different from the kinetic model prediction (32%) because of non-equilibrium considerations. At 80 μM MK-801, amplitude reduction ≈90%.
16. In the presence of DL-2-amino-5-phosphonovalerate [DL-AP5 (100 μM)] to block NMDA receptors, the AMPA receptor–mediated EPSC was unaffected by MK-801 (5 μM; n = 6), demonstrating that MK-801 did not have a presynaptic action. The assumption that all NMDA channels have the same P_o was tested as follows: If glutamate activates a mixed population of NMDA channels, then those with higher values of P_o will be preferentially blocked. This relation will bias the population toward lower P_o channels as more channels are blocked, and the decay of the EPSC should be slowed. However, the EPSC decay remained constant, even after >50% block by MK-801 (Fig. 1B), suggesting that P_o was similar for all synaptic NMDA channels.
17. With the optimum kinetic model parameters for each EPSC, the model simulated two EPSCs that were evoked 10 s apart in the continuous presence of 5 μM MK-801. The relative amplitude reduction of the second response gave the proportion of channels blocked during the first response.
18. The ratio of the number of high to low P_r terminals is equal to the ratio of the area under the fast to that under the slow exponential component of progressive block. These ratios are equal because the time constant of an exponential component is proportional to P_r and because its amplitude is proportional to the number of terminals and inversely proportional to P_r. Thus, the exponential amplitude multiplied by the time constant (or the area under the exponential) is proportional to the number of terminals and is independent of P_r. The ratio of the efficacy of high to low P_r terminals is equal to the ratio of the amplitude of the fast to that of the slow exponential component.
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Functional Stoichiometry of Shaker Potassium Channel Inactivation

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Shaker potassium channels from *Drosophila* are composed of four identical subunits. The contribution of a single subunit to the inactivation gating transition was investigated. Channels carrying a specific mutation in a single subunit can be labeled in a heterogeneous population and studied quantitatively with scorpion toxin sensitivity as a selection tag. Linkage within a single subunit of a mutation that removes the inactivation gate to a second mutation that affects scorpion toxin sensitivity demonstrates that only a single gate is necessary to produce inactivation. The inactivation rate constant for channels with a single gate was one-fourth that of channels with four gates. In contrast, the rate of recovery from inactivation was independent of the number of gates. It appears that each of the four open inactivation gates in a Shaker potassium channel is independent, but only one of the four gates closes in a mutually exclusive manner.

In the first quantitative theory of Na^+ and K^+ channel gating, Hodgkin and Huxley postulated that multiple gating particles existed and the overall conductivity depended on the positions of all the particles (*1*). Now, with our growing understanding of the proteins that make ion channels, we can begin to attach physical meaning to the notion of multiple gating particles. We asked how inactivation gating in Shaker K^+ channels depends on the movements of individual inactivation gates contributed by each of the four identical subunits.

The best understood gating transition is N-type inactivation in Shaker K^+ channels, the spontaneous closing that occurs after voltage-dependent channel opening. The NH_2-terminus of the Shaker channel causes inactivation by forming a cytoplasmic gate, like the "ball and chain" proposed by Armstrong and Bezanilla to explain inactivation in Na^+ channels (*2–4*). The Shaker K^+ channel consists of four identical subunits. It is not known whether the channel has four separate inactivation gates, one from each subunit, and if so, whether more than one gate is required to produce inactivation. Peptides corresponding to part of the NH_2-terminus of a Shaker K^+ channel can aggregate to form multimers (*5*), raising the possibility that a single gate could be formed through the coassembly of four NH_2-termini.

We studied the number and independence of inactivation gates by exploiting the channel's susceptibility to a scorpion toxin (*6*). A mutation of Asp^{431} to Asn (D431N) affects toxin sensitivity in a recessive manner: the mutation must be present in all four subunits to render the channel insensitive (*7*). Thus, if wild-type and D431N mutant subunits are coexpressed, channels with four mutant subunits can be distinguished from channels containing at least one wild-type subunit because a single wild-type subunit confers toxin sensitivity.

