Characterization of a Mammalian cDNA for an Inactivating Voltage-Sensitive K⁺ Channel

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Summary

A cDNA clone encoding a K⁺ channel polypeptide with 72% amino acid sequence identity to Drosophila Shal was isolated from rat hippocampus. Functional expression of the cDNA in Xenopus oocytes generated 4-aminopyridine-sensitive K⁺ channels displaying rapid inactivation kinetics. The fastest component of inactivation was slowed by the deletion of 3 basic residues in the amino-terminal region. Northern blots revealed that the mRNA encoding this K⁺ channel polypeptide was expressed at a similar level in the brain and in the heart. In situ hybridization revealed that the mRNA encoding this K⁺ channel appeared concentrated in the hippocampus, dentate gyrus, and habenular nucleus in the brain. Thus, this K⁺ channel polypeptide is likely to form some of the A-type K⁺ channels expressed in the mammalian nervous system and heart.

Introduction

In the nervous system voltage-sensitive K⁺ channels play an essential role in sensory transduction, signal propagation, and processing (Hille, 1984; Jan and Jan, 1989). In particular, the voltage-sensitive and rapidly inactivating A-type K⁺ channel (Hagiwara and Saito, 1959; Connor and Stevens, 1971; Neher, 1971; Rogawski, 1985) has been proposed to be involved in controlling action potential frequency (Connor, 1978; Segal et al., 1984) and waveform (Kaczmarek and Strumwasser, 1984), the establishment of a delay in the initiation of burst of action potentials (Byrne, 1980; Cottrell, 1983), and the postsynaptic modulation of synaptic potential amplitude (Daut, 1973; Cassell et al., 1986). In addition, the activities of these K⁺ channels may be modulated by different neurotransmitter actions (Levitan, 1985; Aghajanian, 1985; Nakajima et al., 1986; Atkins et al., 1990). Thus, this subset of K⁺ channels contributes to the control of timing, duration, frequency, and number of action potentials, which is important for the coding and processing of information in the nervous system. Furthermore, the biological role of these K⁺ channels in controlling excitability and synaptic efficacy has been implicated in learning and memory (Nelson et al., 1990).

The A-type K⁺ channels in the nervous system exhibit characteristics such as rapid activation in response to membrane depolarization followed by a relatively rapid phase of inactivation. The time course of the process of inactivation can vary greatly depending on the A-current being examined, but inactivation is usually complete within hundreds of milliseconds. In addition, these currents are blocked in a dose-dependent manner by 4-aminopyridine (4-AP). A-type K⁺ currents that are sensitive to 4-AP have also been detected in cardiac cells, including Purkinje fibers (Coraboeuf and Carmeliet, 1982; Kenyon and Sutko, 1987), the crista terminalis (Giles and Van Ginneken, 1985), quiescent atrioventricular node cells (Nakayama and Irisawa, 1985), ventricular myocytes (Josephson et al., 1984; Tseng and Hoffman, 1989), and human atrial cells (Escande et al., 1987). They may moderate action potential waveform and duration, prevent arrhythmogenic slow-response action potentials, and help maintain a high resting membrane potential in latent pacemaker cells. In Purkinje cells, this transient outward current is modulated by norepinephrine, a neurotransmitter involved in the neural control of heartbeat (Nakayama and Hozzard, 1988). Indeed, some of the antiarrhythmic drugs are blockers of K⁺ channels (Bacaner et al., 1986; Imai and Giles, 1987; Cook, 1988). However, the proteins constituting components of the A-type K⁺ channels in the mammalian nervous system and heart remain largely uncharacterized.

The Shaker locus of Drosophila melanogaster encodes an A-type K⁺ channel (Jan et al., 1977; Salkoff and Wyman, 1981; Ianoisy et al., 1981; Wu and Haughland, 1983; Timpe and Jan, 1987; Papazian et al., 1987; Tcmpe et al., 1987; Iverson et al., 1988; Timpe et al., 1988a, 1988b). At least five different proteins are derived from the Shaker gene by alternative splicing (Schwarz et al., 1988; Pongs et al., 1988; Kamb et al., 1988). The gene products share a common core sequence but differ at their amino- and carboxyl-terminal regions, and they produce channels with different inactivation kinetics in Xenopus oocytes (Timpe et al., 1988a, 1988b; Iverson et al., 1988). The “core” sequence is composed of the S4 sequence, five potential transmembrane sequences, and the highly conserved, partly hydrophobic H5 sequence. From this arrangement and the absence of defined signal peptide sequences it is proposed that the amino- and carboxyl-termini of these polypeptides are located intracellularly. Recent studies have shown that the amino-terminal region is involved in inactivation, perhaps as part of the cytoplasmic inactivation gate (Hoshi et al., 1990; Zagotta et al., 1990).

Voltage-sensitive K⁺ channels with extensive sequence similarity to Shaker have been isolated from mammalian brain (Tempel et al., 1988; Baumann et al., 1988; Stühmer et al., 1989; Grupe et al., 1990; Swanson et al., 1990). They show extensive amino acid sequence identity with Shaker K⁺ channels over the putative membrane-spanning core region, but not at the amino or carboxyl termini. Although these proteins
may constitute a component of the A-type channel if they coassemble with an inactivating subunit (Christie et al., 1990; Isacoff et al., 1990; Ruppersberg et al., 1990), most of these mammalian homologs, when expressed individually in Xenopus oocytes, produce noninactivating or very slowly inactivating currents. The exception to this generalization is the mammalian Shaker K' channel homolog RCK4, which elicits an inactivating A-type current in Xenopus oocytes (Stühmer et al., 1989). This K' channel is encoded by a 4.4 kb mRNA expressed in rat brain (Beckh and Pongs, 1990). Since the major A-type K' current has been detected in fractions of rat brain mRNA of larger size when injected into Xenopus oocytes (Rudy et al., 1988), most likely there are other mammalian genes in addition to RCK4 that also encode A-type K' channels.

