EXPERIMENTAL EVIDENCE CONSISTENT WITH AGGREGATION KINETICS IN THE SODIUM CURRENT OF *MYXICOLA* GIANT AXONS

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ABSTRACT Aggregation kinetics, in contrast to the Hodgkin-Huxley equations, predict that if an axon is subjected to a brief perturbing depolarization of large amplitude, the resulting perturbed current will cross over the response to a conventional maintained depolarization, and then remain smaller for the remainder of the depolarizing step. This has been experimentally tested using voltage-clamped *Myxicola* giant axons, compensated for series resistance and bathed in 10% Na^+ sea water to minimize possible artifacts. Under such conditions perturbed and unperturbed currents are observed to cross over in a manner qualitatively consistent with the behavior predicted by an aggregation model. We suggest, therefore, that the aggregation concept may warrant further experimental and theoretical investigation.

INTRODUCTION

A variety of chemical kinetic models have been introduced over the last 30 years to account for the behavior of the ionic currents in excitable biological membranes. With few exceptions, these have involved a sequence of reversible first-order reactions with voltage-dependent rate constants. Often, purely sequential schemes have been modified to include alternate or cyclical pathways (c.f. Armstrong and Gilly, 1979). Although they describe the basic voltage-clamp data reasonably well, purely kinetic formulations are generally not based on well-defined physical assumptions, and thus must be regarded as involving an arbitrary definition of kinetic states for which little independent evidence exists.

An additional difficulty arises if we wish to incorporate intramembrane charge movements into a kinetic scheme. The exact relationship between gating currents and sodium channel kinetics is generally considered complex, perhaps because these two currents reflect occupancies of different states. It has, however, been demonstrated that sodium currents can be substantially slowed by heavy water substitution without any significant change in intramembrane charge movement (Schauf and Bullock 1979, 1981). Although, in principle, one can explain this observation with kinetic schemes in which the charge movement is exclusively associated with transitions that involve a sequence of multiple nonconducting precursors to the open state of the sodium channel, such an explanation does not provide an interpretation in molecular terms, and is thus difficult to test independently.

In the previous paper, Baumann (1981) discussed in some detail the unusual kinetic behavior that can result from a particular model in which a reversible, first-order, voltage-dependent reaction step (converting inert to active subunits) is followed by a reversible, voltage-independent, step-by-step assembly of active subunit molecules into a channel-

forming aggregate (Baumann and Mueller, 1974; Baumann, 1979; Baumann and Easton, 1980). Such a model is attractive in that it specifies a testable physical mechanism for the observed dissociation between ionic and asymmetry currents. The present study describes an attempt to apply one of the suggested protocols to voltage-clamped *Myxicola* giant axons.

METHODS

Myxicola giant axons were internally dialyzed with Cs^+ glutamate solutions (550 mM Cs glutamate; 50 mM CsF; 1 mM Hepes; pH 7.30 ± 0.05) to block the K^+ conductance and were voltage-clamped by procedures described elsewhere (Schauf et al., 1977; Bullock and Schauf, 1978). Because certain of the experimental features predicted by the aggregation model can be artifactually produced by a large series resistance (R_s) error, considerable efforts were made to rule out this possibility. The computations of series resistance effects are discussed in the Results and compared with the observed behavior. Here we wish to stress only the experimental precautions taken to avoid possible artifacts.

Axons were initially examined in normal sea water (430 mM NaCl; 50 mM $MgCl_2$; 10 mM $CaCl_2$; 20 mM Tris; pH 7.30 ± 0.05). Under such conditions peak inward current averaged 2 mA/cm^2. Series resistance was determined by measuring the voltage transient in response to a current step, using the corrections for clamp rise time described by Binstock et al. (1975). Values for R_s ranged from 7.6–11.1 ohm cm^2. The series resistance compensated for was determined by dividing the measured dip in the voltage trace by the peak inward sodium current. Compensation for all but 1.5–2.5 ohm cm^2 could be routinely applied while maintaining stability. During the compensation procedure, current records were continuously monitored and axons were not regarded as adequately compensated unless a point could be found (short of clamp instability) where a further increase in feedback did not result in any changes in the time course of the sodium current at any voltage. In cases where this could not be done the axon was discarded.

