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Tetraethylammonium blockade distinguishes two inactivation mechanisms in voltage-activated K^+ channels

(quaternary ammonium ions/open channel block/ball-and-chain model)

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ABSTRACT **Voltage-activated K^+ channels are a family of closely related membrane proteins that differ in their gating behavior, conductance, and pharmacology. A prominent and physiologically important difference among K^+ channels is their rate of inactivation. Inactivation rates range from milliseconds to seconds, and K^+ channels with different inactivation properties have very different effects on signal integration and repetitive firing properties of neurons. The cloned Shaker B (H4) potassium channel is an example of a K^+ channel that inactivates in a few milliseconds. Recent experiments have shown that removal of an N-terminal region of the Shaker protein by site-directed deletion practically abolishes this fast inactivation, but the modified channel does still inactivate during a prolonged depolarization lasting many seconds. Here we report that this remnant inactivation must occur by a distinct mechanism from the rapid inactivation of the wild-type Shaker channel. Like the inactivation of another K^+ channel [Grissmer, S. & Calahan, M. (1989) *Biophys. J.* 55, 203–206], this slow inactivation is retarded by the application of a channel blocker, tetraethylammonium, to the extracellular side of the channel. By contrast, the fast inactivation of the wild-type Shaker channel is sensitive only to intracellular application of tetraethylammonium. Intracellular tetraethylammonium slows down the fast inactivation process, as though it competes with the binding of the inactivation particle.**

Recent experiments on the rapidly inactivated Shaker K^+ channel suggest that the N-terminal part of this protein may act as a blocking particle that blocks current through the channel and thus produces fast inactivation (1, 2). The model of a blocking particle tethered to the rest of the channel (picturesquely termed the "ball-and-chain" model) was proposed many years ago to explain the properties of inactivation of voltage-activated Na^+ channels (3, 4). Inactivation of both Na^+ and Shaker K^+ channels can be removed by mild protease treatment of the intracellular side of the membrane (1, 5, 6). Inactivation of both types of channels can also be mimicked by the application of intracellular compounds that block current through the channel after the activation gates have opened (7–13). One such compound is a soluble 20-amino acid peptide with the sequence of the N terminus of the Shaker protein, which mimics the fast inactivation of this channel when applied to the intracellular side of a Shaker channel from which a region near the N terminus has been genetically removed (14). These results suggest that the N terminus of the Shaker protein is a strong candidate for the tethered "ball" and that the inactivation process is localized to the intracellular portion of the channel.

These results on the rapid inactivation of Shaker stand in contrast to studies of the much slower inactivation process seen in a delayed-rectifier K^+ channel in lymphocytes. This inactivation process, which occurs over a period of hundreds of milliseconds to seconds, can be retarded by blocking the external mouth of this channel with tetraethylammonium (TEA) (15). Grissmer and Cahalan (15) showed that this slowing was consistent with a model in which the channel could inactivate from the open state but not from the blocked state. This result is surprising when compared with the Shaker studies showing clearly that the inactivation particle interacts with the channel from the internal side. To learn the basis for this discrepancy, we examined the effect of TEA on the inactivation of the Shaker H4 K^+ channel.

MATERIALS AND METHODS

Vectors and cDNA. The cDNA coding for the Shaker H4 K^+ channel [which is nearly identical in sequence to the Shaker B channel (16, 17); kindly provided by M. Tanouye, University of California, Berkeley] was subcloned adjacent to the human cytomegalovirus promoter (18) in the GW1-CMV expression vector (kindly provided by British Bio-Technology, Oxford, U.K.).

Cells. Cells of the human cell line 293 were obtained from the American Type Culture Collection. These are transformed fibroblastoid cells of human embryonic kidney origin.

DNA Transfection. The plasmid DNA for the expression construct was introduced into the 293 cells using a calcium phosphate transfection method (19). On the morning of transfection, 5 ml of fresh Dulbecco's modified Eagle's medium (DMEM)/F12 medium (JRL Scientific, Woodland, CA) with 10% (vol/vol) fetal bovine serum (Sigma) was added to a 25-cm^2 flask containing growing 293 cells at a density of 5–10 × 10^4 cells per cm^2. The medium and incubator P_{CO_2} were previously adjusted to give a final pH value between 7.30 and 7.40. After 3 h of incubation in the fresh medium, a precipitate was prepared by mixing 5 μg of the plasmid DNA in 250 μl of 0.25 M $CaCl_2$ with an equal volume of 2× HBS (1× HBS = 140 mM NaCl/25 mM Hepes/1.4 mM Na_2HPO_4, adjusted to pH 7.12 with NaOH). The precipitating mixture was pipetted vigorously and added immediately to the culture medium. After 3 h of incubation at 37°C, a fine precipitate had settled onto the cell layer. At the end of this incubation, the medium was gently aspirated and the cells were glycerol-shocked by adding 1 ml of prewarmed 15% (vol/vol) glycerol in phosphate-buffered saline (PBS; GIBCO). The glycerol solution was removed, and exactly 40 sec after application a rinse of 5 ml of PBS was added to the flask. The PBS was aspirated, and cells were suspended by trituration in fresh DMEM/F12 medium with 10% fetal bovine serum. The cell suspension was placed into 35-mm

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Abbreviation: TEA, tetraethylammonium.

