A peptide from the *Drosophila* Shaker K⁺ channel inhibits a voltage-gated K⁺ channel in basolateral membranes of *Necturus* enterocytes

*(small intestine/K⁺ channel reconstitution/lipid bilayers)*

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**ABSTRACT** A synthetic peptide composed of the first 22 amino acid residues of the *Drosophila* Shaker K⁺ channel inhibits a voltage-gated K⁺ channel in basolateral membrane vesicles from *Necturus* enterocytes reconstituted in planar phospholipid bilayers when added to the solution bathing the inner surface of this channel but not when added to the solution bathing its outer surface. A modified peptide in which the leucine in the 7 position is replaced with phenylalanine is also an effective inhibitor, but replacement of the leucine-7 with lysine markedly reduces but does not abolish the effectiveness of the peptide as an inhibitor. These results are analogous to those reported for the Shaker K⁺ channel [Hoshi, T., Zagotta, W. N., & Aldrich, R. W. (1990) *Science* 250, 533-538; and Zagotta, W. N., Hoshi, T. & Aldrich, R. W. (1990) *Science* 250, 568-571.] and suggest that the molecular anatomy of the receptor at the inner face of the *Necturus* K⁺ channel with which the peptide interacts to bring about inhibition of that channel may be similar to that of the Shaker K⁺ channel.

The reconstitution of basolateral membranes from *Necturus* enterocytes in planar phospholipid bilayers has disclosed the presence of Ca²⁺-independent, high-("maxi") conductance, voltage-gated K⁺ channels that can be blocked by the addition of Ba²⁺ or the venom of the scorpion *Leiurus quinquestratus* to the solution facing the extracellular surface of this channel. While the relation between single channel current, *I*<sub>s</sub>, and voltage, *V*<sub>m</sub>, is linear over a wide range, the open-time probability of this channel, *P*<sub>o</sub>, is markedly affected by *V*<sub>m</sub>; *P*<sub>o</sub> is close to unity when the solution bathing the intracellular face of the channel is electrically negative with respect to the extracellular solution, but the channel is rapidly deactivated when this electrical polarity is reversed (1).

Hoshi *et al.* (2) and Zagotta *et al.* (3) have presented compelling evidence that the spontaneous and voltage-independent inactivation of K⁺ channels encoded by the Shaker (Sh) gene of *Drosophila melanogaster* can be attributed to the interaction of a positively charged cytoplasmic domain consisting of the first 20 amino acids of the amino terminus of this protein ("inactivation-inducing peptide") with a receptor region at the inner surface of the membrane ("inactivation region"). In addition, Zagotta *et al.* (3) have demonstrated that a synthetic peptide corresponding to this cytoplasmic domain also inactivates a voltage-gated K⁺ channel from rat brain (RBK1) that has extensive homology with the Shaker K⁺ channel except in the amino- and carboxyl-terminal regions.

The present studies were designed to determine whether the "Sh inactivation-inducing peptide" described by Zagotta *et al.* (3) affects the voltage-gated K⁺ channel in basolateral membranes of *Necturus* enterocytes.

**METHODS AND MATERIALS**

The methods for isolating a basolateral membrane fraction highly enriched in Na⁺,K⁺-ATPase activity from mucosal scrapings of *Necturus maculosa* small intestine, the reconstitution of these membrane vesicles in planar phospholipid bilayers, and the collection and analysis of data on single channel activities have been described previously (1). Briefly, planar phospholipid bilayers consisting of phosphatidylethanolamine and phosphatidylserine (1:1) dissolved in decane were formed over a 0.33-mm aperture in a Delrin cup that was inserted into a cut-away polyvinyl chloride block as described by Alvarez (4). The cup formed the cis compartment and the remainder of the block formed the trans compartment. Channels were incorporated into the bilayer by simply extruding a small sample of a suspension of vesicles directly onto the bilayer from the trans compartment by employing a micropipette. As in our previous studies (1), in every instance the orientation of the reconstituted channel was such that its "intracellular mouth" faced the cis compartment and the *Leiurus* venom-sensitive mouth faced the trans compartment. In all experiments, the cis compartment contained *~*150 mM KCl and the trans compartment contained *~*5 mM KCl; the solutions in both compartments were buffered at pH 7.0 with 10 mM Hepes (Tris salt). At the conclusion of every experiment, the two solutions were withdrawn and their K⁺ concentrations were determined by flame photometry; K⁺ activities were calculated by using the activity coefficients reported by Robinson and Stokes (5).

