Gating currents in *Shaker* K^+ channels

Implications for activation and inactivation models

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ABSTRACT We have studied ionic and gating currents in mutant and wild-type *Shaker* K^+ channels to investigate the mechanisms of channel activation and the relationship between the voltage sensor of the channel and its inactivation particle. The turn on of the gating current shows a rising phase, indicating that the hypothetical identical activation subunits are not independent. Hyperpolarizing prepulses indicate that most of the voltage-dependence occurs in the transitions between closed states. The open-to-closed transition is voltage independent, as suggested by the presence of a rising phase in the off gating currents. In *Shaker* channels showing fast inactivation, the off gating charge is partially immobilized as a result of depolarizing pulses that elicit inactivation. In mutant channels lacking inactivation, the charge is recovered quickly at the end of the pulse. Internal TEA mimics the inactivation particle in its behavior but the charge immobilization is established faster and is complete. We conclude that the activation mechanism cannot be due to the movement of identical independent gating subunits, each undergoing first order transitions, and that the inactivation particle is responsible for charge immobilization in this channel.

INTRODUCTION

Voltage-dependent ion channels respond to changes in the electric field across the membrane by rearrangements of charges or dipoles within the transmembrane segments of the channel molecule. These intramolecular charge displacements, which can be measured as gating currents, are thought to induce a series of conformational changes that convert the closed channel into a conducting pore (Armstrong, 1981, Bezanilla, 1985). Channel gating has been interpreted as a series of transitions between kinetically distinct closed states that must be populated before the channel opens. According to this view, depolarizations shift the equilibrium towards the open state whereas hyperpolarizations shift the equilibrium towards the first closed state. Gating currents are particularly sensitive to the voltage-dependent transitions between closed states because most of the charge seems to move between these states. Therefore gating current information is crucial for the establishment of working kinetic models for activation of ion channels.

On sustained depolarization, several types of channels enter a long-lived nonconducting state known as the inactivated state. In Na channels, it has been observed that the inactivation process is associated with a partial immobilization of the gating charge transferred during channel activation (Armstrong and Bezanilla, 1974). Charge immobilization has been defined as the failure to quickly recover the on gating charge at the end of a depolarizing pulse. In fact, if the membrane is repolarized long enough, all the charge must move back because a subsequent depolarizing pulse moves the same amount of charge. The kinetics and extent of charge immobilization closely resemble the properties of ionic current inactivation, which has led to the proposal that these two phenomena represent the same mechanism.

Based on gating charge immobilization and the effect of internally perfused proteases on the squid Na channel, a mechanistic model of the inactivation process was advanced by Armstrong and Bezanilla (1977). It was proposed that a positively charged domain of the channel, located intracellularly, enters into the open pore in a voltage-independent manner and effectively blocks ion flow. Support for this proposal has come from elegant experiments using mutant *Shaker* channels by Hoshi et al. (1990). These authors have identified a portion of the NH_2 terminus of the molecule as the putative inactivation particle. Additionally, synthetic peptides based on sequence of this region induce time-dependent inactivation in noninactivating mutants (Zagotta et al., 1990).

We have measured ionic and gating currents from *Shaker* channels and a mutant lacking inactivation (Bezanilla et al., 1991), and showed a close correlation between the presence of the inactivating particle and immobilization of the gating charge. It was also shown that an open pore blocker, tetraethylammonium, is able to induce charge immobilization in noninactivating chan-

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nels and that the TEA-induced charge immobilization is complete.

In the present communication, we have confirmed and extended these findings on the inactivation mechanism of *Shaker* channels. Additionally, we have compared the ability of kinetic models based on independent or coupled gating subunits to describe the gating currents from *Shaker* K channels.

METHODS

RNA synthesis and oocyte injection

RNA was synthesized from each cDNA construct by linearization of the Bluescript plasmids (Stratagene, Madison, WI) with EcoRI and transcription with T7 RNA polymerase (Promega, La Jolla, CA) as described by Timpe et al., (1988). Highly concentrated RNA was injected into mature oocytes under Barth's solution at room temperature. Defolliculation was performed in 4 mg/ml collagenase (Sigma Chemical Co., St. Louis, MO) for ~2 h at room temperature. After injection, the oocytes were kept at 16°C and were tested for expression ~4 d after injection.

Two types of constructs were used. The wild type *Shaker* B construct (ShB WT; Schwarz et al., 1988) was the SacII-EcoRI fragment from the original *Shaker* B cDNA sub-cloned into Bluescript. The mutant channel construct (ShH4-IR, Yellen et al., 1991) was similar, but it contained a deletion of amino acids 6 to 46 (Hoshi et al., 1990) to remove inactivation.

Open oocyte vaseline-gap voltage clamp with internal perfusion

Ionic and gating currents were recorded using a modification of the vaseline-gap voltage clamp method used for muscle and nerve fibers and developed for frog oocytes by Taglialatela et al. (1992*b*). The oocyte is defolliculated and mounted in a multicompartment chamber that divides the egg into three electrically independent sections physically separated by vaseline seals (Fig. 1 *A*). The three compartments, denoted external, guard and internal pool, are in a configuration that allows constant exchange of solutions. The upper hole has a diameter of ~700 μm, whereas the lower opening is close to 800 μm in diameter. The oocyte is opened before mounting it in the chamber by cutting it with a small needle. The internal pool is in contact with the interior of the oocyte and is used for current injection. The external pool corresponds to the extracellular which is under voltage control.

