an enzyme solution [collagenase (0.5 mg/ml) and protease (0 1 mg/ml), 5 min]. The experimental superfusion solution contained. NaCl, 145 mM; KCl, 4 mM; $CaCl_2$, 1 mM; CsCl, 1 mM; $BaCl_2$, 0.5 mM; Hepes, 10 mM, glucose, 10 mM; pH 7.4. $NiCl_2$ (5 mM) was added to this solution to block the Ca^{2+} channels and the Na^+-Ca^{2+} exchanger. Another solution designed to prevent Ca^{2+} influx into the cells was nominally Ca^{2+}-free, and NaCl was replaced by LiCl. The pipette filling solution had the following composition. CsCl, 120 mM; Hepes, 20 mM; tetraethylammonium, 20 mM; DM-nitrophen, 2 mM; $CaCl_2$, 0.5 mM; potassium adenosine triphosphate, 5 mM; pH 7.2. Most experiments were carried out at 21° to 23°C, some at 31° to 32°C. The gigaohm-seal technique in the whole-cell configuration was used to control the membrane voltage and to equilibrate the cytosol with the pipette filling solution. Cells were held at a holding potential of −40 mV during the loading procedure (8 to 12 min). Electrodes were pulled from borosilicate glass and had a series resistance between 0.8 and 1.5 megohms. Cell length was measured with a video-dimension analyzer (60 cell-length measurements per second)

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Restoration of Inactivation in Mutants of *Shaker* Potassium Channels by a Peptide Derived from ShB

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Site-directed mutagenesis experiments have suggested a model for the inactivation mechanism of *Shaker* potassium channels from *Drosophila melanogaster*. In this model, the first 20 amino acids form a cytoplasmic domain that interacts with the open channel to cause inactivation. The model was tested by the internal application of a synthetic peptide, with the sequence of the first 20 residues of the ShB alternatively spliced variant, to noninactivating mutant channels expressed in *Xenopus* oocytes. The peptide restored inactivation in a concentration-dependent manner. Like normal inactivation, peptide-induced inactivation was not noticeably voltage-dependent. Trypsin-treated peptide and peptides with sequences derived from the first 20 residues of noninactivating mutants did not restore inactivation. These results support the proposal that inactivation occurs by a cytoplasmic domain that occludes the ion-conducting pore of the channel.

DELETIONS AND POINT MUTATIONS near the amino terminal of the ShB potassium channels of *D. melanogaster* dramatically show inactivation (*1*). These data plus the lack of voltage dependence of the inactivation rates and the ability of internal proteolytic agents to modify inactivation suggest a mechanism of inactivation similar to the ball and chain model originally proposed by Armstrong and Bezanilla (*2*) for voltage-gated Na^+ channels. In ShB channels, the first 20 amino acids in the NH_2-terminus are proposed to form a structural domain that interacts with part of the open channel to cause inactivation. This structural domain, or "ball" region, is connected to the rest of the protein by a "chain" sequence of 60 or more amino acids that tether the inactivation ball near its receptor. According to this model of inactivation, the putative ball region should be able to interact with the rest of the channel and produce inactivation even when it is not covalently attached to the rest of the channel protein. We tested this hypothesis by examining the effects of a peptide corresponding to the ball region (first 20 amino acids of ShB: MAAVAGLYGLGEDRQHRKKQ) (*3*) on the gating of ShB channels expressed in *Xenopus* oocytes. The experiments were performed with mutant ShB channels that contain, near their NH_2-terminus, a large deletion that effectively removes fast inactivation (*4*).

ShBΔ6-46 is a 41–amino acid deletion mutant of ShB that does not inactivate with a rapid time course (*1*). Application of the ShB peptide to the cytoplasmic side of ShBΔ6-46 channels accelerates their inactivation rate (Fig. 1). We tested the effects of different concentrations of peptide on macroscopic currents in inside-out patches elicited by voltage steps to 0 mV and +50 mV. The peptide-induced inactivation occurs rapidly and can be readily reversed when peptide-free solution is perfused into the bath, indicating that the peptide only weakly associates with the channel. At each voltage, the rate of the macroscopic inactivation is dependent on the peptide concentration and increases with increasing concentrations of peptide, as expected for a simple bimolecular reaction. In addition, the macroscopic inactivation rate at a given peptide concentration is dependent on the voltage. At the more positive voltages, where the channels activate more rapidly, peptide-induced inactivation occurs more rapidly. However the rate of the inactivation transition induced by peptide observed in single-channel recording is independent of voltage. These results indicate that the inactivation produced by the peptide is coupled to activation, as is the case for the normal inactivation process. Application of the ShB peptide to the extracellular side did not have any effect on the currents recorded from ShBΔ6-46 channels.

