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<p>Whether the kinetics and thermodynamics of electric-field initiated alteration of nerve membrane to the conduction of specific ions through ion channels (gating) can be validly described by linear, equilibrium models will be determined by examining the time reversal properties of ion channel current fluctuations. The kinetics of gated channels derived from analysis of stochastic current fluctuations and those derived from linear macroscopic determinations (membrane complex admittance determinations) will be compared to ascertain whether the assumed relationship exists. <i>Keywords:</i></p>			
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ANNUAL REPORT ON CONTRACT NOOO-14-87-K-0055

PRINCIPAL INVESTIGATOR: Harvey M. Fishman

CONTRACTOR: University of Texas Medical Branch

CONTRACT TITLE: Gating Kinetics and Ion Transfer in Channels of Nerve Membrane

Research Objectives

Alteration of a membrane to the conduction of specific ions by an electric field is known as "gating." The research objective of this project is to determine whether gating mechanisms of ion channels are properly described by models that assume linear rate processes and equilibrium thermodynamics. Measurement of stochastic fluctuations from ion channels via patch clamp techniques and analysis of the time-reversal of the statistical properties of the fluctuations are the means by which these objectives are sought.

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Progress Report

1. TIMREV: a computer program for analysis of the time reversibility of fluctuations from transition-state models and ion channels.

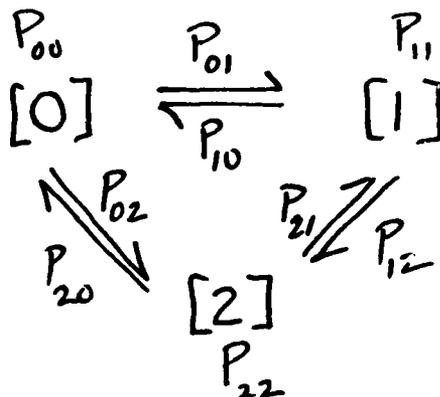


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We have developed a program that evaluates a generalized autocorrelation function of a noise signal derived from a transition-probability model of an ion channel or from actual conductance fluctuations recorded from a membrane preparation. Noise generated by systems that are at thermodynamic equilibrium (ones that obey microscopic reversibility), show the same statistical properties when evaluated forward or backward in time whereas the statistical properties of fluctuations from nonequilibrium systems are not time reversible. Thus a thermodynamic distinction can be made by examination of the generalized autocorrelation function:

$$G^{1,2}(\tau) = \frac{1}{(T_2 - T_1) - \tau} \int_{T_1}^{T_2 - \tau} f^1(t) f^2(t + \tau) d\tau$$

to see if the time-reversed autocorrelation function,  $G^{2,1}(\tau)$  is the same or different from the time-forwarded correlation function,  $G^{1,2}(\tau)$ . As an example, consider the 3-state transition model below:



In this model each state has a probability, and an amplitude or level associated with the state and transition probabilities to and

from each state. For this model the following must be true;

$$P_{00} + P_{01} + P_{02} = 1$$

$$P_{10} + P_{11} + P_{12} = 1$$

$$P_{20} + P_{21} + P_{22} = 1$$

Selection of the probabilities and amplitudes, shown below yielded a reversible and an irreversible, 3-state model which were tested for time reversal.



Fig. 1 shows the forward and time-reversed correlation functions for the above reversible and irreversible transition-state models as evaluated by TIMREV.

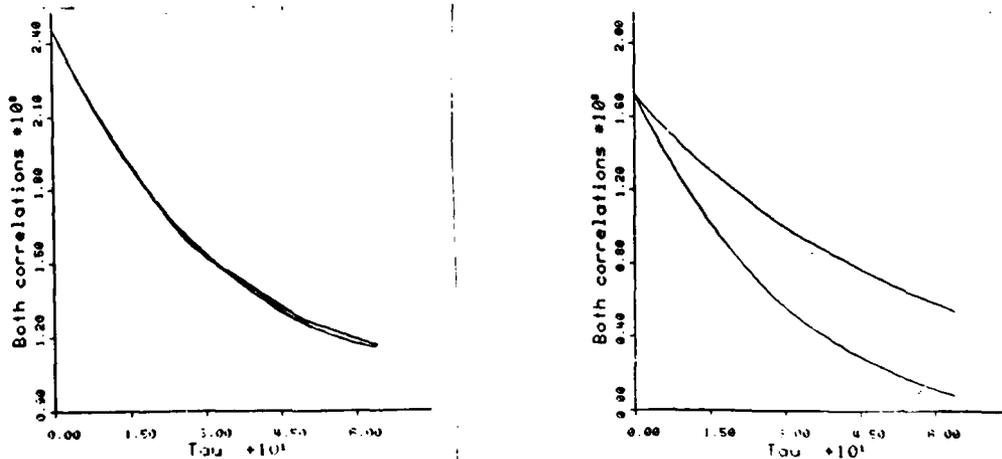


Figure 1: Left, reversible model. Right, irreversible model

From the similarity and dissimilarity for the two cases, as the theory predicted, for the time-forwarded and reversed correlations functions is confirmed in these plots. This program has been tested extensively and is now ready for evaluation of real data from membranes, which is the next step.