The electronic configuration is based on three voltage clamps: the

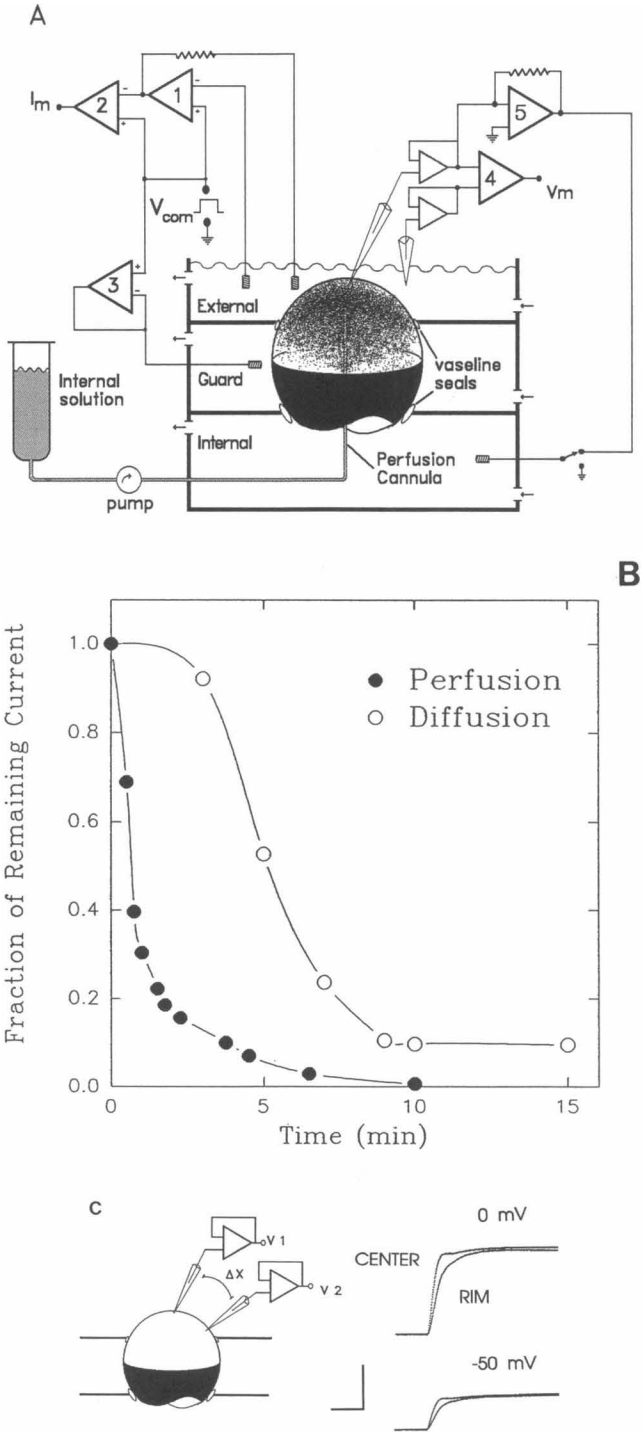

FIGURE 1 (*A*) Schematic diagram of the experimental chamber and electronics. An oocyte is shown in contact with the internal, external and guard compartments. The internal solution is pumped directly into the oocyte through a metal perfusion cannula. Amplifiers 1 and 2 record transmembrane currents. Amplifier 3 keeps the guard compartment at Vcom. Amplifiers 4 and 5 monitor Vm and inject current into the internal compartment. *Im*, membrane current, *Vm*, membrane voltage, *Vcom*, command potential. (*B*) Performance of the internal perfusion system. Values of peak current are plotted as a function of time. For the open symbols no internal perfusion was used. The internal solution contained NMG, a Ca^{++} chelator (EGTA), and a pH buffer (Hepes); the external solution contained Na-methanesulphonate, Ca^{++}, Mg^{++} and 3,4 diaminopiridine (3,4-DAP). For the filled symbols, the oocyte was perfused with the same solution. No 3,4-DAP was present in the external solution (*C*) Accuracy and speed of the open-oocyte voltage clamp. The voltage difference between a reference electrode (V1) and the external pool and the difference between a probe electrode (V2) and the external pool are plotted as a function of time in the lower panel. The upper panel illustrates the experimental arrangement. Calibration: vertical, 40 mV; horizontal, 0.5 ms.

primary clamp controls the external pool to the command and holding voltages whereas the secondary clamp maintains the interior of the oocyte to ground. An additional passive clamp matches the guard potential to the external pool potential. The arrangement insures minimum current flow between the external and guard pools, which are effectively electrically isolated. The internal potential was recorded with a conventional microelectrode arrangement, and was used as part of the feedback of the secondary clamp. The transmembrane potential was obtained differentially with the aid of an external electrode. Membrane currents are recorded from the external pool with a standard current-to-voltage converter with various feedback resistors. The connection between the electronic circuitry and the three pools used Ag-AgCl pellets inmersed in 3 M KCl and connected to the pools of the chamber with 1 M NaCl agar bridges with an internal platinum wire to improve their frequency response.

The system has been modified to include an internal perfusion system running through the internal and guard compartments (Fig. 1 *A*) based on a small stainless steel needle (33 gauge) attached to an epoxy base. The needle, connected to a syringe pump that allows flow rates of 5–50 μl/h, is positioned within the egg. Ideally, the needle sits close to the oocyte membrane, but this can vary considerably, depending on the size of the oocyte. The positioning of the tip of the internal needle is critical for the optimal performance of the perfusion system.

Only oocytes with high expression (> 100 μA/oocyte) were used for the gating current measurements. For ionic currents, oocytes with lower levels of expression (< 10 μA/oocyte) were used. Experiments were performed at 18–19°C.

Solutions

Solutions for ionic current recordings were (in mM): External pool, 120 Na-Methanesulphonate, 2.5 KCl, 1.8 $CaCl_2$, pH 7.6. Internal pool and perfusate, 110 K-glutamate, 10 Hepes-NMG (*N*-methyl glucamine), 10 EGTA-NMG pH 7.3. Guard pool, 120 Na-Methanesulphonate, 10 Hepes-NMG, 1.8 $CaCl_2$. In some experiments, ionic currents were recorded in Ringer's solution.

Solutions for gating currents were (in mM): external pool, 120 TEA-Methanesulphonate, 10 Hepes-NMG, 1.8 $CaCl_2$. Guard pool, 120 Na(or NMG)-Methanesulphonate, 10 Hepes-NMG, 1.8 $CaCl_2$. Internal pool and perfusate, 120 NMG-glutamate, 10 Hepes-NMG, 10 EGTA-NMG pH 7.3, or 120 TEA-glutamate, 10 Hepes-NMG, 10 EGTA-NMG pH 7.3.

RESULTS

Performance of the voltage clamp and internal perfusion

The homogeneity and speed of the voltage clamp system was tested with a pair of intracellular electrodes that measure the difference in the internal voltage between the center of the clamped membrane (V1, reference electrode) and any point around it (V2, probe electrode). Fig. 1 *C* shows the experimental arrangement, and the value of the membrane voltage at the center (V1) and at the rim of the hole (V2) for voltage pulses to −50 mV and 0 mV from a holding potential of −80 mV. The steady-state difference between the center and the edge is 4% for the 0 mV pulse that elicited 4 μA of ionic current. The transient difference reaches 4% after 300 us.

The effectiveness of the internal perfusion system was tested by monitoring the decrease of the potassium outward current as K^+ is removed from the intracellular space. In Fig. 1 *B*, the peak K^+ current is plotted vs time, after the beginning of the perfusion at a flow rate of ~ 10 μl/h. Decline of the current is much faster than that seen when the internal solution is exchanged by diffusion. Although the K current amplitude decreases sharply with exchange of toxins and K^+ ions by diffusion, we have observed a residual outward current present even after extended periods of time (1–2 h). On the other hand, the perfusion system can eliminate almost all of the ionic current in a 10–15 min period.

Ionic and gating currents properties

Figs. 2 and 3 show the general characteristics of the ionic and gating currents measured from ShB-WT and ShH4-IR channels expressed in *Xenopus* oocytes. Depolarizations for 40 ms to different test potentials from a holding potential of −80 mV were recorded for both types of channel. The wild type ionic currents, shown on the left panel of Fig. 2 *A*, display the typical characteristics of an A-type potassium current, with threshold activation around −50 mV and fast inactivation in the millisecond time range.