The rate of recovery from peptide-induced inactivation, however, appears to be slower than that from normal fast inactivation. During repeated voltage pulses at a frequency where ShB currents completely recover in the interval between pulses, the currents in the presence of peptide tend to decrease in amplitude progressively because the channels accumulate in the peptide-blocked state. This difference in recovery rates probably arises because the synthetic peptide has a greater binding affinity for the channel than does the normal NH_2-terminal ball domain. This difference may result from the absence of covalently linked residues, which normally destabilize the inactivated state. Alternatively, the peptide could bind to additional sites on the channel, which could cause slow inactivation. In either case, the slower recovery rate from peptide-in-

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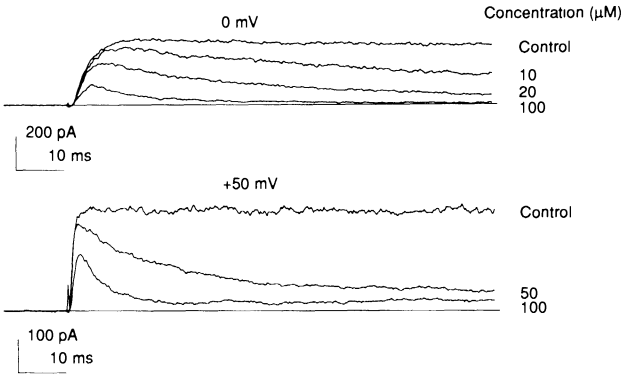

Fig. 1. Effects of intracellular ShB peptide on macroscopic currents from ShBΔ6-46 expressed in *Xenopus* oocytes. Macroscopic currents from an inside-out patch before and after the application of crude ShB peptide to the bath. Currents were recorded with voltage steps to 0 mV from a holding voltage of −110 mV (top) and +50 mV from a holding voltage of −100 mV (bottom). The data were low-pass filtered at 1.2 kHz and digitized at 100 μs per point.

duced inactivation probably does not represent a fundamental difference in the mechanisms of normal and peptide-induced inactivation.

Peptide-induced inactivation is similar to normal inactivation in single channels. Although the ShBΔ6-46 channels normally exhibit long bursts of openings, application of 100 μM peptide reduces the number of openings per burst and the open durations (Fig. 2). The general behavior of single channels with added peptide is similar to normal ShB channels with initial short bursts of openings separated by long-lived closed states. The open durations of ShBΔ6-46 channels in the presence of 20 and 50 μM peptide are independent of voltage and shorter than those without peptide (Fig. 2B), indicating that the peptide produces an inactivation transition that occurs from the open state with a voltage-independent rate. The mechanism of peptide-induced inactivation, therefore, cannot involve the movement of charged residues in the membrane electric field, such as the penetration of the peptide deep within the mouth of the channel. This inactivation process resembles the normal fast inactivation in ShB channels in several respects. Both forms of inactivation are voltage-independent and coupled to activation. As suggested by our data from macroscopic currents (Fig. 1), the rate of the peptide-induced inactivation is dependent on the peptide concentration. When the reciprocal of the mean open duration is plotted as a function of the peptide concentration (Fig. 2C), we see that a peptide concentration of about 100 μM produces a rate of inactivation comparable to the normal fast inactivation in ShB. A linear concentration dependence is consistent with a mechanism whereby inactivation occurs by the binding of a single peptide to the channel. A linear least-squares fit to the plot of the reciprocal of the mean open time as a function of the peptide concentration indicates that the peptide binds with a rate of 4.3×10^6 s^{-1} M^{-1} (*5*). This rate is substantially slower than the diffusion limited rate ($\sim 10^8$), indicating that the interaction of the inactivation-inducing peptide with the channel is not controlled simply by the peptide colliding with the channel, but involves some effects of orientation or conformation.

