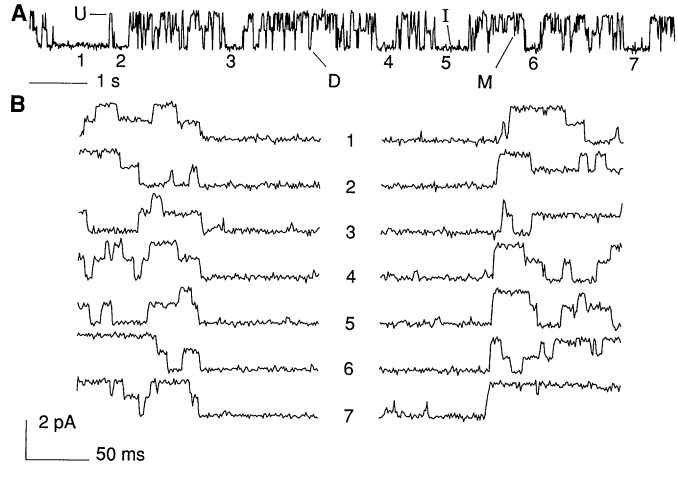


Fig. 1. Time asymmetry of a single *Torpedo* electroplax Cl^- channel. (**A**) A single-channel record at a slow time scale, with the four states of the channel labeled (U, D, M, and I), and the seven inactivated intervals numbered. (**B**) The same trace at an expanded time scale. Only the beginning and end of each inactivated state is shown, to allow identification of the states immediately preceding and following the inactivated interval. Single Cl^- channels were isolated by patch-recording from a planar bilayer (*9*) containing *Torpedo* Cl^- channels (*2*). All solutions used contained 1 m*M* $CaCl_2$, 0.1 m*M* EDTA, 150 m*M* KCl, and 10 m*M* Mops (4-morpholine propanesulfonic acid), *p*H 7.4. Voltage was held at −80 mV on the "cis" side, to which the *Torpedo* vesicles were added.

Howard Hughes Medical Institute, Graduate Department of Biochemistry, Brandeis University, Waltham, MA 02254.

fluxes, is related to the externally delivered free energy, ΔG, coupled to the cycle of conformational change:

$$J_+/J_- \leq \exp(-\Delta G/RT) \quad (1)$$

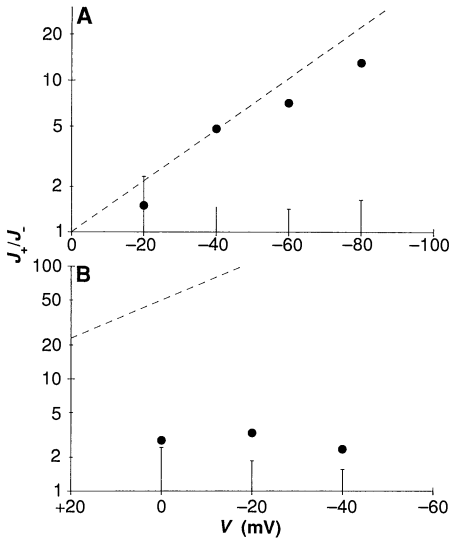
where R is the gas constant and T is the absolute temperature.

An understanding of this channel's nonequilibrium gating requires an attack on two separate issues, namely, (i) the source of free energy needed to maintain the cyclic steady state and (ii) the specific mechanism by which the channel's gating process is coupled to the free energy source. Plausible energy sources are few. All these experiments were carried out in a minimal reconstituted system with planar lipid bilayer membranes used to separate solutions containing only buffered Cl^- salts (*4*). No chemical sources of energy were available, such as a phosphorylation-dephosphorylation cycle, as might operate in the intact cell. In fact, the only available thermodynamic driving forces in the system are the transmembrane electrochemical gradients of the aqueous ions, Cl^- and K^+. Since this channel allows Cl^- to permeate specifically and since its operation is unaffected by the type of cation present (*4*), we postulate that the observed cyclic steady state is driven by the electrochemical potential difference for Cl^- across the membrane, that is, that the conformational changes among I, U, and M are in some way coupled to the downhill movement of Cl^- through the channel.