ShB-WT gating currents are shown on the right panel of Fig. 2 *A*. In contrast to the ionic currents, they can be detected at more negative potentials. For small depolarizations, the gating current rises very fast and then relaxes with a single exponential time constant. For large depolarizations, a slow, rising phase appears both at the beginning and at the end of the pulse. This rising phase cannot be explained by the limited speed of the clamp, because the slowest clamping time at the edge of the control region is much faster than the rising phase of the gating current. These kinetic characteristics do not agree with the predictions of a model based on the action of independent subunits (see below).

Fig. 2 *B* illustrates the relationships between the conductance and voltage, and the charge displaced as a function of voltage (obtained from the integral of the gating current). A considerable amount of charge is transferred before any channel has opened. The midpoint of the *Q–V* curve is ~ −30 mV whereas for the *G–V* curve it is ~ − 10 mV, and they exhibit different slopes.

As with Na channel gating currents, the gating charge moved at the end of the voltage jump, Q_{off}, measured as the integral of the current for a finite period of time (20 ms) is smaller than that at the beginning of the voltage jump (Q_{on}) (Fig. 2 *C*). The difference between Q_{off} and

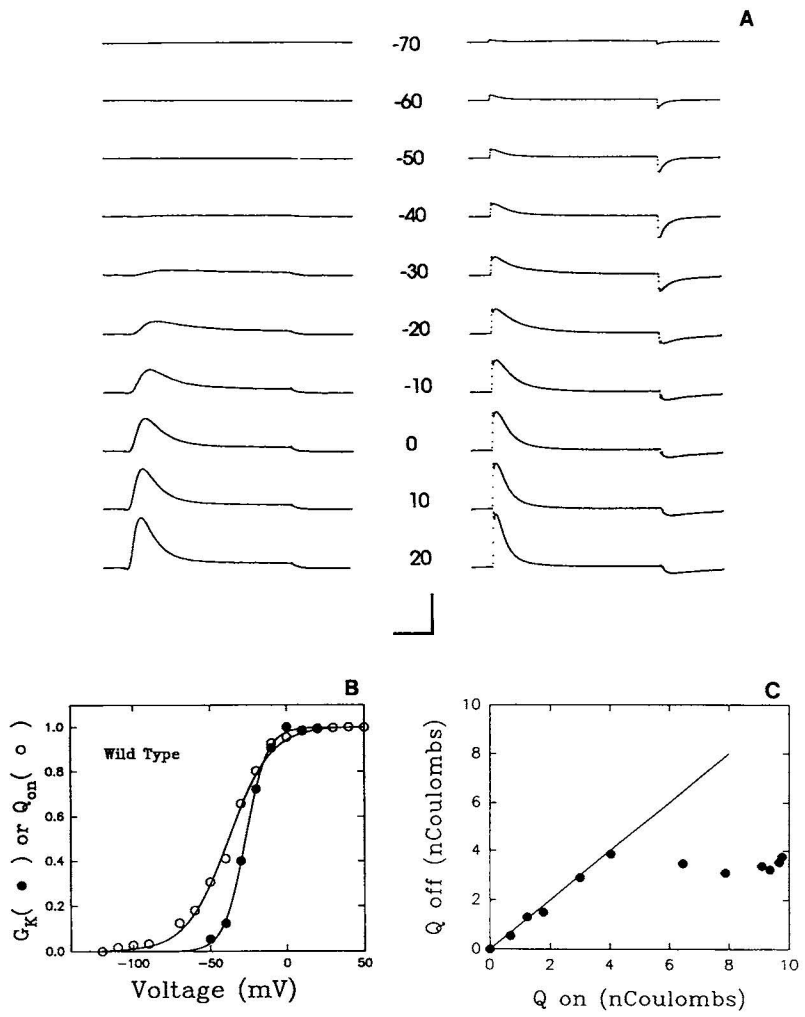

FIGURE 2 Ionic macroscopic and gating currents from wild-type *Shaker* channels. (*A*) Families of ionic (*left*) and gating (*right*) currents taken from a holding potential of −80 mV and a subtracting holding potential of −140 mV. Each number represents the test potential for each trace. Calibration: gating current, 0.5 μA; ionic current, 3 μA; time, 10 ms. (*B*) Normalized conductance vs voltage and transferred charge vs voltage curve for the On gating charge. The gating current was integrated for the whole duration of the test pulse to obtain the charge. (*C*) Ratio of the integral of the gating current during the test pulse and after the end of the pulse for voltages between −70 and 20 mV.

Q_{on} indicates that the charge has been immobilized. The immobilized charge is eventually recovered after membrane repolarization, but it takes several tens of milliseconds to return completely.

Ionic and gating currents obtained from the mutant ShH4-IR are shown in Fig. 3. The ionic current does not inactivate, as previously reported (Hoshi et al., 1990) (Fig. 3 *A, left*). The On gating currents are similar to those of wild type (Fig. 3 *A, right*), except for small differences in the overall kinetics: the mutant channel is slightly slower than the wild type. The *G–V* and *Q–V* curves show different slopes and are shifted from each other ~30 mV (Fig. 3 *B*). However, the noninactivating mutant does not show charge immobilization: the Off gating charge is recovered quickly and in full, as illustrated in Fig. 3 *C*. This indicates that the same region of the NH_2-terminal that blocks the channel and induces inactivation is also responsible for immobilization of the gating charge.

The Off gating currents of both the mutant and wild type channels display a marked slow rising phase, particularly at potentials where the open state is populated. A likely explanation is that the transition from the last closed state to the open state is voltage independent, as suggested by single channel analysis (Zagotta and Aldrich, 1990).

Fig. 4 illustrates the effects of conditioning prepulses on the On gating current of *Shaker* channels. Fig. 4 *A* shows the gating current traces obtained by pulsing to 20 mV after prepulses to −100, −60, −50, and −40 mV.