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dishes containing protamine-treated glass coverslips [flame-sterilized coverslips were incubated for 5 min with a filter-sterilized protamine solution (1 mg/ml) and then rinsed with sterile PBS before plating]. Cells were grown on these coverslips for 1–4 days before patch clamping.

Transfection efficiencies were 30–60% as measured by cotransfection and staining for β-galactosidase (20).

Patch Recording. Excised patch recordings (21) were made 16–96 h after transfection with the following solutions: external, 155 mM NaCl/5 mM KCl/3 mM $CaCl_2$/1 mM $MgCl_2$/10 mM Hepes, pH 7.4; internal, 160 mM KCl/1 mM EGTA/0.5 mM $MgCl_2$/10 mM Hepes, pH 7.4. The specified amount of TEACl was added to the appropriate solution.

RESULTS AND DISCUSSION

The cDNA coding for this channel protein was subcloned adjacent to a the strong cytomegalovirus promoter (18) and introduced by transient transfection into a fibroblastoid cell line. Patch recording from transfected cells shows that hundreds to thousands of channels are present in a patch, resulting in macroscopic currents resembling those seen in whole cell membranes. We exposed excised outside-out membrane patches from transfected cells to a concentration of external TEA (30 mM) that blocks approximately half of the peak outward current (Fig. 1*A*). In contrast to the slowing effect that Grissmer and Cahalan (15) observed for the slow inactivation of the lymphocyte K^+ channel, we observe no effect of external TEA blockade on the rate of inactivation. Instead, the current is uniformly reduced at all times.

Internal TEA blockade, however, produced a substantial slowing of the fast inactivation of the Shaker channel (Fig. 1*B*). The simplest interpretation of this result is that internal TEA and the inactivation particle compete for binding to the same site. The effect of such competition can be described by the following kinetic scheme (in which we ignore the voltage-dependent activation steps, which are rapid and complete and thus do not contaminate measurements of inactivation at the positive voltages studied. O is the open state, B is the blocked state, I is the inactivated state, and κ is the inactivation rate constant:

$$\begin{array}{ccc} & & \mathrm{O} \xrightarrow{\kappa} \mathrm{I} \\ & & \\ \mathrm{TEA} & \rightleftharpoons & \uparrow \\ & & \mathrm{B} \end{array}$$

Since in the steady state the fast inactivation process is nearly complete, we have approximated the rate of return from inactivation as zero. If TEA binding and dissociation is much more rapid than the inactivation process, this scheme predicts that the apparent inactivation time constant (τ_{app}) will be increased by exactly the same factor by which the current is reduced [$\tau_{app} \approx (f\kappa)^{-1}$, where f is the fraction of channels that remain unblocked]. The ≈2-fold reduction in current produced by TEA in Fig. 1*B* was accompanied by an ≈2-fold slowing in the inactivation. We tested this relationship systematically by varying the TEA concentration. Internal TEA behaved just as the competition model predicts: the degree of