Channel activity was monitored by a List EPC-7 amplifier, visualized with a Nicolet digital oscilloscope, and recorded in digital form on a video tape employing an analog-digital converter (Medical Systems, Greenvalle, NY, model PCM-2). For analysis, the analog signal derived from the digitized data was passed through an eight-pole Bessel filter with the corner frequency (~3 decibels) set at 500 Hz and then digitized at a sampling frequency of 2000 Hz by using a Labmaster DMA interface (Axon Instruments, Burlingame, CA). Data were analyzed by using the p-CLAMP program (Axon Instruments). Open-time probabilities were determined from computer-generated values with the open-closed discriminator halfway between the zero-current and full open-current levels. As is the convention, a positive current represents the flow of a cation from the cis to the trans compartment. The "clamping voltage," *V*<sub>m</sub>, is the electrical potential of the cis compartment with respect to that of the trans ("ground") compartment.

Peptides were synthesized in the Analytical Chemistry Center at the University of Texas Health Science Center at

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Houston. The parent sequence was Met-Ala-Ala-Val-Ala-Gly-Leu-Tyr-Gly-Leu-Gly-Glu-Asp-Arg-Gln-His-Arg-Lys-Lys-Gln-Gln-Gln (ShB peptide). All substitutions were performed at position 7, replacing the leucine with a glutamic acid (ShB-L7E), a lysine (ShB-L7K), a phenylalanine (ShB-L7F), or a glycine (ShB-L7G) residue. The peptides were subsequently purified by reverse-phase high-performance liquid chromatography (HPLC) on a 10 × 100 mm RP-300 Aquapore C8 column (Applied Biosystems). The sample (10 mg/0.5 ml of 0.1% trifluoroacetic acid) was eluted with a 0-70% (vol/vol) acetonitrile gradient in 0.1% trifluoroacetic acid (5 ml/min for 30 min). The structure of the reverse-phase purified peptide was confirmed by fast atom bombardment mass spectroscopy and comparison of the parent mass to the predicted mass for each molecule. All experiments were performed with the HPLC-purified product. Concentrations were estimated on the basis of the weight of the purified peptide.

In six experiments, the peptide was trypsinized by incubation with immobilized trypsin (from bovine pancreas, bound to agarose beads; Sigma) in 10 mM Tris–Hepes at pH 7.0 for 16 hr at room temperature. After the incubation, the trypsin/agarose beads were removed by centrifugation in a table-top centrifuge.

Following the addition of peptides to the cis or trans compartment, the solution in that compartment was vigorously stirred for 1 min; a uniform concentration is achieved well within that period.

Phospholipids were obtained from Avanti Polar Lipids and mixtures were freshly prepared daily.

All experiments were carried out at room temperature (22°C).

Statistical variance is expressed as the SEM and n designates the number of experiments.

RESULTS

The effect of the addition of a synthetic peptide corresponding to the first 22 amino acids of the ShB K+ channel (2) to the cis compartment at 50 μM is illustrated in Fig. 1A; in every experiment (n = 6), channel activity was completely inhibited within 5 min after the addition of this peptide to the cis compartment. It should be noted that in all instances the channel passed through an intermittently active state before complete inhibition of activity. But once the completely inhibited state was achieved it persisted for many minutes and could not be reversed by clamping Vm at negative values as large as −90 mV.

This inhibition was completely prevented by incubation of the peptide with trypsin, which specifically cleaves peptide bonds at lysine and arginine residues.