Whereas the peptide retains its activity after boiling, treatment of the peptide with trypsin with subsequent boiling to inactivate the trypsin abolishes nearly all of the inactivation-inducing activity (Fig. 3A). This result demonstrates that the active component of the solution of synthetic peptide is proteinaceous. In addition, it suggests a possible mechanism by which intracellular trypsin can disrupt inactivation in normal ShB channels. However, trypsin cleavage at any of the potential sites in the putative chain domain might also be expected to remove inactivation. Because the trypsin cleavage sites all occur in the hydrophilic end of the peptide, this result also demonstrates that the hydrophobic portion of the peptide sequence (first 11 amino acids) is insufficient to produce inactivation.

The peptide-induced inactivation is dependent on the sequence of the peptide in the same way that normal inactivation is dependent on the sequence of the NH_2-terminal domain. Peptides were synthesized with the sequence of the first 20 amino acids of the mutants ShBΔ6-46 (MAAVALREQQLQRNSLDGYG) and ShB-L7E (MAAVAGEYGLGEDRQHRKKQ), mutations in ShB that disrupt inactivation (*1*). Neither peptide affected the inactivation rate of ShBΔ6-46 channels when applied at 50 μM (Fig. 3B). The one amino acid substitution completely abolishes its inactivation-inducing activity, as it does in the mutant channels ShB-L7E. This similarity in the effects on inactivation of a single amino acid substitution suggests that the mechanism of the peptide-induced inactivation is similar to that of normal inactivation. These mutant peptides frequently produced a time-independent, small depression in the amplitude of the macroscopic currents but we did not study this effect further. To examine wheth-

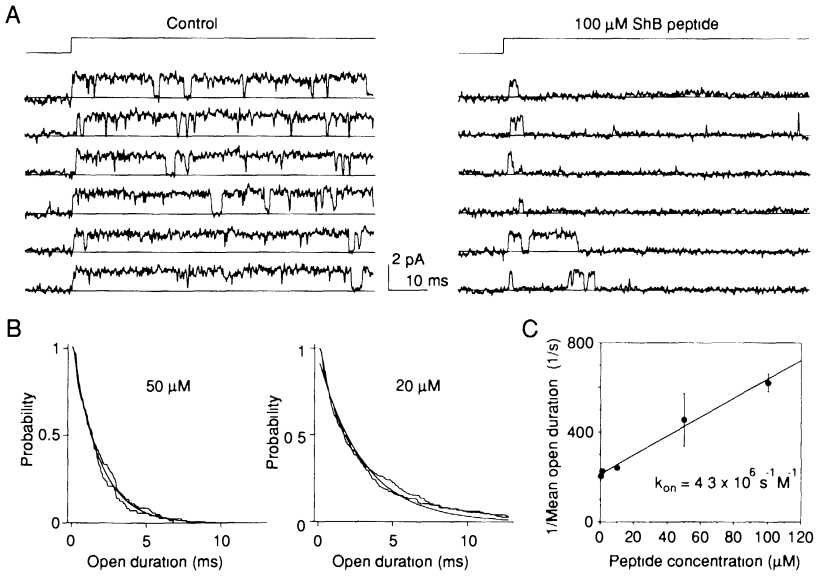

Fig. 2. Effects of the ShB peptide on the gating of single ShBΔ6-46 channels. (**A**) Representative single-channel openings from a ShBΔ6-46 channel in an inside-out patch before (left) and after (right) application of 100 μM crude ShB peptide to the bath solution. The openings were elicited by voltage steps to +50 mV from a holding voltage of −120 mV. The data were filtered at 1.5 kHz and digitized at 50 μs per point. The voltage protocols are shown at the top. (**B**) Open duration histograms of ShBΔ6-46 channels at 0 and +50 mV in the presence of 50 μM (left) and 20 μM (right) crude ShB peptide. The open duration histograms are displayed as tail distributions and show the probability that a given open duration is greater than the time indicated on the abscissa. The histograms at each peptide concentration are fitted with a single exponential function with a mean of 1.7 ms for 50 μM and a mean of 3.0 ms for 20 μM. (**C**) Plot of the reciprocal of the mean open duration as a function of the concentration of crude ShB peptide. Error bars are ±SEM, $n = 2$. The k_{on} was determined from a least-squares linear regression and has a value of $4.3 \times 10^6 \pm 0.25 \times 10^6$ s^{-1} M^{-1} (mean ± SEM).