At this point a complete I_{Na} (V,t) family was obtained and the external solution was changed to 115 mM Na^+ ASW (25% Na^+) by Tris (hydroxymethyl) aminomethane substitution. A more sensitive criterion for adequate R_s compensation was that the current records at a particular membrane potential differed only by a scaling factor from those recorded in 430 mM Na^+. Particular attention was paid to the invariance of Na^+ tail kinetics under these conditions. In all cases in which the original criteria were satisfied, reduction of [Na^+] did not alter the time-course of I_{Na}.

The experiments themselves were performed in 43 mM Na^+ ASW (10% normal) in which the largest inward tail currents following the perturbations used were <0.5 mA/cm^2 with peak inward currents of 0.1–0.2 mA/cm^2. Again the sodium currents differed only by a scaling factor from those recorded in full [Na^+]. Signal averaging of 2–4 sweeps was used to improve the signal-to-noise ratio. Unless otherwise stated, experiments were done at 5°C and data acquisition was usually complete within 10–15 min. Data were recorded digitally with 10 μs resolution and stored on magnetic tape for later analysis. Leakage and capacity currents were subtracted electronically using appropriate analog circuitry.

The protocol is extremely simple and follows that outlined in Fig. 3*e* of the preceding paper (see also the inset to Fig. 4 of this paper). The axon was held at a potential of −80 mV and depolarized to an initial command voltage V_c between −30 mV and +20 mV. After a variable period of time (Δt) the axon was perturbed by a depolarization (V_{pt}) to between +100 mV and +200 mV for a period of time T_{pt} (usually between 50 and 200 μs). The potential then returned to V_c for a time sufficient for Na^+ inactivation to be complete. In the following discussion the unperturbed current will be termed $I_{Na}(V_c, t)$ and the perturbed current $I^*_{Na}(V_c, V_{pt}, t)$.

RESULTS

Effects of Uncompensated Series Resistance

Before presenting the experimental results it is necessary to consider the manner in which an appreciable uncompensated series resistance might alter the response of the Hodgkin-Huxley (HH; 1952) axon to such brief voltage perturbations. Fig. 1 illustrates the responses of the