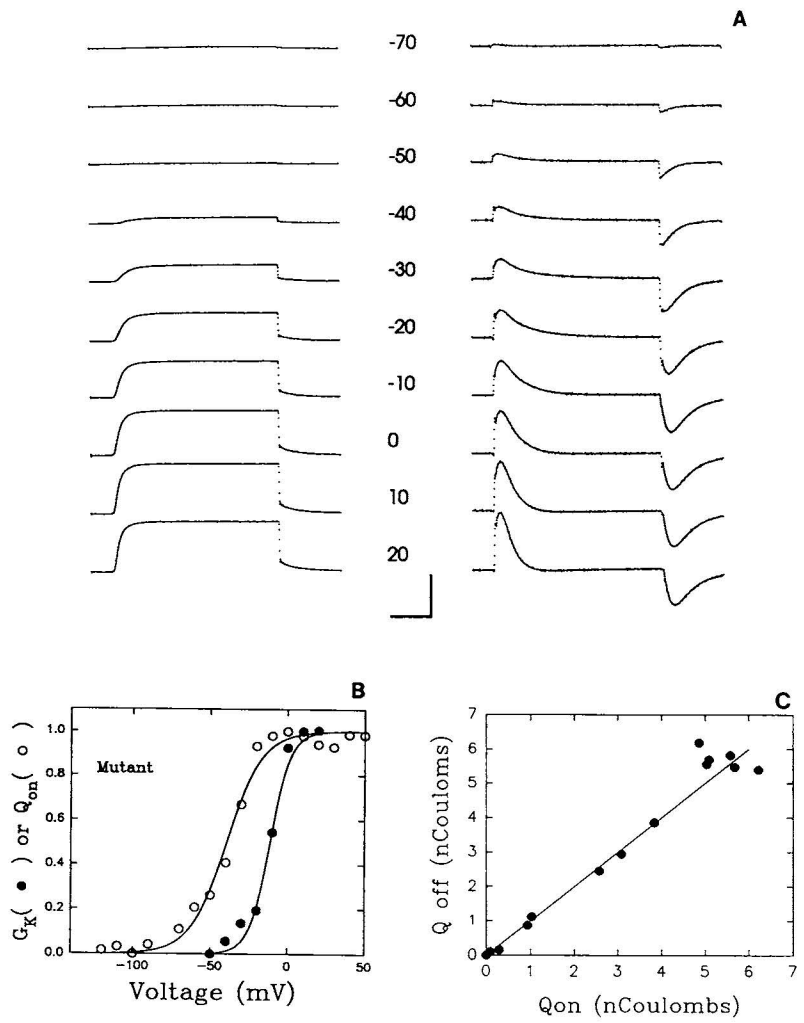

FIGURE 3 Ionic macroscopic and gating currents from mutant *Shaker* channels without the NH_2-terminal segment. For *A*, *B*, and *C*, refer to the legend of Fig. 2. Calibration: gating current, 1.5 μA; ionic current, 4.2 μA; time, 10 ms.

The integral of each gating current is plotted as a function of the prepulse voltage in Fig. 4 *B*. There is little change in the on charge for all prepulses up to −60 mV. After −50 mV, the gating charge is sharply reduced. As the initial slow rising phase disappears, the kinetics of the gating currents change. The amount of charge displaced by each prepulse can be computed by taking the difference of charge for each successive prepulse (Fig. 4, *inset*). These results suggest that the initial transitions among closed states do not involve large charge transfers. Therefore, these transitions must be weakly voltage dependent. They also indicate that most of the voltage dependence should be located within the intermediate closed transitions, given that the closed to open transition is voltage independent (Zagotta and Aldrich, 1990).

Correlation between inactivation and charge immobilization

Additional evidence linking the inactivation process with the phenomenon of charge immobilization comes from the experiments illustrated in Fig. 5. The time course of ionic current inactivation, was similar to the time course of the charge immobilization at 0 mV (Fig. 5 *A*), indicating that inactivation and charge immobilization are intimately coupled.

Fig. 5 *B* compares the steady state properties of ionic current inactivation and the extent of gating charge immobilization at different potentials. The experimental protocol is the same for both measurements: a set of prepulses of different voltages was applied, followed by a brief repolarization to −80 mV before depolarizing the

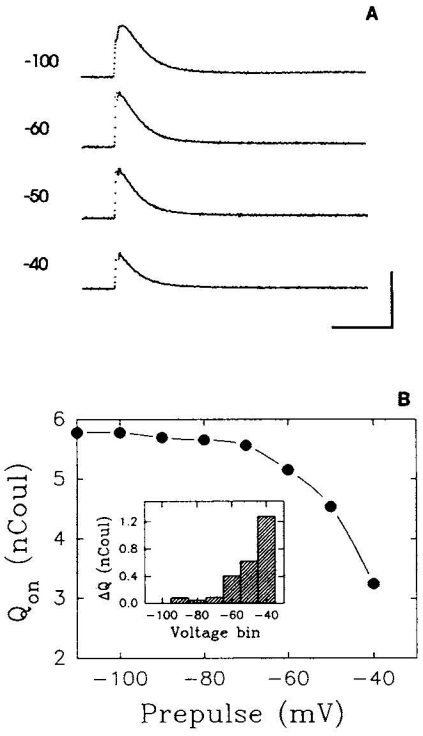

FIGURE 4 Effect of initial conditions on gating charge. (*A*) On gating currents obtained at 0 mV with a 50 ms prepulse of varying amplitude. Each number represents the value of the conditioning prepulse. (*B*) Plot of the integral of the On gating current as a function of the conditioning prepulse. (*Inset*) Charge difference of the On gating current between consecutive prepulses. Calibration: gating current, 1.0 μA; time, 10 ms.

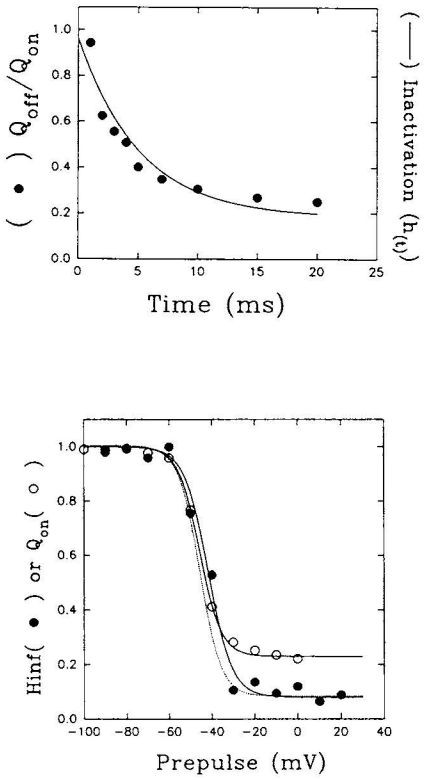

FIGURE 5 Correlation between gating charge immobilization and ionic current inactivation. (*A*) The time course of ionic current inactivation is similar to the time course of the Off gating charge immobilization. The inactivation time course was obtained by fitting a single exponential to the ionic current decay (continuous line). Off gating charge immobilization was obtained, in a different oocyte, from the ratio of the integral of the On and Off gating currents at different test pulse durations. (*B*) The voltage dependence of the steady-state inactivation resembles the voltage dependence of the charge immobilization. For inactivation and charge immobilization a test pulse was preceded by a varying prepulse, separated by a 2-ms interpulse to −80 mV. Steady-state inactivation was obtained with a test pulse of 20 mV and a prepulse of 20 ms duration. Charge immobilization was obtained, in a different oocyte, from the integral of the On gating current for a test pulse to 0 mV with prepulses of 50 ms. Solid lines are Boltzmann fits to the data according to the equation,

$$f(V) = \frac{1-\alpha}{1+\exp\left[\frac{(V-V_{1/2})}{k}\right]} + \alpha,$$

where $f(V)$ is either steady-state inactivation or charge immobilization, $V_{1/2}$ is the voltage at the midpoint of the curve, k is the slope factor and α is the noninactivating component. Parameters of the fit: *open symbols* (Q_{on}), $V_{1/2} = -45$ mV, $k = 5.1$, $\alpha = 0.23$, *filled symbols* (h_{inf}), $V_{1/2} = -42$ mV, $k = 4.3$, $\alpha = 0.08$. The dotted line represents the fit to the Q_{on} data scaled to that of the steady-state inactivation (h_{inf}).

membrane to a constant test voltage. In this case, although the voltage dependence of the *h* infinity parameter and of the relative value of Q_{on} are very similar, the steady-state level does not reach the same level, i.e., there seems to be more ionic current inactivation than there is charge immobilization.