Scorpion toxin inhibits by binding at the extracellular face of the channel, whereas the inactivation gate, formed by the NH_2-terminus, is located on the intracellular side of the membrane. Toxin sensitivity and inactivation gating are independent; mutations affecting one property do not affect the other. We therefore used the recessive nature of the mutation at position 431 to ask if only a single gate can cause inactivation. The approach is illustrated in Fig. 1A. The sketch shows the heteromultimeric channels that can result from the coexpression of two different subunits. If an inactivation gate is present only on the toxin-sensitive subunit, then by applying toxin to the population of channels we can determine whether a single gate is sufficient for inactivation. Channels with at least one gate will be blocked because the gate is linked to the toxin-sensitive subunit. Therefore, if a single gate produces inactivation, then all of the toxin-sensitive channels will inactivate and the insensitive channels will not. In such an experiment, some of the channels in the mixed population inactivated and some did not (Fig. 1B). When toxin was applied, the entire inactivating component was blocked and the sustained component was insensitive (Fig. 1C). This outcome exactly matches our expectation if a single gate causes inactivation.

If subunits without an intact inactivation gate were unable to coassemble with the wild-type subunits, then the channels expressed in the experiment described above would have either four gates or no gates; the experiment of Fig. 1 (A through C) does not exclude this possibility. By

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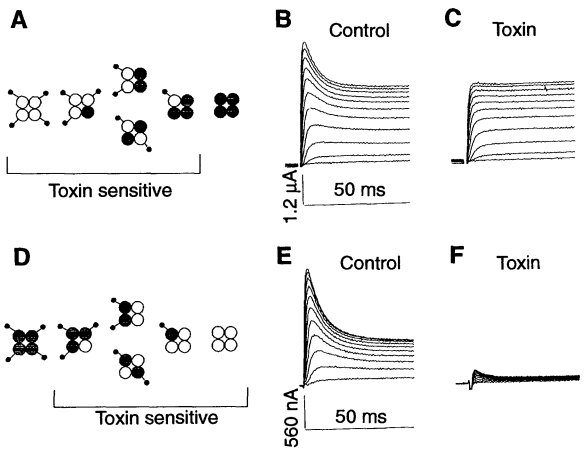

Fig. 1. Analysis of the number of gates required to produce inactivation. (**A**) Wild-type subunits are shown as empty circles (to indicate toxin sensitivity) with a connected ball and stick (to indicate an intact inactivation gate). Subunits with a D431N mutation to remove toxin sensitivity (*7*) and a deleted inactivation gate (*3*) are designated as filled circles. All possible combinations of the two subunits in a tetrameric channel are shown. (**B** and **C**) Currents resulting from coexpression of the subunits described in (A) in the absence (B) or presence (C) of 66 nM toxin. (**D**) Possible combinations of subunits in a second experiment in which the toxin sensitivity was incorporated only on the subunit without an inactivation gate. (**E** and **F**) Currents resulting from the experiment described in (D) in the absence (E) and presence (F) of toxin. The inactivation gate was removed by deleting amino acids 6 to 46 (*10*). The Asp→Asn mutation at position 431 was made by site-directed mutagenesis (*7*). RNA mixture ratios were adjusted empirically to obtain the desired fraction of inactivating and sustained currents. Currents were recorded in *Xenopus* oocytes with a two-electrode voltage-clamp amplifier (Warner Instruments) 1 to 2 days after RNA injection (*11*). The membrane was held at −80 mV and stepped for 50 ms to potentials between −40 and 60 mV in 10-mV increments. The bath solution contained 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 0.3 mM $CaCl_2$, and 5 mM Hepes (pH 7.6). The apparent dissociation constant, K_d, for the wild-type channel is about 0.6 nM, and for the channel with a single sensitive subunit K_d is about 3 nM. All experiments were done at room temperature (21° to 23°C). Data were filtered at 2 kHz (8-pole Bessel) and sampled once every 250 μs.