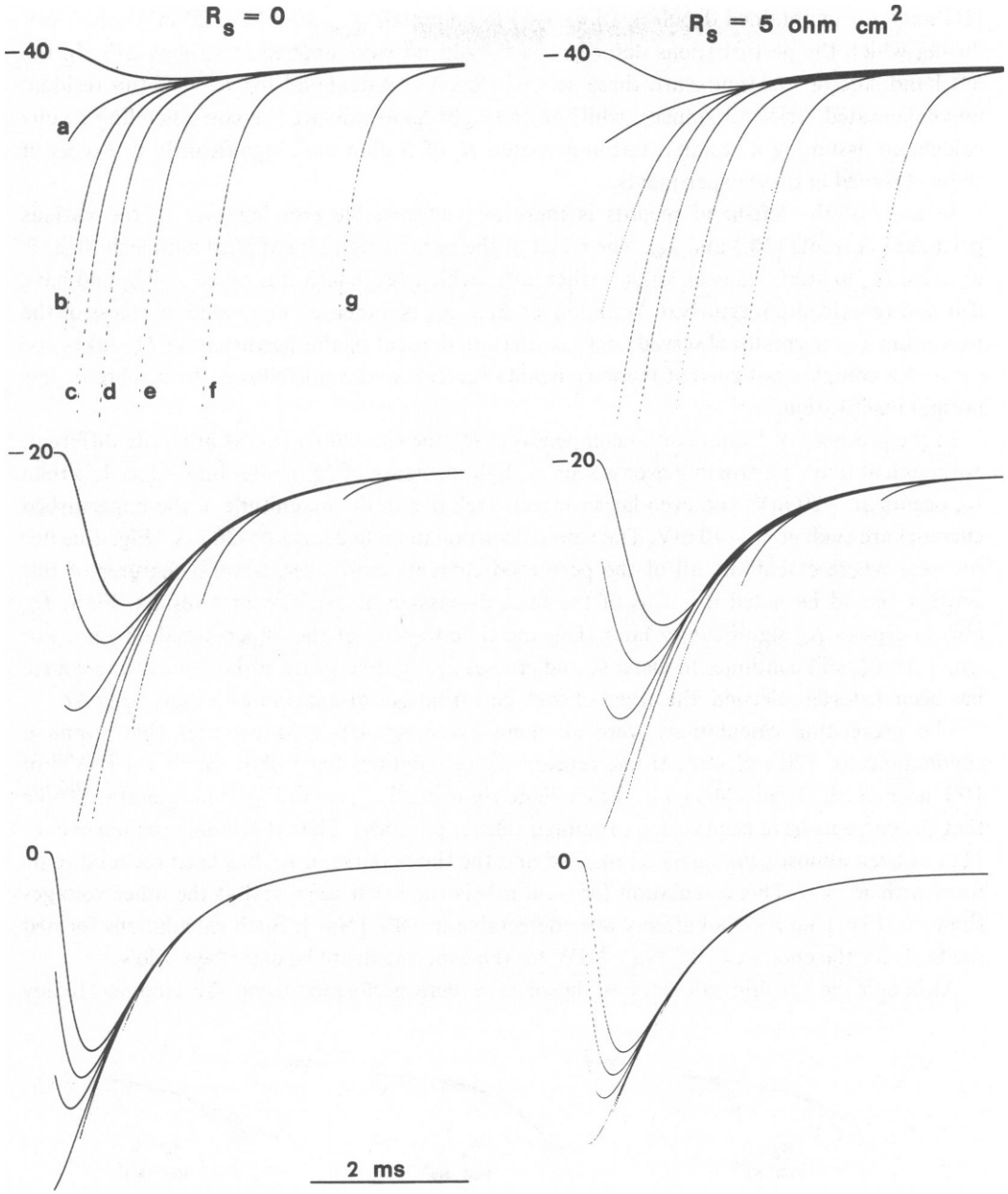

FIGURE 1 Response of the Hodgkin-Huxley axon to various voltage perturbations. The three sets of curves on the left-hand side of this figure were calculated ($\bar{g}_{NA}$ = 120 mS/cm^2) in the absence of any series resistance, while the corresponding curves on the right-hand side assumed an uncompensated R_s of 5.0 ohm cm^2. From top to bottom are shown corresponding calculations for voltage steps to −40, −20, and 0 mV. In each of the six examples illustrated calculations were performed in the absence of a perturbation, and for a brief step to +200 mV at various times after the initial step depolarization. From left to right the first two curves (labeled by lower case letters in the example in the upper left) are for Δt = 150 μs and perturbations lasting 25 μs (*a*) and 50 μs (*b*). The five remaining calculated responses all assume a 100-μs perturbation applied at various Δts ranging from 150 μs (*c*) to 4.1 ms (*g*).

HH axon for maintained depolarizations (V_c) to potentials of -40 mV, -20 mV, and 0 mV during which the perturbations described in the legend were applied at various Δt's. In the left-hand side of the figure are three sets of responses calculated for $R_s = 0$ (no residual uncompensated series resistance), while on the right-hand side are the corresponding results calculated assuming a residual uncompensated R_s of 5 ohm cm^2, significantly in excess of those observed in these experiments.

In none of the left-hand records is there any appreciable crossing over of the various perturbed currents (I^*_{Na}) and I_{Na}. The result of the perturbing pulse applied with a short Δt is to cause I^*_{Na} to attain its peak value earlier, after which I^*_{Na} inactivates much as it would have if it had remained unperturbed. With longer Δt's, g_{Na} is increased to a value in excess of the maximum g_{Na} normally observed at V_c so that at the end of the perturbation I^*_{Na} takes the form of a complex tail current (time constants τ_m, $2\tau_m$, and $3\tau_m$) followed by a more or less normal inactivation.

In the presence of 5 ohm cm^2 uncompensated R_s, the calculated results are quite different. Although at 0 mV no crossing over is seen, a slight decrease of I^*_{Na} to absolute values less than I_{Na} occurs at -20 mV, and even larger effects (relative to the magnitude of the unperturbed current) are evident at -40 mV. The worst distortion in fact occurs at -30 mV (Fig. 2-center records) where essentially all of the perturbed currents cross over. Several features of this artifact should be noted in terms of the later discussion of experimental results. First, I^*_{Na} always crosses I_{Na} significantly later than the time-to-peak of the unperturbed current. For small Δt, I^*_{Na} still continues to increase and crosses I_{Na} relatively late, although its time-course has been distorted. Second, the effect of such perturbations disappears with increasing Δt.