2. Axoballs: a new model ion channel preparation from the squid giant axon.

We discovered a novel processes (injury-induced vesiculation) following neural injury of giant axons of cephalopods (see Fig. 2). After the cutting, tearing or stretching of an axon, membranous vesicles form in an axon, become larger by fusing, and migrate to the site of injury, thereby closing off the damaged region from the external medium. This redistribution process involves mobilization of membrane from sources very close to or at the subaxolemmal surface. Injury-induced vesiculation does not occur in divalent-free media, but addition of  $\text{Ca}^{+2}$  or  $\text{Mg}^{+2}$  at physiological concentrations (10 mM) elicits the process in injured axons.

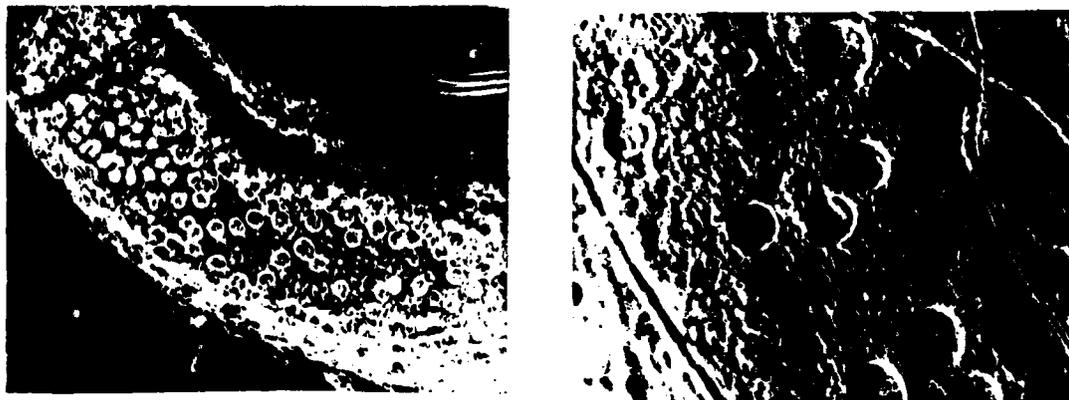


Figure 2: Injury-induced vesiculation in a squid giant axon.

Before sealing occurs in transected axons, some large vesicles emerge from the cut ends and form large spheres, designated "axoballs." Application of a patch-clamp technique to axoball membrane showed stochastic currents fluctuating between two discrete levels over a range of membrane voltages with a conductance (12pS) indicative of a single, cation channel (Fig. 3). A variety of other stochastic waveforms from other channel types was also observed, suggesting that the redistributed membrane contains ion channels, which may play a role in the restoration process at sites of injury. A possible application of harvested axoballs may be to provide a model membrane preparation for the study of ion channels derived from native (squid axon or other) membrane without any biochemical intervention.

In their passage through the constricted open end of a cut axon, some balls fuse and consequently emerge from an axon much larger (maximum size observed, 250  $\mu\text{m}$  in diameter) than the balls in an axon. Thus, axoball size does not correlate directly with axonal size. The attachment of axoballs to axoplasm provided a stable anchor that allowed access to their surfaces and isolation of a patch by an electrolyte-filled glass pipet. Voltage clamp of patches of the external surface of an axoball demonstrated its membranous structure.