TEA-induced charge immobilization

Replacing NMG in the internal perfusate with the quaternarium ion tetraethylammonium (TEA), which blocks the ionic current, produces complete charge immobilization in wild type channels (Fig. 6 *A*). This enhanced charge immobilization is present even in the absence of the inactivating particle (Fig. 6 *C*), as the extent of the immobilization is the same for mutant H4-IR channels or for ShB WT channels.

A plot of the Q_{off}/Q_{on} ratios as a function of time shows that the TEA-induced charge immobilization occurs in both inactivating and noninactivating channels

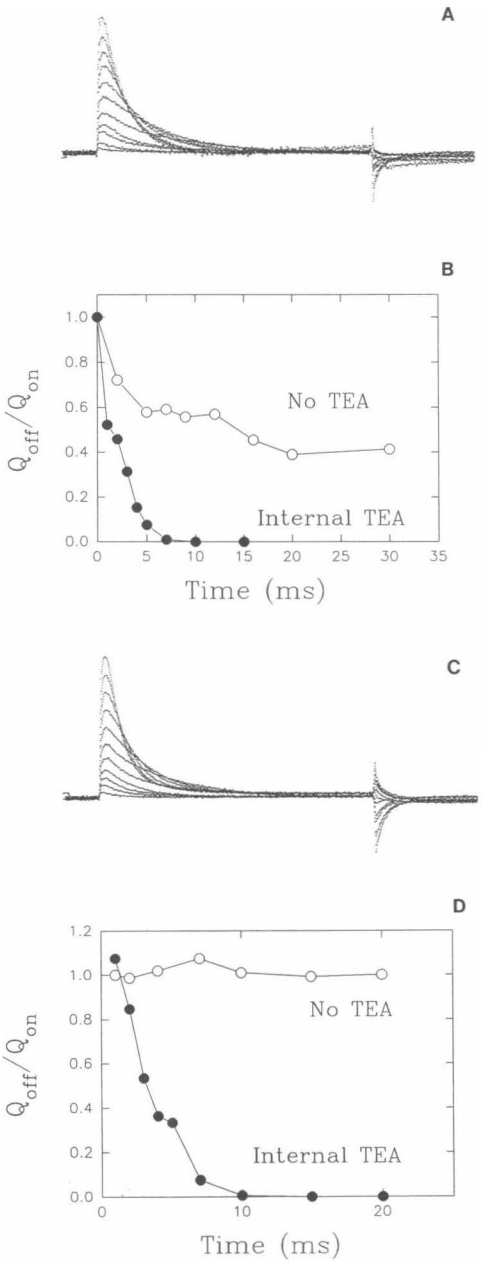

FIGURE 6 Effect of internal TEA on charge immobilization (*A* and *C*) Gating current traces from *Shaker* wild type channels (*A*) and mutant noninactivating *Shaker* channels (*C*) in the presence of 120 mM internal TEA. The holding potential was −80 mV, the subtracting holding potential was −140 mV. Calibration (*left*) vertical, 1 μA; (*right*) vertical, 0.5 μA, Time, 10 ms (*B* and *D*) Ratio of the Off and On gating charge as a function of the pulse duration. *B*, wild type shaker channels. (*D*) noninactivating *Shaker* channels. (*Open symbols*) gating currents in 120 mM *N*-methyl glucamine. (*Filled symbols*) gating currents in 120 mM TEA. Each curve in *B* and *D* comes from currents recorded in different oocytes.

(Fig. 6, *B* and *D*). This time course appears to be faster than the normal charge immobilization or the normal kinetics of inactivation in wild type channels, being closer to the activation time course. This result indicates that TEA must enter the open channel in order to produce charge immobilization. Additionally, it suggests that the interaction of TEA (and probably of the inactivating particle) with the voltage sensor of the channel must take place within the channel pore.

Modeling of gating currents

In this communication we have limited our modeling to the activation process with special reference to the Cole-Moore shift, which is a sensitive indicator of the transitions between closed states. In the model proposed by Zagotta and Aldrich (1990), there is a voltage independent transition between the open state and the last closed state and the inactivated states can only be reached from the open state and the closed states near the open state. The voltage dependence of the activation is modeled as the movement of four identical independent charges in a first-order process, as it is in the Hodgkin and Huxley (1952) model for the potassium channel. When this model is written sequentially, the rate constants are related as 4α, 3α, 2α, and α for the forward transitions from the first closed towards the open state and 4β, 3β, 2β, and β for the backward transitions in the direction of the open to the first closed state. In the right panel of Fig. 7 the gating current has been simulated using this scheme with a Cole-Moore protocol. It is clear that this model is not able to reproduce the main features of the experimental gating currents shown in Fig. 4 *A*, and in particular the model fails to predict the prominent rising phase observed with a prepulse to −110 mV. This is due to the fact that the first transition is faster than the following ones and, as the conditioning pulse shifts the population of channels to the first closed state, the movement of the charge will get faster instead of slower. If we allow the relation between the rate constants between closed states to vary, we find a better agreement with the experimental data as shown in the left panel of Fig. 7. In this case, the first transition is slower than the second transition, giving origin to the rising phase. The interpretation of this result is that the gating subunits, if identical, cannot be considered independent. If the ratio of the first transition to the second is $<4/3$, it means that the movement of one charge is more difficult when none of them have moved as compared to when one has already moved (Vandenberg and Bezanilla, 1991). The model presented in the left panel of Fig. 7, however, still cannot

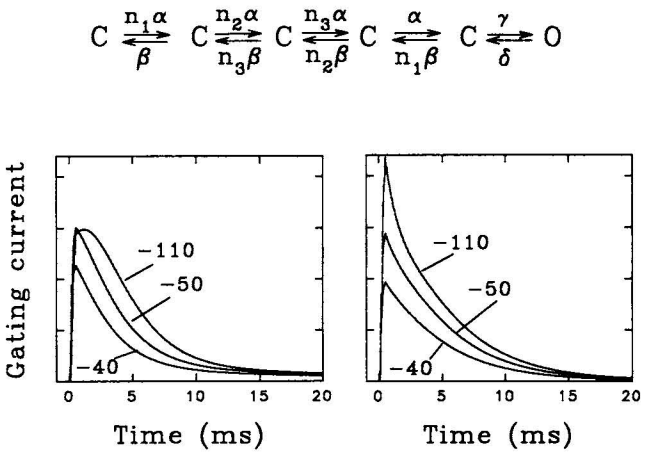

FIGURE 7 Predictions of models based on independent or coupled subunits. The model is illustrated in the top part of the figure. The equations for the voltage dependent rates were:

$$\alpha = \alpha_0 \exp(zxV/kT) \text{ and } \beta = \beta_0 \exp[-z(1-x)V/kT].$$