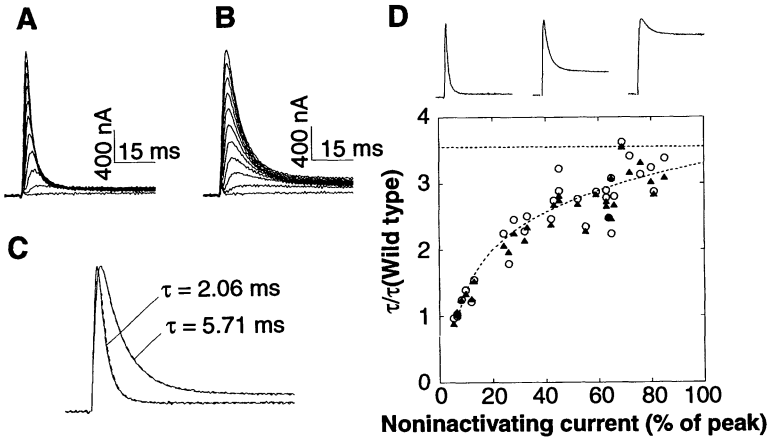

Fig. 2. Rate of inactivation in channels containing fewer than four gates. (**A**) Wild-type Shaker K^+ channel currents were recorded with a two-electrode voltage-clamp amplifier as described (Fig. 1). Capacitive currents were removed by subtracting traces recorded after fully blocking K^+ currents with toxin. (**B**) Currents obtained by subtracting the traces in Fig. 1C from those in Fig. 1B. (**C**) The 60-mV (top) traces from (A) and (B) were scaled to the same peak amplitude and superimposed. A single exponential function was fit to the decay phase of each superimposed trace (dashed line) (*12*). (**D**) Wild-type Shaker K^+ channel subunits with an intact inactivation gate were expressed at various ratios with subunits in which the inactivation gate had been removed. The mixture ratio ranged from all wild type with four inactivation gates to nearly all mutant with no inactivation gates. Typical 60-mV current traces are shown above the graph for wild-type channels alone (left) and two mixtures containing consecutively smaller fractions of wild-type subunits. The inactivating current was fit to a single exponential, as shown in (C), by two separate methods: a least squares fit (circles) and by dividing the area measured under the transient by the initial amplitude. The time constant from each experiment was normalized to the estimated mean for the wild-type channel (2.05 ± 0.05 ms, from the three leftmost points) and plotted against the noninactivating current (percentage of peak). Each pair of points corresponds to a separate experiment. The dashed line is at 3.57 on the *y* axis, the expected ratio if the microscopic inactivation rate constant is proportional to the number of gates (one to four) and if recovery is independent: 3.57 = (0.48 + 0.02)/(0.12 + 0.02) from τ_{inact} = 2 ms for channels with four gates and τ_{rec} = 50 ms (see Eq. 1). The dashed curve assumes random assembly of subunits (binomial distribution of channels with zero to four gates) and a microscopic inactivation rate constant of 0.1 ms^{-1} per gate and a recovery rate constant of 0.03 ms^{-1} (*13*). The experimental conditions were identical to those described in Fig. 1.

switching the subunit that carries the toxin sensitivity, we demonstrated that the different subunits must coassemble. In this experiment (Fig. 1D), separate subunits were responsible for toxin sensitivity and inactivation gating. In this case, the sustained current as well as a large fraction of the inactivating current was sensitive to toxin (Fig. 1, E and F). This result is remarkable because the inactivation gate is carried by a subunit that is itself toxin-insensitive (it contains Asn^{431}). The outcome is a direct consequence of subunit coassembly: hybrid channels are toxin-sensitive because they have at least one toxin-sensitive subunit and they inactivate because they have at least one inactivation gate. Taken together, these two experiments demonstrate inactivation in channels with only one inactivation gate and rule out the necessity of multiple gates, either individually or in aggregate, for N-type inactivation.