The preceeding calculations were all done assuming 430 mM external [Na^+] and a conductance of 120 mS/cm^2. If one repeats the calculations for 5 ohm cm^2 but a [Na^+] of 10% normal, the results shown in the extreme right-hand curves in Fig. 2 are obtained (note that the currents have been scaled to comparable amplitudes). The artifactual crossing over of I^*_{Na} has been almost completely eliminated and the time-course of I_{Na} has been restored to its form with $R_s = 0$. This calculation for -30 mV is the worst case, and at the other voltages shown in Fig. 1 no residual effects were detectable in 10% [Na^+]. Such calculations formed the basis for the choice of 10% Na^+ ASW for the experiments to be described below.

Although the specific calculations shown here were performed using the Hodgin-Huxley

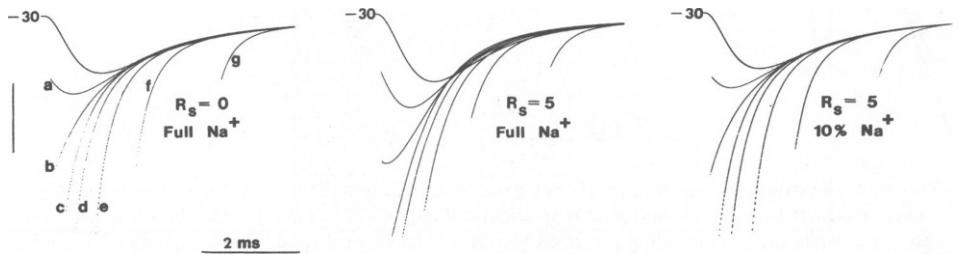

FIGURE 2 Similar calculations for a depolarization to -30 mV, except that now the responses of the Hodgkin-Huxley axon are calculated in 430 mM [Na^+] with no series resistance (*A*), in 430 mM [Na^+] with 5 ohm cm^2 residual uncompensated series resistance (*B*), and in 43 mM [Na^+] (*C*) for comparison. Same perturbations (*a–g*) as in the previous figure. Note that the currents in *A* have been multiplied by a factor of 10 so as to correspond visually with those calculated in normal [Na^+].

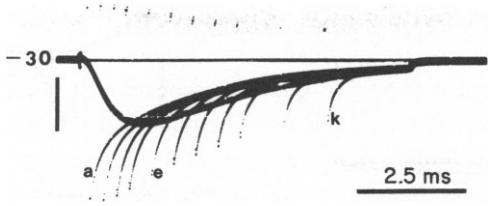

FIGURE 3 Experimental result of the application of the perturbation protocol to *Myxicola* axons compensated for series resistance and bathed in 43 mM Na^+ SW. The axon shown was depolarized to −30 mV, after which perturbations to +200 mV lasting 100 μs were applied after intervals (Δt) of 0.1 (labeled as (*a*), 0.4, 0.7, 1.0, 1.5; (*e*), 2.0, 2.5, 3.0, 3.5, 4.5; and 5.5 ms (*k*). The current calibration is 0.05 mA/cm^2.

parameters, similar behavior is obtained if one substitutes appropriate parameters for *Myxicola* axons, while retaining the essential independent kinetic formalism of Hodgkin and Huxley (Goldman and Schauf, 1973). A more important question concerns the predictions of different kinetic schemes (e.g. Armstrong and Gilly, 1979) in the presence or absence of a residual uncompensated series resistance. As yet we have made no attempt to quantitatively assess these effects.

Experimental Effects of Voltage Perturbations

The experimental effects of large voltage perturbations on the Na^+ currents in *Myxicola* axons are illustrated in Figs. 3–6. Fig. 3 shows the unperturbed Na^+ current for $V_c = -30$ mV, and $I^*_{Na}(t)$ measured following a 100 μs step to +200 mV applied with Δt ranging from