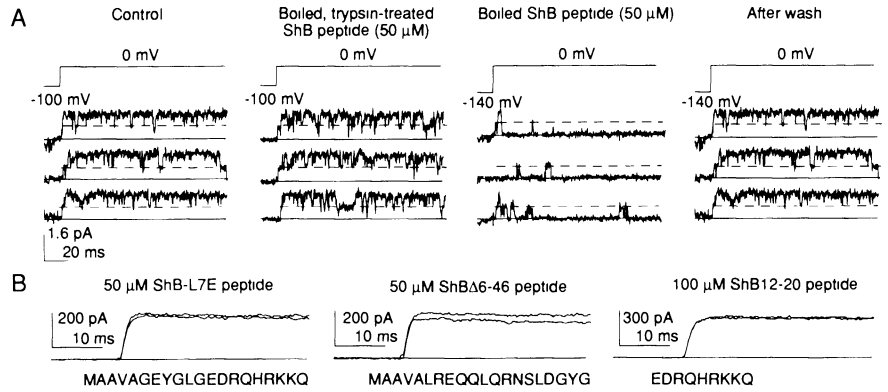

Fig. 3. Effects of altered peptides on ShB channels. (**A**) Effects of boiling (30 min) and trypsin treatment (0.0025%; 5 hours) on the inactivation-inducing activity of the ShB peptide. Representative traces of ShBΔ6-46 channels in an inside-out patch containing two channels are shown before treatment; after application of 50 μM trypsin-treated, boiled ShB peptide; after application of 50 μM boiled ShB peptide; and after wash. The openings were elicited by voltage steps to 0 mV from a holding voltage of −100 mV. The data were filtered at 1.1 kHz and digitized at 50 μs per point. The voltage protocols are shown at the top. (**B**) Effects of mutant peptides on macroscopic currents from ShBΔ6-46. For each of the mutant peptides, the macroscopic currents recorded before and after the application of peptide to the bath solution are overlaid. The HPLC-purified, mutant peptides ShB-L7E and ShBΔ6-46 were applied at 50 μM, and the crude mutant peptide ShB12-20 was applied at 100 μM. Macroscopic currents were recorded from inside-out patches with voltage steps to 0 mV from a holding voltage of −100 mV. The data were filtered at 1.3 kHz and digitized at 100 μs per point.

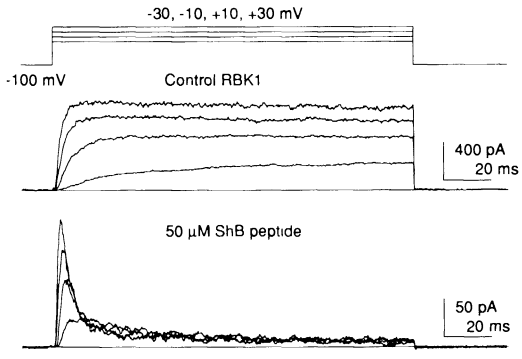

Fig. 4. Effects of intracellular ShB peptide on macroscopic currents from RBK1 expressed in *Xenopus oocytes*. Macroscopic currents were recorded from an inside-out patch before (top) and after (bottom) the application of 50 μM crude ShB peptide to the bath solution. Currents were recorded with voltage steps to −30, −10, +10, and +30 mV from a holding voltage of −100 mV. The data were filtered at 1.2 kHz and digitized at 100 μs per point.

er the hydrophilic portion of the peptide is sufficient for inactivation, we tested the effects of a peptide with the sequence of amino acids 12 to 20 of ShB (EDRQHRKKQ), with an acyl group on the NH_2-terminus and an amide group on the COOH-terminus to eliminate charged atoms at the ends. Application of this peptide at up to 100 μM has little effect on the inactivation rate of ShBΔ6-46 channels (Fig. 3C). This is consistent with results from the deletion mutation ShBΔ6-9 showing that removal of four of the hydrophobic residues on the NH_2-terminal side of this sequence disrupts inactivation. Similarly, applications of lysine (50 μM) or arginine (50 μM) to the intracellular side did not have any noticeable effects on the macroscopic currents from ShBΔ6-46 channels.