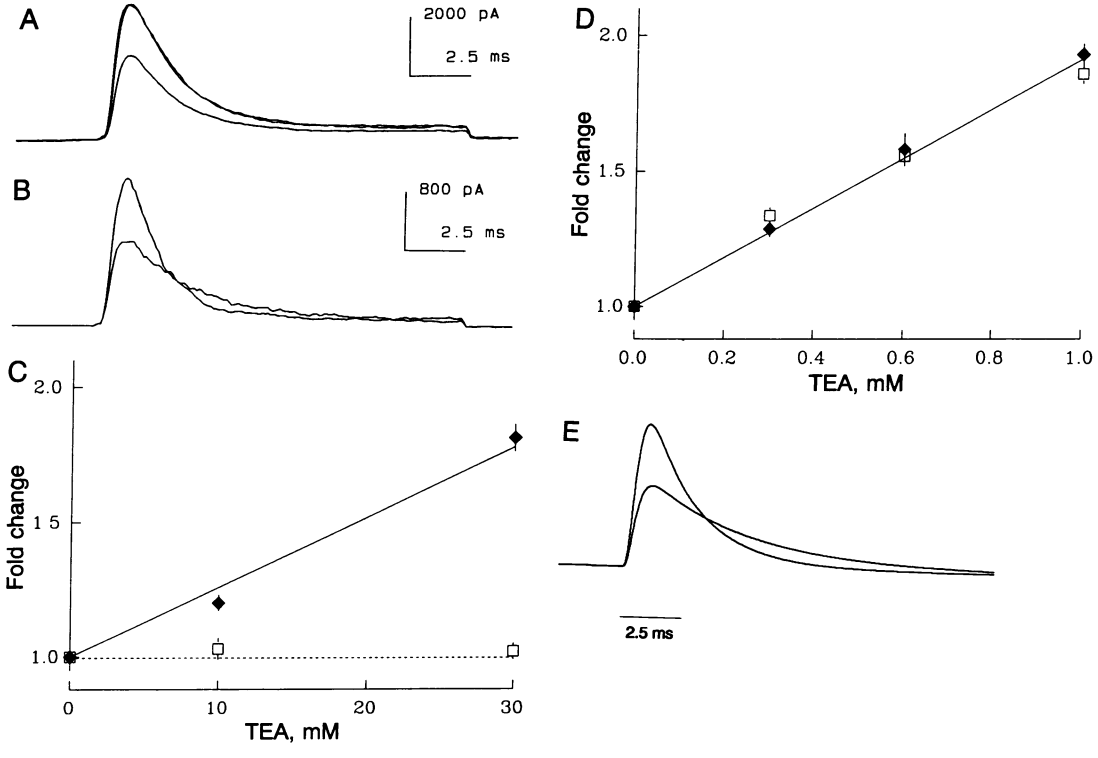

FIG. 1. Effects of TEA blockade on fast inactivation of the Shaker H4 K^+ channel. (*A*) Shaker outward current evoked by a voltage step from −80 mV to +60 mV, with and without the addition of 30 mM TEACl. Outside-out patch from a transfected cell. The current in TEA is shown twice, once at normal scale and again scaled up by 1.6 times to match the peak current of the control. (*B*) Effect of addition of 1 mM TEA Cl to the internal (bath) solution, in a recording from an inside-out patch. (Voltage step as in *A*.) (*C*) External TEA concentration dependence of reduction in peak current (diamonds) and slowing of inactivation (squares). Both parameters are expressed as the fold change from control ($I_{control}/I_{expt}$ or $\tau_{expt}/\tau_{control}$). Inactivation time constants (τ) were measured by fitting an exponential to the declining phase of the current; the measured value was constant in the voltage range +20 to +60 mV. Error bars represent SEM of 3–10 determinations. The solid line corresponds to an EC_{50} of 37 mM; the dashed horizontal line indicates no effect. (*D*) Internal TEA concentration dependence of reduction in peak current (diamonds) and slowing of inactivation (squares). Details are as in *C*. The straight line corresponds to an EC_{50} of 1 mM. (*E*) Simulation of the effect of TEA blockade on inactivation. The computer model used the kinetic scheme described in the text and rate constants derived for the Shaker channel in *Drosophila* myotubes by Zagotta and Aldrich (22). The simulation was performed with and without a concentration of blocker that reduced the current by 50%. The channel could neither close nor inactivate from the blocked state.

slowing matches the degree of block (Fig. 1*D*). External TEA had no effect on the inactivation rate over a range of concentrations that produced substantial block (Fig. 1*C*).

Internal TEA produces a blockade of the K^+ current but also leads to larger currents at later times ("crossover"). This result seems surprising but it is actually a direct prediction of the competition model (12, 13, 15, 23–25). Channels that are blocked by TEA are protected from inactivation. Although neither blocked channels nor inactivated channels can carry current, the blocked channels are in rapid equilibrium with the pool of open channels and thus are only temporarily nonconducting. Inactivated channels, on the other hand, do not return rapidly to the open channel pool and are (on the time scale of the pulse) "permanently" lost. Fig. 1*E* shows simulated currents using the kinetic scheme above with measured rates for Shaker channel gating (22); the simulated currents exhibit the same crossover seen experimentally.

The success of the competition model suggests that TEA and the inactivation particle may bind to the same site at the inner mouth of the channel. Alternatively, they may bind to slightly different sites with some overlap that prohibits both from binding at once. This latter possibility is more likely true, since a site-directed mutation of the Shaker channel that alters internal TEA affinity by 10-fold has little or no effect on fast inactivation (26). In either case, the competition model suggests that the inactivation particle acts by directly occluding the pore, since there is good evidence that TEA and its analogs do so (7–9).