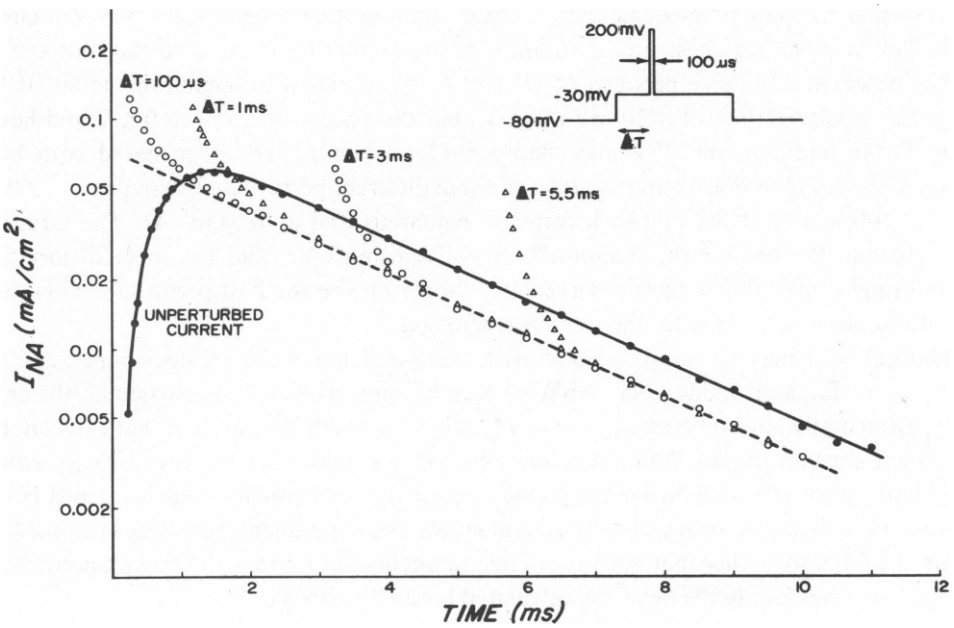

FIGURE 4 Semilogarithmic plots of the data shown in Fig. 3 for the unperturbed current (solid symbols) and for perturbed currents (open symbols) with Δt's of 0.1, 1.0, 3.0, and 5.5 ms. The solid line was drawn by eye and shifted vertically so as to best fit the open symbols. The figure inset illustrates the pulse protocol.

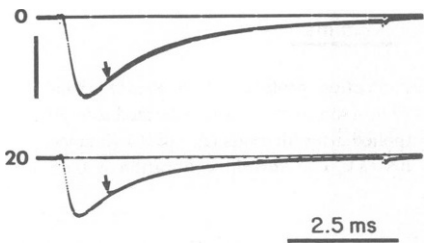

FIGURE 5

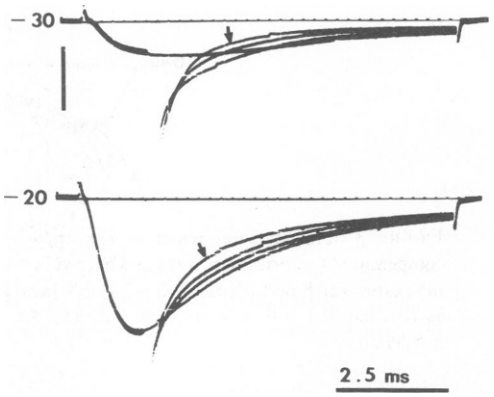

FIGURE 6

FIGURE 5 Effect of a 100 μs perturbation to +200 mV applied after a Δt of 1.0 ms in an axon depolarized to 0 and +20 mV. The perturbed currents (arrow) are not significantly different from the unperturbed currents. The current calibration is 0.10 mA/cm^2.

FIGURE 6 Effect of increasing the duration of a perturbation to +200 mV on the perturbed sodium currents at two different potentials. Pertubations of 50, 100, or 200-μs duration were applied with a Δt of 1.0 ms (the 200-μs perturbed current is indicated by the arrow). The current calibration is 0.05 mA/cm^2.

100 μs to 5.5 ms. In all cases $I^*_{Na}(t)$ crosses over I_{Na} and $I^*_{Na} < I_{Na}$ for the remainder of the post-perturbation current. Furthermore, the crossing over remains apparent even for Δt's in excess of 4 ms. A semilogarithmic plot of the data for several values of Δt (Fig. 4) shows that $I^*_{Na}(t)$ has a complex time-course with a rapid initial decline resembling a Na^+ tail current and a late inactivation whose time constant is similar to that for the unperturbed current.