The ability of the synthetic peptide to induce inactivation in mutant ShB channels is exquisitely sensitive to the primary structure of the peptide. A change in only a single residue can disrupt the ability of the peptide to induce inactivation. To test whether the ability of the inactivation-inducing peptide to cause inactivation is also sensitive to the structure of the channel, we applied the peptide to other types of K^+ channels. A *Shaker* homolog from rat brain (RBK1) has extensive homology with the *Shaker* K^+ channel (*6*), but it deviates in the NH_2- and COOH-terminal regions. RBK1 channels expressed in *Xenopus* oocytes inactivate much more slowly than *Shaker* channels, requiring many seconds for inactivation. We have applied the ShB inactivation-inducing peptide to the internal surface of patches expressing RBK1 channels (Fig. 4). That the peptide (50 μM) induces inactivation in these channels as it did in mutant ShB channels suggests that the "inactivation receptor," or region of the channel where the peptide interacts, is conserved among at least some K^+ channels regardless of their native inactivation rate. This conservation may arise because the ball region interacts with a region of the channel important in forming the ion-conducting pore.

We have shown that inactivation can be restored in mutant ShB channels that have their normal inactivation disrupted by a deletion mutation near their NH_2-terminus by applying a synthetic peptide with a sequence derived from the first 20 amino acids of ShB. The peptide-induced inactivation resembles the normal fast inactivation of ShB channels in several respects. In particular, the peptide-induced inactivation is coupled to activation and occurs with a rate that is independent of voltage. Furthermore, alterations that disrupt normal inactivation when made in the NH_2-terminal of ShB also disrupt the peptide-induced inactivation when made in the peptide. Thus, the normal inactivation mechanism is likely to be similar to the mechanism for peptide-induced inactivation and does not require that the NH_2-terminal domain be attached to the rest of the channel protein.

A physical model consistent with these observations is that inactivation occurs when a cytoplasmic domain of the protein occludes the pore. This model makes several predictions that can be tested by mutagenesis and electrophysiological experiments. The channel would be expected to contain a binding site, or receptor, for the NH_2-terminal "ball" domain. The affinity of the mutated receptor could be assayed by examining the rates of normal inactivation and peptide-induced inactivation.

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3. Single-letter abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
4. RNA was transcribed from cDNA and injected into oocytes from *Xenopus laevis* as in (*1*) Recordings were also made as in (*1*). Peptides were synthesized by Multiple Peptide Systems (San Diego, California) with the sequence of the first 20 amino acids of ShB and the mutants ShB-L7E and ShBΔ6-46 (*1*). An amide group was placed at the COOH-terminus to prevent its charge from influencing the peptide activity. A peptide with the sequence of amino acids 12 to 20 of ShB was synthesized by the Protein and Nucleic Acid Facility at Stanford University, with an acyl group on the NH_2-terminal and an amide group on the COOH-terminal to eliminate charged atoms at the ends. The crude peptides were more than 50% pure, as determined by absorbance at 214 nm after reversed-phase high-performance liquid chromatography (HPLC). The peptides were purified with reversed-phase HPLC on a C4 column with a gradient of acetonitrile and 0.05% trifluoroacetic acid and contained only a single peak of 214-nm absorbance after purification. Experiments were performed with both crude and HPLC-purified peptides. The concentration of the crude peptide was estimated by weighing the crude product and assuming 100% purity. To the extent that the peptide was impure, the indicated concentrations are an overestimate. The concentration of the HPLC-purified peptide was estimated by measuring absorbance

at 214 nm and comparing to a standard curve generated from crude peptide. On the basis of macroscopic and single-channel data, the inactivation-inducing activity of the HPLC-purified ShB peptide was approximately twice as great as that of the same concentration of crude ShB peptide. This increased activity probably results from the removal of impurities (nonactive peptides and organic molecules) that absorb at 214 nm.

5. For a mechanism where the peptide binds to the open state, the mean open duration of the channel in the presence of peptide is described as $\tau_{open} = 1/(\beta + [peptide] \times k_{on})$, where k_{on} is the rate at which the peptide binds to the open state of the channel and β is the sum of all the other rates leaving the open state. This equation can be rearranged in the form, $1/\tau_{open(peptide)} = [peptide] \times k_{on} + 1/\tau_{open(control)}$ and $\tau_{open(control)} = 1/\beta$. Therefore, the reciprocal of the mean open duration in the presence of peptide $[\tau_{open(peptide)}]$ should be linearly related to the peptide concentration, if a single peptide bind's to the open state of the channel. The slope of this relationship is k_{on}.
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