We next tested whether the rate of inactivation depends on the number of gates. Figure 2A shows a family of currents carried by wild-type Shaker K^+ channels. Difference currents (Fig. 2B) were obtained by subtracton of the toxin-blocked traces (Fig. 1C) from the control traces (Fig. 1B). These currents are carried by channels that have, on average, fewer than four inactivation gates. The 60-mV traces from Fig. 2, A and B, are scaled to the same amplitude and superimposed in Fig. 2C. The channels with fewer gates inactivated with slower kinetics.

To measure how much slower inactiva-

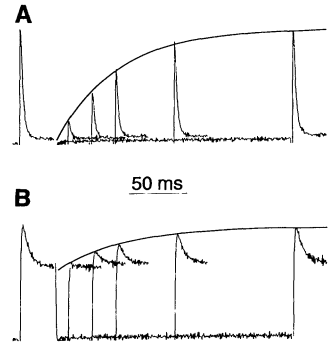

Fig. 3. Independence of the rate of recovery from inactivation from the number of inactivation gates. (**A**) Wild-type Shaker K^+ currents were recorded with a two-microelectrode voltage clamp under the ionic conditions described in Fig. 1. Membrane potential was held at −70 mV. An initial 30-ms conditioning pulse to 20 mV was followed after a variable period at −70 mV by a second test depolarization. One conditioning pulse and five test pulses are shown. The curve is an exponential function (τ_{rec} = 50 ms) fit to the peak of the currents. (**B**) The same experiment described in (A) was done on channels resulting from the coexpression of wild-type subunits and subunits without an inactivation gate. The curve is an exponential with τ_{rec} = 50 ms.

tion is when a channel has only one rather than four gates, we did experiments with several different mixture ratios of the two subunits (Fig. 2D). The wild-type subunit was expressed in the presence of successively higher concentrations of subunits without a gate. A vast excess of subunits without a gate ensures that most channels will have no gate and occasionally a channel will have one subunit with a gate. By studying the small fraction of inactivating channels, we estimated the inactivation rate for channels with only one gate. The traces above the graph in Fig. 2D are from wild-type channels and two successive dilutions of wild-type subunits (coexpressed with subunits without the gate). As the fraction of inactivating channels became smaller, the rate of inactivation became slower. To show this effect graphically, we quantified inactivation with a single exponential fit (Fig. 2C). The resulting time constant, τ_{inact}, normalized by that measured for wild-type channels, was plotted as a function of the noninactivating current (percentage of peak). (The noninactivating current was ~5% of peak current for wild-type channels because inactivation was incomplete.) The Y value at 100% noninactivating current corresponds to the time constant ratio for channels with a single gate. By extrapolating from the measured values, we found that τ_{inact} for a one-gate channel was about 3.5 times that for a four-gate channel.

We also measured the rate of recovery of channels from inactivation (Fig. 3). An initial conditioning pulse caused the channels to inactivate, and a second test pulse showed what fraction recovered during the intervening period. Wild-type channels with four gates (Fig. 3A) and channels with about one gate per channel (8) (Fig. 3B) recovered over the same time course ($\tau_{rec} \approx$ 50 ms). The recovery rate did not depend on the number of gates. This finding indicates that the gates must close in a mutually exclusive manner. If more than one gate was closed at once, the rate of recovery from inactivation would, in general, depend on the total number of gates.

The value of τ_{inact} for channels with a single gate was about 3.5 times that for wild-type channels with four gates. However, we wanted to know how the number of gates affects the microscopic inactivation rate constant, k_{inact}, which differs from τ_{inact} because the recovery rate will contribute to τ_{inact}. For the simple reaction rate model for inactivation

$$O \underset{k_{rec}}{\overset{k_{inact}}{\rightleftharpoons}} I \quad (1)$$

where O and I are the open and inactivated states and k_{inact} and k_{rec} are the rate constants for inactivation and recovery; $\tau_{inact} = (k_{inact} + k_{rec})^{-1}$ and $\tau_{rec} = k_{rec}^{-1}$ (9). If we assume that k_{rec} is independent of voltage, then we can use the value obtained at −70 mV (Fig. 3) to express the ratio of inactivation rate constants for channels with four gates, $k_{inact}(4)$, to those with one gate, $k_{inact}(1)$, in terms of experimentally determined time constants

$$\frac{k_{inact}(4)}{k_{inact}(1)} = \frac{[\tau_{rec} - \tau_{inact}(4)]}{[\tau_{rec} - \tau_{inact}(1)]} \frac{\tau_{inact}(1)}{\tau_{inact}(4)} \quad (2)$$