Experimental gating currents recorded from the noninactivating mutant for a pulse to 0 mV following the prepulses to −110, −50, and −40 mV were fitted to the model using ScoP and ScoPFit. In the left panel n_1, n_2 and n_3 were left as free parameters and the values were 9.1, 11.5, and 10.8, respectively. $\alpha_0 = 52.4$, $\beta_0 = 5.6$, $z = 1.97$, $x = 0.84$, $\gamma = 5{,}384$, $\delta = 54$. In the right panel, the fit was done to the Hodgkin-Huxley type of model where n_1, n_2 and n_3 were forced to 4, 3, and 2, respectively, and the fitted parameters were $\alpha_0 = 207.98$, $\beta_0 = 11.6$, $z = 1.97$, $x = 1$, $\gamma = 291$ and $\delta = 50$. All simulations were performed assuming a noninstantaneous settling time for the voltage pulse due to the limited bandwidth of the clamp (tau = 0.1 ms).

precisely account for the voltage dependence of the Cole-Moore effect, probably due to the presence of numerous early transitions not included in the present model, that may be weakly or not voltage dependent.

DISCUSSION

We have presented gating current measurements on potassium channels expressed in oocytes injected with mRNA using the cut-open oocyte voltage clamp technique modified for internal perfusion. The technique allows recording of very fast currents with low noise and high recording bandwidth. The fast rise of the gating currents for small depolarizations agrees with the measured speed performance of the clamp (see Fig. 1 *C*) and constitutes an internal control indicating that the slow rising phase observed at large depolarizations is an intrinsic property of the charge movement of the channel. The perfusion system was shown to increase dramatically the rate of intracellular ion exchange, and will prove useful for the application and exchange of a wide range of compounds, from ions to macromolecules.

Molecular origin of charge immobilization

There is a direct correlation between the ability of a channel to inactivate (this is, fast inactivation) and the presence of charge immobilization in the Off gating charge. In the wild-type *Shaker* channel, about one third of the total charge displaced at the onset of the test pulse returns immediately at the end of a depolarizing test pulse. However, channels in which the putative "inactivation particle" (or ball) at the NH_2-terminus of the channel has been deleted show no evidence of charge immobilization, either for very long or very depolarized test pulses. This has been interpreted on the basis of the "ball and chain" hypothesis (Armstrong and Bezanilla, 1977), where the NH_2-terminal segment (positively charged) not only binds to the inner mouth of the channel to block ion flow, but also interacts with the voltage sensor of the channel to effectively lock it in an open, yet nonconducting conformation (Bezanilla et al., 1991). The nature of this interaction remains to be determined, but given the result that internal TEA produces charge immobilization, we suggest that the immobilization mechanism does not depend on the intrinsic structure of the NH_2-terminal segment.

The proposal that the inactivation particle is responsible for both inactivation and gating charge immobilization is supported by the correlations between time course and steady-state properties of these two processes. Inactivation and charge immobilization have similar time courses and voltage dependence (Fig. 5). Therefore, the interaction between the inactivation particle and its binding site in the internal mouth of the pore causes both inactivation and charge immobilization, presumably from the same physical location in the channel.

Comparison with other gating current measurements

Gating currents recorded with the cut-open oocyte voltage-clamp technique from *Shaker* channels expressed in oocytes share many characteristics with those recorded from squid giant axon using axial wire voltage clamp techniques (Bezanilla et al., 1982; White and Bezanilla, 1985). They also closely resemble gating currents from DRK1 channels expressed in oocytes (Taglialatela et al., 1992*a*). The On gating charge shows a conspicuous rising phase that develops with increased depolarizations. It is not present for small depolariza-

tions, appearing at potentials higher than −40 mV. The Off gating current also displays a slow rising phase that not only develops with depolarizations, but is also dependent on the duration of the test pulse. For short pulses (< 5 ms), the charge returns as a single exponential process, but for longer pulses a marked rising phase becomes apparent. These properties indicate the presence of a voltage independent transition from the open to the last closed state. Because for small depolarizations or short pulses, the open state is not reached, no rising phase is expected at the Off gating currents.

As in the case for Na, Ca, and K channels in several preparations, the charge vs voltage curve and the conductance (or fraction of open channels) vs voltage curve are displaced 25 to 30 mV in the voltage axis. Such displacement clearly indicates the movement of a sizable fraction of the total charge before any ionic current can be detected, and points to the fact that several closed states (connected by voltage-dependent transitions) must be populated before reaching the open state.

Recently, Stuhmer et al. (1991) have reported gating current measurements, recorded using patch-clamp techniques from chimaeric *Shaker* A2/RCK1 channels expressed in *Xenopus* oocytes. In contrast to the present results and those of Bezanilla et al. (1991), it was found that the Off gating charge was completely immobilized, independently of the ability of the chimaeric channel to inactivate. These results can be explained by the presence of internal TEA in their internal solutions, and the fact that the chimaera used has an unusually slow deactivation. As reported previously (Bezanilla et al., 1991), TEA induces an artificial immobilization of the charge due to its ability to enter the internal mouth of the channel where it may probably interact with the voltage sensor of each channel subunit. Although control experiments using internal Tris indicated that TEA did not alter gating (Stuhmer et al., 1991), tris ion blocks Na (Keynes et al., 1991) and K channels (Perozo, unpublished data). In their study, no rising phase was found in the On gating current within the recording bandwith (10 KHz). This difference could be due to intrinsically faster kinetics of the tested chimaeras which is evident in the ionic current traces. Nevertheless, the steady-state properties of the transferred charge are very similar to those reported here.

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DISCUSSION

Session Chairman: H. Ronald Kaback *Scribes:* Christopher Penington and Alexandra Klinger

HARVEY POLLARD: In order to define a capacitive transient one must observe that the charge acquired (Q_{on}) is the same as the charge coming off (Q_{off}). However, you show that Q_{on} does not equal Q_{off} for some systems. What process do you go through computationally or experimentally to convince yourself that the capacitance transient is being measured in both wild-type and mutant?

FRANCISCO BEZANILLA: The fact that the charge doesn't come back in the integration period used in the analysis of the tail current doesn't mean it doesn't come back at all. It does come back, but at a slow rate. By integrating for a longer time one can recover all the charge, but it may be hard to resolve. At more negative potentials, the recovery is faster and we start distinguishing two processes: the fast and the slow recovery. When the recovery potential is very negative (< -100 mV), the slow process is fast enough to be above the baseline noise, and in that case a recovery of full charge can be observed.