Mutations that abolish the fast inactivation of Shaker channels produce a channel that remains open much longer than wild-type Shaker, but still inactivates during a very long pulse lasting seconds (1). This slow inactivation resembles the slow inactivation of delayed rectifier channels such as the lymphocyte K^+ channel studied by Grissmer and Cahalan (15), and we wondered how the TEA sensitivity of this process would compare to that of the normal fast inactivation in Shaker and to that of the delayed rectifier. We tested the effects of both external and internal TEA on the remnant slow inactivation of a Shaker deletion mutant (Shaker H4 Δ6-46; see Fig. 2) and found that this process was affected by external TEA in a fashion resembling the delayed rectifier inactivation. External TEA reduces the current and slows down the inactivation (Fig. 2*A*), while internal TEA simply reduces the current equally at all times. The remnant inactivation is, therefore, not simply a crippled version of the normal fast process, but rather a separate process.

We also examined the TEA concentration dependence of this slowing effect, and it also appears compatible with the kinetic scheme for competition (Fig. 2*C*). Since there is no effect of trypsin on the external side of the Shaker channel (1) and no candidate for an external "ball," it may be preferable to consider another physical model, consistent with the same kinetic scheme, to explain the effect of external TEA on slow inactivation. A "foot-in-the-door" model, in which a channel cannot close when occupied by a blocking or permeant ion, has been used to explain ion effects on gating in several types of ion channels (8, 12, 13, 27–29). This model supposes that the blocker (TEA in this case) binds within a part of the pore that becomes constricted upon inactivation, thus preventing inactivation.

We have demonstrated that Shaker K^+ channels have two distinct inactivation processes that can be differentiated by their sensitivity to internal and external TEA. The fast inactivation process appears to be due to a ball-and-chain mechanism, and internal TEA appears to compete with the binding of the ball. This fast inactivation mechanism thus resembles the fast inactivation of Na^+ channels, which is also retarded by internal blockers (12, 13, 23). On the other hand, the slower inactivation revealed by disruption of the fast process (1) is insensitive to internal TEA but slowed by external TEA. This is clearly different from the fast process but similar to the slow inactivation of delayed rectifier K^+ channels. It thus seems likely that slow Shaker inactivation and delayed rectifier K^+ channel inactivation occur by a common mechanism, which is different from the specialized mechanism that produces the fast inactivation of Shaker K^+ channels and Na^+ channels.

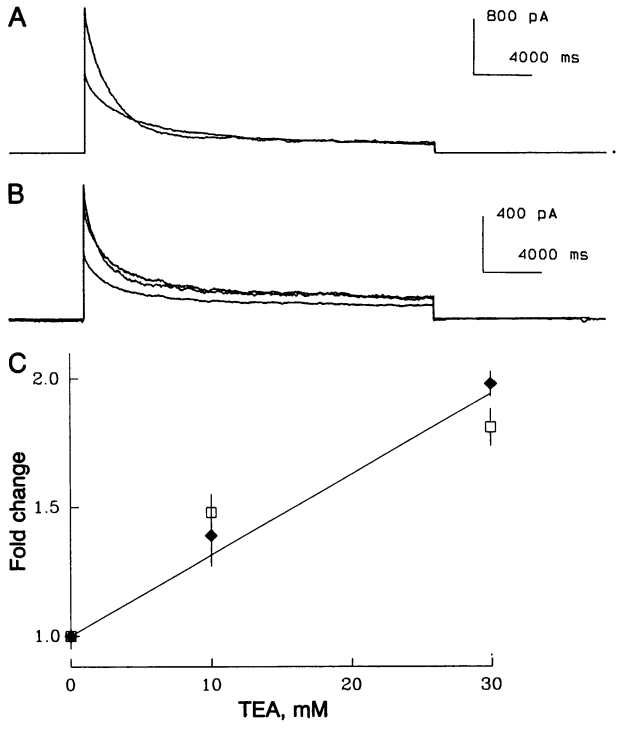

FIG. 2. Effects of TEA blockade on the slow inactivation of a mutant Shaker channel with an N-terminal deletion that abolishes fast inactivation. The deletion mutation (Δ6-46) described by Hoshi *et al.* (1) was introduced into the Shaker H4 cDNA and expressed in mammalian cells as in Fig. 1. We refer to this as the Shaker H4 Δ6-46 channel. (*A*) Slow inactivation of Shaker H4 Δ6-46 currents during a prolonged depolarization to 0 mV and the effect of 30 mM external TEA (outside-out patch). (*B*) Slow inactivation of Shaker H4 Δ6-46 currents and the effect of 1 mM internal TEA (inside-out patch). The current in TEA is shown twice, once at normal scale and again scaled up by 1.75 times to match the peak current of the control. (*C*) External TEA concentration dependence of reduction in peak current (diamonds) and slowing of inactivation (squares), as in Fig. 1*C*. The straight line corresponds to an EC_{50} of 33 mM.

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