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**Fig. 1.** Effects of the synthetic ShB peptide (A), ShB-L7F (B) and ShB-L7K (C) on single channel activity after the addition of these peptides to the cis solution at 50 μM. The "holding voltages," Vm, are given below each set of tracings. c designates the closed state and o designates the open state.
the addition of this peptide to the trans compartment. The single-channel conductance, \(g_c\), before and after the addition of this peptide to the trans compartment averaged 183 ± 14 pS and 188 ± 2 pS, respectively, and the reversal potentials, \(V_r\), averaged −78 ± 5 mV and −64 ± 3 mV, respectively (n = 6). The Nernst equilibrium potential for \(K^+\), \(E_K\), calculated from the \(K^+\) activities in the cis and trans solutions at the conclusion of these experiments averaged −73 ± 3 mV. Thus, under control conditions, \(V_m\) did not differ significantly from \(E_K\) and in the presence of the peptide the permeability of the channel for \(K^+\) over that for \(Cl^-\) averaged 16. While we cannot explain the effect of this peptide added to the trans solution on the relation between \(P_o\) and \(V_m\), the important point is that it completely inhibits the channel only when exposed to its intracellular face.

Hoshi et al. (2) and Zagotta et al. (3) have demonstrated that the blocking ability of the inactivation-inducing peptide of the ShB \(K^+\) channel not only is dependent upon the presence of positively charged groups in the 17–19 positions but also requires the presence of hydrophobic residues positioned closer to the amino terminus and, in particular, the leucine-7 residue. To determine whether this is true for the \(Necturus\) \(K^+\) channel, we synthesized peptides in which leucine in position 7 was replaced with phenylalanine (more hydrophobic), glycine (less hydrophobic), lysine (positive), or glutamate (negative).

The peptide with a phenylalanine substitution (ShB-L7F) was a very effective inhibitor (n = 6) (Fig. 1B). On the other hand, the peptides with glutamate (ShB-L7E) or lysine (ShB-L7K) substitution were ineffective at concentrations as high as 100 \(\mu\)M and for periods as long as 15 min (Figs. 1C and 3). In the experiments examining the effects of cis ShB-L7E, \(g_c\) and \(V_m\) averaged 179 ± 15 pS and −76 ± 2 mV, respectively, under control conditions, and 166 ± 24 pS and −70 ± 3 mV, respectively, in the presence of the peptide; the final value of \(E_K\) in these experiments averaged −67 ± 3 mV (n = 6). The relations between \(I_c\) and \(V_m\) were obtained in 4 of 8 experi-

![Fig. 2](image-url)  
**FIG. 2.** Effects of the synthetic ShB peptide added to the trans solution on the relation between \(P_o\) and \(V_m\) (A) and the relation between \(I_c\) and \(V_m\) (B).

On the other hand, as illustrated in Fig. 2A, the addition of this peptide to the trans compartment did not block the channel but instead shifted the threshold for deactivation to more positive values of \(V_m\); i.e., the sensitivity of the gating mechanism to \(V_m\) was decreased. As illustrated in Fig. 2B, the relations between \(I_c\) and \(V_m\) were linear before and after

![Fig. 3](image-url)  
**FIG. 3.** Effects of 50 \(\mu\)M ShB-L7K or ShB-L7E added to the cis solution on the relations between \(P_o\) and \(V_m\) (A and C, respectively) and the relations between \(I_c\) and \(V_m\) (B and D, respectively).
ments in which we examined the effect of cis ShB-L7K. In these experiments, \( g_c \) and \( V_m \) averaged 191 ± 17 pS and −80 ± 1 mV, respectively, under control conditions, and 157 ± 17 pS and −79 ± 1 mV, respectively, in the presence of the peptide; \( E_K \) averaged −75 ± 2 mV. Thus, neither ShB-L7E nor ShB-L7K significantly affects the high selectivity of this channel for K⁺ over Cl⁻ after addition to the cis solution. In addition, ShB-L7E does not affect the single-channel conductance. The “flickering” observed in the presence of cis ShB-L7K (Fig. 1C) together with the decrease in \( g_c \) (Fig. 3B) are consistent with the notion that this positively charged molecule behaves as a “fast” blocker of the channel mouth (6, 7); flickering was not observed after the addition of ShB-7LE to the cis solution.