RICHARD HORN: With your high resolution method, you might be able to see a component in your gating current records due to the rate of inactivation. Do you?

BEZANILLA: I believe it is too small to see. The differential equations for this process, even considering the case that inactivation is not voltage dependent, will still give eigenvalues derived from the inactivation process. However, you don't see it very well, presumably because the eigenvector is too small.

HORN: You showed that the rising phase is not an artifact of subtraction. However, your records show that the rising phase increases with the amplitude of the step. Could this be an artifact due to saturation of your amplifier?

BEZANILLA: In the unsubtracted records that we just showed, we see a rising phase in the gating current when the amplifier is not saturated as well as when it is saturated. It doesn't make any difference. There is a rising stage in both cases. Also, for very large negative potentials, and in conditions when there is no charge moving, you don't see any rising phase.

MICHAEL GREEN: Concerning the slow rising phase of the off gating current, does the "last closed state" have to be the same as the first state reached on returning from the open state, i.e., is there a path through a different set of states for closing and opening (hysteresis)?

BEZANILLA: We think that in the inactivated case, which is the normal *Shaker* B, the path of return is different. If only a few channels are open, the return is very fast. However, if you drive most of the channels into the open state the return to the closed state is very slow. This means that it must be coming back by a different pathway, and that pathway seems to be eliminated by clipping off the amino terminus.

ANTHONY AUERBACH: How much faster do rates get as you proceed through the activation pathway? Is the return path to the resting state symmetrical in the sense that the steps are slowed as you get nearer to the closed state?

BEZANILLA: Determining these rates requires a lot of fitting. We have done this for the activation process as a function of conditioning potentials as shown in Fig. 7. In the return path we see that the rates get faster the farther they are from the open state.

AUERBACH: As you get closer to the resting state these closing steps become faster?

BEZANILLA: Right. But you have to remember that there's a final step in the opening, between the last closed state and the open state, which is probably voltage independent, and that seems to be slow.

AUERBACH: But could you tell from the rising phase on the "off" gating currents whether that was symmetrically related to the rising phase on the "on" gating currents?

BEZANILLA: Not really, because you are dealing with two different processes due to the asymmetry imposed by the uncharged step near the open state.

RONALD KABACK: As I understand it, the NH_2-terminal segment is the inactivating particle and TEA mimics it. Is the expression too low for you to look at the biochemistry of the interaction? It would seem to me that a photolabeled TEA derivative could be used to identify the actual gating particle.

BEZANILLA: We haven't explored other TEA derivatives, but we have looked at the affinity. It's only 0.5 mM.

KABACK: There are some very interesting reagents that have a sulfhydryl group on one end, a reducible middle, and a photoreactive group on the other end. You can photolabel and then reduce so you pass a label from a labeled ligand to its binding site. It's quite sensitive. These photolabels are commercially available.

BEZANILLA: That would be great. But then you have to look at the protein, and we can't purify enough protein from the injected oocytes to do biochemistry.

DIANE PAPAZIAN: There was a nice paper from Isacoff et al. (1991. *Nature* [*Lond.*]. 353:86–90) recently that implicates a loop between S4 and the next putative transmembrane segment as, at least, part of the spot where the NH_2-terminal interacts in the inactivated state. It seems likely that TEA might interact in the same region. We're going to try and test that.

KABACK: What's the binding constant for the NH_2-terminal peptide? That might be more useful than TEA.

PAPAZIAN: I don't know if it has been measured.

BOB GUY: There are at least two explanations for the sigmoidal shape of your activation gating currents: (*a*) the entire movement of each voltage sensor occurs virtually instantaneously and the movement of the first makes movement of the second more probable; or (*b*) each voltage sensor moves independently but the movement involves several steps; the first of which is the slowest. These models can be experimentally distinguished by either measuring the noise of the gating current or by using the type of potassium channel that Hess's group have developed, in which four potassium channel subunits are covalently linked and the voltage sensor is always in the activated conformation in three of these subunits.

BEZANILLA: I agree. If you have four identical independent particles moving in one step the only conclusion we can make is that they have to interact. But of course, each one of them can move in several steps where each step is getting faster.

GUY: John Clay has data on squid potassium channels indicating that TEA acts differently than do TEA analogues with a longer alkyl chain. He claims that TEA can bind when the channel is deactivated. How do you reconcile his results with yours?

BEZANILLA: I'm not sure the results are comparable. I think John Clay was just looking at ionic currents, because gating current in squid is not affected by TEA. We have not tried derivatives. What we see in the case of the inactivating particle, which does have hydrophobic regions, is that in fact the channel can partially close. This is good evidence that the channel can inactivate before it is completely open, before all the subunits have moved. But we don't know if TEA can similarly go into a channel in a partially closed state. It could be different with a TEA derivative that has the alkyl chain. That is an interesting point.

OLAF ANDERSEN: Is all the gating charge associated with functional channels? It seems that you need very large movement of charge in order to achieve a given current. You have a charge movement of ~10 microCoulombs for ~200 μA. This means that you transfer ~300 e/pA. Is that a problem? That is, do you have nonfunctional channels that still contribute to the gating currents?

BEZANILLA: We can't answer that with the present data. You noticed in the Methods section that gating currents were recorded in oocytes with more than 100 μA of ionic current and ionic currents were recorded from oocytes with less than 10 μA. The correct ratio can only be obtained with measurements of ionic and gating currents in the same oocyte, and you must know the single channel current and the open probability (which is much less than one in attached patches) to calculate the number of channels. We can only guess that is much lower than 300 when we consider a large expression of ionic current and the open probability. Schoppa et al. (1992. *Biophys. J.* 61:426*a*. [Abstr.]) have measured ionic and gating current in the same macropatch. Using fluctuation analysis of the ionic current, they calculated the numbers of channels in the patch. They have found about twelve charges per channel. That doesn't mean we may have a different situation here. We don't know.

ALAN FINKELSTEIN: How many voltage-dependent closed states do you use in your model?

BEZANILLA: Four transitions and five states.

FINKELSTEIN: Am I correct in assuming that if you consider a model where the entire S4 region moves as a unit rather than in sequential steps, you would not get more than four states?

BEZANILLA: If the subunits are considered to be identical then the kinetic scheme can be reduced to five states (Vandenberg and Bezanilla, 1991).

FINKELSTEIN: And for nonidentical subunits?

BEZANILLA: Then the kinetic scheme is much more complicated and can not be reduced to five states. A "multidimensional" kinetic model must be used that is not very easy to treat. Your question is quite relevant in the case of the Na^+ channel because there is good reason to believe that the four subunits are different. However, in the case of the K^+ channel we have better grounds to assume that they are identical.