We tested this hypothesis by measuring the channel's time asymmetry at varying electrochemical gradients for Cl^-. Channel records were collected at varying transmembrane voltages, in the presence or absence of transmembrane Cl^- concentration gradients. The relation between the asymmetry ratio and electrochemical potential in symmetrical 250 m*M* Cl^- solutions is shown in Fig. 2A. The observed asymmetry increases with Cl^- electrochemical potential gradient, which in this case is wholly determined by transmembrane voltage. At a low voltage (−20 mV) the observed ratio is not significantly different from unity, and thus the system is near equilibrium. As voltage is increased beyond −40 mV, an increasingly strong asymmetry around the conformational cycle is observed. In Fig. 2, the dashed lines represent the asymmetry expected for "perfect coupling," in which the transmembrane movement of a single Cl^- occurs obligatorily with each complete turn around the cycle.

In a similar experiment (Fig. 2B) a large Cl^- concentration gradient was used across the membrane (250 m*M*/5m*M*). These conditions allowed the recording of channels at zero voltage, where a small but significant asymmetry was observed. However, in contrast to the symmetrical Cl^- case, the asymmetry ratio fell far below that expected for perfect stoichiometric coupling between Cl^- movement and cyclic gating.

Fig. 2. Dependence of the asymmetry ratio on Cl^- electrochemical gradient. Positive cycles were defined as U→M→I→U, negative cycles as U→I→M→U. (**A**) Symmetrical 250 m*M* KCl solutions, buffered with 10 m*M* of either Bicine-HCl [*N*,*N*-bis(2-hydroxyethyl)glycine–HCl] or Hepes-KOH [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid–KOH], in the *p*H range 7.4 to 8.5. (**B**) Asymmetrical Cl^- solutions. The "cis" side contained 250 m*M* KCl, and the "trans" side contained 5 m*M* KCl + 245 m*M* potassium acetate; both solutions contained 10 m*M* Bicine, *p*H 8.5. Dashed lines represent theoretical maximum asymmetry ratios, with one Cl^- moving per cycle. Error bars on the abscissa represent the 95% confidence limits under the hypothesis that the gating is at equilibrium, according to the binomial sign test. Each point represents a set of four to seven experiments, containing 23 to 130 cycles.



How are the movements of Cl^- through the channel coupled to the channel's conformational changes? For heuristic purposes, let us consider a channel with only a single pore. We imagine that this pore has two "gates" flanking a Cl^- binding site located in the diffusion pathway (Fig. 3A). For simplicity, we postulate that both gates can close simultaneously only when Cl^- is bound (*5*). Moreover, we consider the case of a large transmembrane Cl^- gradient, in which the "lower" solution is Cl^- free. Under these conditions, transitions between states 1 and 2, 1 and 4, and 3 and 4 will be freely reversible. However, transitions from state 2 to state 3 will be effectively one way. The Cl^- trapped in state 2 will rapidly dissociate off the site as soon as the lower gate opens (state 3). Since the lower solution is Cl^- free, the back-transition to state 2 cannot occur. Thus, with this extreme concentration gradient for Cl^-, the model cycles in a clockwise sense, with a single Cl^- being