Equation 2 shows how the ratio of inactivation rate constants differs from the ratio of time constants. The two ratios are proportional through a correction factor. In our experiments, τ_{rec} was ~50 ms (Fig. 3), $\tau_{inact}(4)$ was 2 ms (Fig. 2, wild type), and $\tau_{inact}(1)$ was ~7 ms (Fig. 2D). Substituting these values into Eq. 2 gives a value close to 4 for the ratio of inactivation rate constants. This simple outcome agrees with our most naive expectation of how the inactivation mechanism must work. The open gates apparently do not interfere with each other; the microscopic inactivation rate is nearly proportional to the number of gates. All of our observations here can be fully explained if our mutations act solely to change the local "concentration" of the gate near the pore on the inside.

From our findings we draw three conclusions about the mechanism of inactivation. First, channels with only a single gate still inactivate. Second, the microscopic inactivation rate for channels with a single gate is approximately one-fourth that of channels with four gates. Third, the rate of recovery from inactivation does not depend on the number of gates. Taken together, these conclusions indicate that the Shaker K^+ channel acts as if there are four equivalent inactivation gates that move independently of one another, but when one gate closes, it prevents the others from closing.

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6. The toxin is an isoform of charybdotoxin (M. L. Garcia, M. Garcia-Calvo, P. Jimenez Hidalgo, R. MacKinnon, in preparation) and is responsible for the blocking activity against Shaker [that was attributed to charybdotoxin (*7*)]. All experiments were done with purified toxin. We also did several experiments with recombinant toxin produced in bacteria, and the results were indistinguishable from those obtained with the purified toxin.
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8. When the noninactivating current is 50% of peak, ~76% of the inactivating current should be from channels with one inactivation gate, 21% should have two gates, and less than 3% should have three. In Fig. 3B, in which greater than 50% of the current did not inactivate, an even greater fraction of the inactivating channels should have only one gate.
9. The τ_{rec} was measured at −70 mV, a potential at which the deactivation rate (rate of transition back to the closed state) is fast. At such negative potentials we assume that a channel recovers from state I to state O and then immediately deactivates. In this case the recovery time constant would depend only on k_{rec}.
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12. For a heterogeneous population of channels with different inactivation rates, a best fit single exponential decaying function (fit to the multi-exponential macroscopic decay) will yield a time constant that is similar to (but in general not equal to) the mean of the underlying time constants (weighted according to the distribution of channels).
13. The curve assumes a population of open channels undergoing inactivation according to $O \underset{k_{rec}}{\overset{mk_i}{\rightleftharpoons}} I$, where m is the number of gates, mk_i is the inactivation rate constant for a channel with m gates, and k_{rec} is the recovery rate constant. If $A(m)$ is the fraction of channels with m gates, then the time constant normalized by that for channels with four gates (determined by dividing the area under the transient by the initial amplitude) is given by

$$(4k_i + k_{rec}) \left[\sum_{m=1}^{4} \frac{mA(m)k_i}{(mk_i + k_{rec})^2} \Big/ \sum_{m=1}^{4} \frac{mA(m)k_i}{mk_i + k_{rec}} \right]$$

and the noninactivating current (percentage of peak) is given by

$$A(m=0) + \sum_{m=1}^{4} \frac{A(m)k_{rec}}{mk_i + k_{rec}}$$

The curve in Fig. 2D corresponds to the first quantity plotted against the second for $A(m)$ given by the binomial distribution and for $k_i = 0.1\ ms^{-1}$ and $k_{rec} = 0.03\ ms^{-1}$. Thus, a simple theory based on a binomial distribution of channels provides a reasonable fit using rate constants that are similar to those measured directly from inactivation and recovery time constants.

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