The observed difference between $I^*_{Na}(t)$ and I_{Na} (t) varies with membrane potential. It is large for depolarizations of −30 mV (Fig. 3), but disappears for steps to 0 mV and beyond (Fig. 5). In addition, the difference between $I^*_{Na}(t)$ and I_{Na} (t) is increased with larger perturbations. Fig. 6 illustrates the effect of three different perturbations (steps to +200 mV for 50, 100, and 200 μs) at two membrane potentials and a constant Δt. The larger the perturbation, the greater the magnitude of the crossing over, and the more distorted the overall time-course. The effects observed at −20 mv for the smallest perturbation are larger than those seen at −30 mv for the same perturbation.

This behavior may be compared both with that predicted in the previous paper, and with that seen artifactually in high Na^+ ASW with an uncompensated R_s. In particular, the degree of experimentally induced crossing over of I^*_{Na} and I_{Na} is much greater than that calculated for a residual uncompensated R_s of 5 ohm cm^2, even in 430 mM Na^+, but is consistent with the magnitude of the effects shown in the previous paper. For experiments done in 43 mM Na^+ no crossing over should be seen even with a residual R_s. The experimental crossing over of I^*_{Na} can occur at or slightly earlier than the time of maximum inward I_{Na} (e.g. the earliest perturbation in Fig. 3), in contrast to the behavior calculated for an R_s artifact.

DISCUSSION

The experimental results of brief voltage perturbations of large amplitude on the Na^+ currents of *Myxicola* axons resemble those that were predicted in the preceeding paper from a

model in which the initial change in electric field produces subunits (active monomers) that then aggregate to form a conducting channel. Unfortunately, the interpretation of the experimentally recorded currents compared with artifactually observed currents must remain largely quantitative. The experimental behavior is in the same direction as the artifacts produced by residual R_s. We have tried to show that experiments performed in 10% Na^+ ASW should not suffer from series resistance errors. However it would clearly have been preferable to have obtained an experimental result that could not even qualitatively result from a series resistance artifact.

It would be desirable to compute the experimental behavior of other kinetic schemes capable of fitting the basic voltage-clamp data, as our calculations apply to only a very specific case. In particular, sodium activation and inactivation have increasingly become regarded as coupled processes. Initially this conclusion was based on the behavior of ionic currents (Goldman and Schauf 1972, 1973; Schauf et al., 1976), but recently coupling has been inferred from the inability to detect intramembrane charge movements associated with Na^+ inactivation and the presence of a concommitant charge "immobilization" (Armstrong and Bezanilla, 1977; Bullock and Schauf, 1979). Kinetic models can describe such data if, for example, it is assumed that channels must open before they can inactivate. Such sequential models of inactivation also seem likely to exhibit crossing over of perturbed and unperturbed sodium currents under the conditions used here since newly opened channels could inactivate immediately, while those not yet activated would be required to open before becoming inactivated. Clearly a detailed comparison of the behavior of such models with that seen as a result of aggregation would be a useful next step in designing further experimental studies.

We previously commented that the aggregation model, in contrast to a simple linear sequence, provides a well-defined explanation for the observed dissociation between charge movement and the kinetics of channel opening that can be achieved with D_2O (Schauf and Bullock 1979, 1981). As Fig. 5 of the latter paper shows, sodium currents can be slowed by 50–60% in the presence of 99.8% D_2O while the ON and OFF asymmetry currents superimpose exactly (the very slight rounding of the rising phase of the ON asymmetry current seen in Fig. 4 of Schauf and Bullock [1979] was not generally seen after D_2O substitution in later studies with lower noise and better temporal resolution). One can easily imagine that the voltage-dependent creation of active monomers may not involve an appreciable interaction with solvent, while their voltage-independent aggregation to form a transmembrane aqueous pathway obviously does. Thus, intramembrane charge movement would not be affected by solvent substitution, while the ionic currents, reflecting occupancy of the conducting state, are slowed because of an energetically unfavorable change in solvent order. Such an inherent separability would exist in an aggregation model even if the assumed details of the precursor production vs. assembly processes were more complex than those specified in the preceeding paper.

The novel kinetic behavior described here, even in combination with the D_2O experiments, do not prove that aggregation is in fact the basis for gating in biological membranes. However, the aggregation concept is consistent with these experimental results and offers the advantage of specifying a particular physical relationship which ought to be independently testable.

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