Fig. 3 (photograph) shows an axoball of about 100  $\mu\text{m}$  diameter, outside the cut end of an axon, from which a patch was isolated (100 gigohms), excised and voltage clamped. The accompanying current records were obtained seconds after establishment of the constant potentials indicated (pipet voltage relative to bath voltage). The representative samples, taken from longer duration

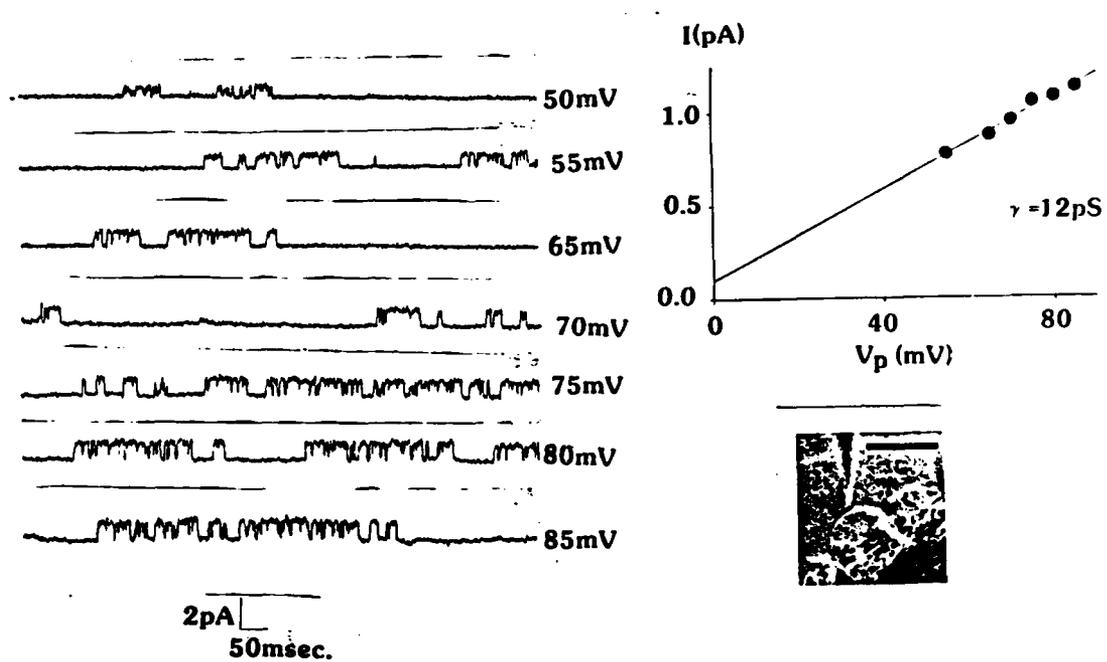


Figure 3: Patch clamp records of a single cation channel from an excised patch of axoball (photo) membrane.

(20 sec) current records, show a binary waveform with random transitions characteristic of the opening and closing of a single channel. The current-voltage relation,  $I(V)$ , obtained from such records over the voltage range of 50 to 85 mV is linear, as shown in Fig. 3. The slope of  $I(V)$  gives an open-channel conductance of 12 pS. Conductances of 11.5 and 13 pS were obtained in two other patches. Furthermore, the single channel conductance, when chloride was replaced by glutamate in both pipet and bath solutions, was similar demonstrating that these data are not from a chloride channel. A voltage-sensitive sodium channel is excluded because tetrodotoxin was present in both solutions. The conductance obtained from the single channel  $I(V)$  is in the range of estimates (2-12 pS) for potassium channels in squid axon obtained previously by spectral analyses of fluctuations measured

from large populations of channels. The conductance is also close to the 10 pS value reported recently in patches of cut-open squid axon. Also, addition of 1 mM  $Zn^{+2}$  to the bath severely limited channel openings. Since addition of this concentration of  $Zn^{+2}$  to the ASW bathing an axon reversibly reduced  $K^+$  currents, in voltage clamp experiments, by 65%, the hypothesis that these are potassium channels is supported. Thus, although the ion selectivity of this channel was not determined, these data indicate a potassium channel. Other channels with multiple conducting states and channels with larger conductances were also observed under different ionic conditions.

These observations suggest that injury-induced vesiculation, fusion and redistribution provide the structures and processes necessary to seal a cut axon. In addition, axoballs carry ion channels that may play a role in the recovery process by establishing current flow between the seal and axolemma, after axoball incorporation into the seal. Sealing after injury also occurs in cardiac muscle and in skeletal muscle fibers bathed in high  $Ca^{+2}$  solutions. In previous work on transected cockroach axons, a partition-like structure formed and complete sealing occurred in 15-20 min. During this period, "vacuoles" similar to axoballs were observed. Recovery of resting ionic conductances and regeneration of severed cockroach axons occurred several days later. The injury-induced vesiculation described here may be a manifestation of the same process that gives rise to sub-axolemmal vacuoles seen during peripheral neuropathy associated with demyelination of axons in mutant hamster and in murine motor neuron disease in the central nervous system. Both of these disorders

involve a redistribution of axonal cytoskeleton and axonal organelles in myelinated nerve fibers.

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