Finally, replacement of leucine-7 with glycine (ShB-L7G) markedly reduced but did not abolish the ability of the peptide to inhibit channel activity when added to the cis solution. As illustrated in Fig. 4, increasing concentrations of this peptide at constant \( V_m \) brought about concomitant decreases in \( P_o \), mean open time (\( \tau_o \)), and single-channel current amplitude (\( n = 6 \)).

The finding that the lysine-containing peptide was ineffective excludes the possibility that the effect of the unmodified peptide is simply due to its net positive charge. And the finding that simply replacing leucine-7 with glycine markedly reduces inhibiting ability indicates that a strong interaction of the peptide with its receptor requires the presence of a hydrophobic side chain in the 7 position.

**DISCUSSION**

Hoshi et al. (2) have demonstrated that site-directed mutagenic deletions affecting a cytoplasmic domain consisting of the first 22 amino acids of the amino terminus of the *Drosophila* ShB K⁺ channel disrupt inactivation of these channels expressed in *Xenopus* oocytes. These studies indicated that normal inactivation is dependent upon the presence of charged arginine and lysine residues in the 17–19 positions and a hydrophobic residue in the 7 position. Zagotta et al. (3) demonstrated that intracellular injection of a synthetic peptide consisting of the first 20 amino acids of the ShB K⁺ channel restored inactivation in a noninactivating mutant channel that lacked residues 6–46, expressed in *Xenopus* oocytes. Treatment with trypsin, which cleaves the peptide at the arginine and lysine residues, or replacement of the leucine in position 7 with glutamate abolished its ability to restore inactivation.

These and other findings by Hoshi et al. (2) are consistent with the “ball and chain” model for Na⁺ channel inactivation proposed by Armstrong and Benzanilla (8, 9), in which the 22 amino acid cytoplasmic domain (the “ball”) is tethered to the channel protein and brings about inactivation when it interacts with a receptor on the inner surface of the channel; as pointed out by Hoshi et al. (2) and Zagotta et al. (3), a most attractive candidate for the “inactivation receptor” is the inner mouth of the channel, inasmuch as inactivation is observed only when the channel is in the open configuration. Additional evidence that the peptide occludes the mouth of the channel has been recently presented by Choi et al. (10).

As noted above, Zagotta et al. (3) found that the ShB K⁺ channel inactivation-inducing peptide also blocked inactivation of a voltage-gated channel from rat brain. These channels are functionally similar inasmuch as they are activated by depolarization of the membrane and then spontaneously inactivate; the main difference is that the ShB K⁺ channel inactivates more rapidly than the RBK1 channel. This finding suggested that “the ‘inactivation receptor’ or region of the channel with which the peptide interacts is conserved among at least some K⁺ channels regardless of their native inactivation rate” (3). More recently, Isacoff et al. (11) demonstrated that this peptide also blocks a delayed-rectifier K⁺ channel cloned from rat brain and expressed in *Xenopus* oocytes.

The K⁺ channel present in the basolateral membranes of *Necturus* enterocytes is functionally very different from the above K⁺ channels; it is active when the membrane is at rest or is hyperpolarized but deactivates when the electrical polarity across the basolateral membrane is reversed (Fig. 2A) (1). Nonetheless, it can be completely, albeit not abruptly, inhibited by the ShB inactivation-inducing peptide.

**FIG. 4.** Effects of ShB-L7G added to the cis solution on the activity of a single channel. The tracings shown were obtained 10 min after increasing the concentration of the peptide to the value given at the left. The total height of the bars at the right of each tracing represents the single-channel current under control conditions and the shaded portion indicates the current amplitude in the presence of the peptide. c indicates the closed configuration and \( \tau_o \) designates the mean open time.
Further, structural modifications of this peptide that impair its ability to inactivate the ShB $K^+$ channel also impair its ability to deactivate the Necturus $K^+$ channel. Thus, cleaving the positively charged residues with trypsin or replacement of the leucine in the 7 position with hydrophilic residues renders the peptide ineffective, whereas replacement of the leucine in the 7 position with a highly hydrophobic residue does not impair its ability to inhibit the channel. These findings suggest that the molecular anatomy of the region of the Necturus $K^+$ channel with which this peptide interacts to bring about deactivation is similar to that of the ShB $K^+$ channel and that this structure may be more widely conserved than originally suspected.

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