IGOR VODYANOY: What do you know about the nonlinearity of oocyte membranes? Is it linear in the region that you measure? I would suspect that it is not symmetrical.

BEZANILLA: As far as we know the Q-V curve is linear. However, if you go to very high potentials you start activating strange ionic currents. So, we have to be careful when that happens. It basically happens when the oocytes are in bad shape. It is important to be within a range where you can trust the current you're measuring. Of course, if you remove most of the permeant ions, the current nonlinearities are really minimized.

VODYANOY: Did you try TEA without channels in oocytes?

BEZANILLA: Yes, we've tried once and we didn't find anything. That was a case where there was no expression.

JOE MINDELL: Do other potassium channel blockers, for example charybdotoxin, have effects on gating current similar to those of TEA?

BEZANILLA: We have tried LQ2 with R. MacKinnon. We have done experiments to eliminate most of the ionic currents, but we have not looked carefully at the shape of the gating current. The part that remains after the ionic current seems to be quite unchanged by LQ2, but a detailed analysis has to be done.

EDUARDO PEROZO: Perhaps you should point out the different kinetic behaviors for gating currents reported using macropatches in relation to our results; maybe advance probable explanations.

BEZANILLA: There have been reports of gating currents measured with the patch technique using excised macropatches. Stuhmer et al. (1991) (reference in paper being discussed) have found that the charge at the off is totally immobilized regardless of whether inactivation is present or not. But this was done with a clone with slow deactivation, which is the first reason why charge shouldn't come back very fast. The second reason is that they used TEA which immobilizes the charge on the return. Patch gating currents recorded from *Shaker* K channels have also been reported (Schoppa et al., 1992). Their measurements show different kinetics than the ones we have reported. They show a faster "on" phase and a slower "off" phase. In fact we have seen kinetics like that in some of our oocytes. We do not know how to explain this variability. Perhaps phosphorylation or the state of sulfhydryl groups could be important. We must realize that the techniques are very different because with the cut-open oocyte the membrane is maintained in contact with the cytoplasm, while in the excised patch the membrane is pulled away from the cytoplasm. We have to wait until we have a clear understanding of how to get consistently the same kinetics from oocyte to oocyte before attempting a detailed modeling of gating.

DAVID BUSATH: You raise a point that the Stuhmer group uses TEA. I gather you use NMG to block the potassium channel in the normal *Shaker* case. What kind of control do you have in order to say that the NMG doesn't also cause charge immobilization?

BEZANILLA: In NMG there is no immobilization of the charge. Our best control, in terms of the effect of solutions, is the mutant W434F from R. MacKinnon (1992. Perozo et al. *Biophys. J.* 61:426*a*.). In this mutant the gating current has exactly the same shape as the current we

record with NMG in the normal clone. In this mutant also, there is no ionic current and the gating is also affected by TEA going into the mouth with similar affinity. Our measurements with W434 were recorded with normal potassium inside and normal sodium outside. These are physiological solutions and the recordings look the same as the recordings done in NMG.

MAURIZIO TAGLIALATELA: I was very surprised when I first saw this similarity of TEA immobilizing charge in noninactivating and inactivating *Shaker* mutants. Does TEA inactivate the macroscopic current in the noninactivating *Shaker* mutant? Is the blockage of the ionic current of the channel mediated through the same site that immobilizes the gating charge? If that is the case, it is very important to consider the fact that in DRK1 the charge does not get immobilized even if you put 100 mM TEA inside.

This makes a very important case for the mouth of the *Shaker*-like channels and DRK1-like channels being completely different. Of course, if you try to model the same thing by putting the results that you obtain in with mutation in *Shaker* and DRK1 or DRK1-like channels, there might be slight differences in the geometry of the mouths.

BEZANILLA: That's beautiful. That is one of the most interesting points that has come out here. We have to look at the sequence and probably exchange those pieces, because the *Shaker* does have the effect of TEA and you have clearly shown that it doesn't in DRK1, so there must be a place where TEA has to fit in the *Shaker* and not in DRK1. We tried TEA on the gating currents of the squid axon and it doesn't do it. It seems to be that the *Shaker* has a special receptor that results in immobilization. Of course TEA blocks the squid axon, but it doesn't immobilize the charge. DRK1 is very similar in behavior to the squid axon delay rectifier.

So I have a question for you: does the inactivating polypeptide produce the same effect in the DRK1 as in the *Shaker*?

TAGLIALATELA: In our hands the polypeptide has never inactivated the current in DRK1.

BEZANILLA: I think that that is in agreement with our results. By the way, the polypeptide doesn't do anything in the squid axon except to decrease the current.

ZIMMERBERG: Now that these channels have been expressed in a number of systems, are there any differences in kinetic processes, such as gating, that indicate any effects of lipid viscosity, or any other membrane specific rather than sequence-specific results?

BEZANILLA: We don't know about the gating, but I can tell you that the ionic currents from those cells infected by the baculovirus have different kinetic and voltage dependence than those recorded in the oocyte. That's why I think there are many other factors that depend on posttranslational modification and perhaps the microenvironment of the inside of the membrane. This must be clarified before we go into strict details of what part of the sequence is doing what.

HORN: That's also true obviously for sodium channels. There are a lot of sodium channels that have been expressed in oocytes that inactivate very slowly, abnormally slowly. When the same channels have been expressed in mammalian cells they have rapid inactivation.

ANDERSEN: But shouldn't one keep in mind that the final product that gets into the membrane in the different systems may be very different even though the same sequence is injected. The posttranslational modifications in the mammalian cells could be quite different from those in oocytes, and I would guess that this is the case. This can cause a problem in comparing results in different systems.

HORN: That probably is true, Olaf. In sodium channels it has been shown that 30% of the molecular weight is due to sugar. There are somewhere on the order of 100 sialic acid residues per channel. The glycosylation pattern is very different in an oocyte than in a mammalian cell.

BOB FARLEY: The previous discussion about the TEA immobilization of charge contrasted between the *Shaker* channel and the DRK1 channel seemed to imply a binding site for TEA on the *Shaker* channel. Will you please comment about the statement that the immobilization apparently does not depend on the intrinsic structure of the amino terminal segment of the protein?

BEZANILLA: TEA can emulate the NH_2-terminal polypeptide, but it can only emulate it if you have the right receptor for it.

FARLEY: So does that mean if you took a series of basic amino acids as some appropriate length amino terminal extension you would expect this to immobilize charge?

BEZANILLA: I would think so. And that is something that could be tried, that is, TEA derivatives or something that has the correct amino group to produce immobilization.

TAGLIALATELA: It might not necessarily be the same site that is blocking the internal mouth where TEA is binding and producing inhibition of the recovery of the charge. This is also suggested by mutation analysis. The T442S mutation reduces by tenfold the affinity for TEA in *Shaker* channels, but it does not remove inactivation.

BEZANILLA: That is true. It is not that the site that accepts TEA and blocks the channel is the same site that receives the influence of TEA to immobilize the charge.