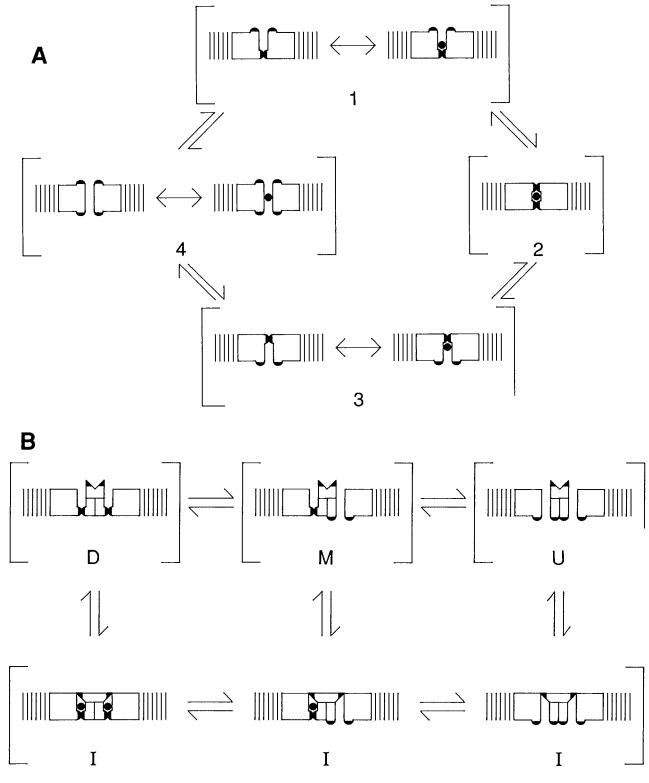


Fig. 3. Models for time-asymmetric cyclic gating. (**A**) A single-pore model capable of exhibiting cyclic gating that violates detailed balance. Two "gates" flank a Cl^- binding site within the conduction pore. When the "upper" gate is open, the site rapidly equilibrates with the upper solution containing Cl^- (state 1). When the "lower" gate is open, the site rapidly equilibrates with the lower solution lacking Cl^- (state 3). By assumption, both gates can be closed only when Cl^- is in the binding site (state 2). When both gates are simultaneously open (state 4), the channel conducts Cl^- across the membrane. (**B**) A "double-barreled" extension of the single-pore model to represent the *Torpedo* Cl^- channel. For simplicity, binding of Cl^- and symmetrical gate positions are not shown. The permutations of the gate positions are labeled to illustrate the states and transitions observed during single-channel recording. Note that the upper gate (pointed bar), which represents the "inactivation" process, closes off both pores simultaneously.

obligatorily moved from high to low chemical potential with each turn of the conformational cycle. Indeed, this model shows a strong resemblance to a conventional solute transport system, such as the glucose carrier, in the presence of a large gradient for the transported solute, if it were possible to distinguish the various conformational states of single transporter molecules. Of course, with a single-pore channel operating in this way, we would not actually be able to observe a time-asymmetric single-channel record; it is the channel "substates" arising from the double-barreled nature of the Cl^- channel that reveal the time asymmetry.

Although useful in rationalizing our results qualitatively, this simple picture is unrealistic. The model does not predict the vastly different asymmetry ratios observed with different Cl^- concentrations (Fig. 2). We have elaborated the basic model in several ways to introduce more realism. First, we have considered a double-barreled model (Fig. 3B), in which the two protochannels always inactivate together, as in the Cl^- channel. Second, we have relaxed the constraint that the "upper" and "lower" gates can simultaneously close only when Cl^- is bound. With these two twists on the simple model, Monte Carlo simulations show that the asymmetry depends on the many rate constants in the model, and that efficiency of coupling between Cl^- movement and gating asymmetry varies with the absolute concentration of Cl^-. We are currently investigating whether these more complex models can be used to understand quantitatively the variations in coupling efficiency we have observed under different Cl^- gradient conditions.

The *Torpedo* Cl^- channel provides an opportunity to explore nonequilibrium channel gating driven by electrochemical gradients. Previous investigations on nonequilibrium gating, based on current flow through open channels, are either wholly theoretical (*6*) or have been described only anecdotally (*7*). The Cl^- channel studied here provides a crisp example of a gating process with a clear, manipulable dependence on electrochemical potential of the permeating ion. It is worth noting that a well-documented and puzzling property of a different channel, the inward rectifier K^+ channel of excitable membranes, is the ability of its gating machinery to "follow" the electrochemical gradient for K^+, opening only when net K^+ movement is inward (*8*). The mechanism presented here, which provides a concrete example of the coupling of ion-channel gating to the transmembrane gradient of the conducting ion, may therefore have a wider relevance than to the Cl^- channel alone.

REFERENCES AND NOTES

1. Preliminary discussions of the data and model are found in E. A. Richard and C. Miller, *Biophys. J.* **53**, 266a (1988); C. Miller and E. A. Richard, in *Cl^- Channels and Carriers in Nerve, Muscle, and Glial Cells*, F. J. Alvarez-Leefmans and J. Russell, Eds. (Plenum, New York, in press).
2. C. Miller, *Philos. Trans. R. Soc. London Ser. B* **299**, 401 (1982); ——— and M. M. White, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 2772 (1984).
3. T. L. Hill, *Free Energy Transduction in Biology* (Academic Press, New York, 1977).
4. C. Miller and M. M. White, *Ann. N.Y. Acad. Sci.* **341**, 534 (1980); M. M. White and C. Miller, *J. Gen. Physiol.* **78**, 1 (1981).
5. A similar treatment can be performed by specifying that both gates can only close on an empty site.
6. P. Läuger, in *Single-Channel Recording*, B. Sakmann and E. Neher, Eds. (Plenum, New York, 1983), pp. 177–198; A. Finkelstein and C. S. Peskin, *Biophys. J.* **46**, 549 (1984).
7. A. Hamill and B. Sakmann, *Nature* **294**, 462 (1981); A. Trautmann, *ibid.* **298**, 272 (1982); S. G. Cull-Candy and M. M. Usowicz, *ibid.* **325**, 525 (1987).
8. R. H. Adrian *et al.*, *J. Physiol. (London)* **208**, 607 (1970); S. Hagiwara and K. Takahashi, *J. Membr. Biol.* **18**, 61 (1974); but see C. A. Leech and P. R. Stanfield, *J. Physiol. (London)* **319**, 295 (1981).
9. O. S. Andersen, *Biophys. J.* **41**, 119 (1983).

10. We thank R. Horn for advice on the statistical analysis of our data and I. Levitan and R. MacKinnon for critical readings of the manuscript. Supported by NIH research grant GM-31768 and training grant NS-07292.

10 October 1989; accepted 28 December 1989

Conserved Residues Make Similar Contacts in Two Repressor-Operator Complexes

CARL O. PABO, ANEEL K. AGGARWAL,* STEVEN R. JORDAN, LESA J. BEAMER, UPUL R. OBEYSEKARE, STEPHEN C. HARRISON

Comparison of a λ repressor-operator complex and a 434 repressor-operator complex reveals that three conserved residues in the helix-turn-helix (HTH) region make similar contacts in each of the crystallographically determined structures. These conserved residues and their interactions with phosphodiester oxygens help establish a frame of reference within which other HTH residues make contacts that are critical for site-specific recognition. Such "positioning contacts" may be important conserved features within families of HTH proteins. In contrast, the structural comparisons appear to rule out any simple "recognition code" at the level of detailed side chain–base pair interactions.

THE λ AND 434 REPRESSORS, WHICH are produced by related *Escherichia coli* phages (*1*), recognize very distinct operator sites, but both use the HTH unit for recognition (*2–4*). Alignment of the amino acid sequences reveals significant similarities (*5*), and there also are clear structural similarities between the proteins: Helices 1 to 4 of λ repressor correspond to helices 1 to 4 of 434 repressor (*4*). In each case, helices 2 and 3 form the conserved HTH unit that participates in operator recognition and 7 of the 20 residues in the HTH unit are identical (Fig. 1). The NH_2-terminal domain of λ repressor (92 residues) is somewhat larger than the NH_2-terminal domain of 434 repressor (69 residues), and 434 repressor lacks an NH_2-terminal arm or any region that corresponds to helix 5 of λ repressor.

Comparing the HTH units from the two cocrystal complexes (residues 33 to 52 of λ repressor and residues 17 to 36 of 434 repressor) shows that these regions are extremely similar (Fig. 2). The α-carbons of these units can be superimposed with root-mean-square (rms) deviations of 0.48 to 0.59 Å (*6*) depending on which subunits are used. These values are similar to the deviations observed when comparing the two halves of the same repressor (0.31 Å rms for λ and 0.59 Å for 434). In this region, the polypeptide backbones of the two proteins are essentially indistinguishable, consistent with previous observations that the HTH units of the λ Cro protein, the *E. coli* CAP protein, and the λ repressor are almost identical (*7, 8*).

Although the structures of the λ and 434 repressors are similar in the HTH regions, there are significant differences if one compares the other helices or compares the relative orientation of the protein dimers. When superimposing helices 1 to 4 of the two repressors, it seems most appropriate to

C. O. Pabo and U. R. Obeysekare, Howard Hughes Medical Institute, Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, MD 21205.
A. K. Aggarwal and S. C. Harrison, Howard Hughes Medical Institute, Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, MA 02138.
S. R. Jordan, Agouron Pharmaceuticals, Inc., 11025 North Torrey Pines Road, La Jolla, CA 92037.
L. J. Beamer, Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, MD 21205.

*Present address: College of Physicians and Surgeons, Columbia University, New York, NY 10032.