In Drosophila, there is genetic and biophysical evidence that genes other than Shaker encode other A-type K' channels (Sole et al., 1987; Baker and Salkoff, 1990). One candidate for an A-type K' channel has been cloned based on its sequence similarity to Shaker cDNAs (Butler et al., 1989). Expression of this cDNA, Shal, in Xenopus oocytes gives rise to relatively rapidly inactivating K' currents (Wei et al., 1990).

Here we describe the cloning of a rapidly inactivating voltage-sensitive K' channel cDNA from rat hippocampus. The deduced protein sequence shows extensive amino acid identity to Drosophila Shal over the amino terminus as well as the putative membrane-spanning region. Because of this strong similarity, this K' channel will be referred to as rat Shall. Rat Shall is encoded by a 6 kb mRNA present in different regions of the rat brain and in the heart. When expressed in Xenopus oocytes, it gives rise to 4-AP-sensitive channels that resemble A-type K' channels. Deletion of 3 basic residues in the amino-terminal region significantly slowed the rate of fast inactivation. Thus, like Shaker K' channels (Timpe et al., 1988b; Hoshi et al., 1990; Isacoff et al., 1990), the amino-terminal region of rat Shall appears to be involved in inactivation.

Results

Novel K' Channel Sequences from Rat Hippocampus Isolated by Polymerase Chain Reaction Using Degenerate Oligonucleotide Primers

To identify novel voltage-sensitive K' channels expressed in mammalian brain, we screened rat hippocampal mRNA by the polymerase chain reaction (PCR) (Girgis et al., 1988; Lee et al., 1988) using K' channel-specific degenerate oligonucleotide primers. The primers corresponded to amino acid sequences that are highly conserved in all known K' channel sequences (amino acids 440-446 and 473-479 of Shaker K' channels). In this screen we identified several sequences consistent with K' channels that had not been identified previously and a sequence identical to RCK4 (Stühmer et al., 1989). One PCR-derived sequence was noted as having 88% amino acid identity, excluding primer sequences, to the Drosophila Shal K' channel (Wei et al., 1990) and only 58%-65% identity with other known K' channel sequences (Stühmer et al., 1989; Frech et al., 1989; McCormack et al., 1990; Swanson et al., 1990). This PCR fragment was used to isolate cDNA clones from a rat hippocampus cDNA library. The sequence of the PCR fragment differed from that of the cDNA by 1 amino acid; whereas the 5' primer sequence included a codon for Val, the corresponding codon in the cDNA was for Leu (amino acid 371) (Figure 1).

Isolation of Rat Shall cDNAs

We isolated 64 cDNA clones from 300,000 phage of a rat hippocampus cDNA library using the Shall-like PCR fragment described above. Analysis by restriction mapping suggested that the majority of these cDNAs were identical. We chose the cDNA clone with the longest 5' untranslated sequence for sequence analysis and expression in Xenopus oocytes. This cDNA was composed of 3350 bp, of which 1890 bp contained a single open reading frame, with 551 bp of 5' untranslated and 909 bp of 3' untranslated sequence (Figure 1). The deduced protein sequence contains 630 amino acids, and the predicted molecular mass of the primary translation product is 71 kd. Analysis of the amino acid sequence predicted six hydrophobic segments, of which five have been modeled to be membrane spanning. Furthermore, the sequence contained an S4 sequence characteristic of voltage-gated ion channels. The proposed S4 sequence of rat Shall had 6 basic residues.

The deduced amino acid sequence of rat Shall revealed a number of potentially biologically significant sites and signatures for posttranslational modification (Figure 1). These included a potential cAMP- and cGMP-dependent protein kinase phosphorylation site in the amino-terminal region (Thr-38) and a potential protein kinase C site in the sequence between S4 and H4 that is highly conserved in K' channels (Thr-316). The functional expression in Xenopus oocytes of two Shaker K' channel proteins joined in tandem (Isacoff et al., 1990) supports the notion that both amino and carboxyl termini are located intracellularly. Because the glycosylation consensus sequences are located in the amino- and carboxyl-termini at Asn-46, Asn-408, and Asn-536, it appears that rat Shall channels lack any N-linked glycosylation consensus sequences that would be exposed to the extracellular side of the membrane. The other known K' channels that do not have such consensus sequences in the presumed extracellular sequences are RCK2 (Grupe et al., 1990), dK1 (Frech et al., 1989), and Drosophila Shal (Wei et al., 1990), although they also contain N-glycosylation consensus sequences in the amino- and carboxyl-termini.

The amino acid sequences predicted from rat Shall and Drosophila Shal (Wei et al., 1990) exhibit 72% identity. In addition, 8.6% of the nonidentical residues in rat Shall are similar to the corresponding amino acids...
Rat Brain cDNA for a Voltage-Gated K⁺ Channel

Figure 1. Nucleotide Sequence and Deduced Amino Acid Sequence of Rat Shal

The deduced amino acid sequence is displayed beneath the nucleotide sequence of the coding strand of the rat Shal cDNA. The amino acids are numbered as groups of ten starting at the Met of the predicted amino terminus. The nucleotides are numbered at the side of the sequence. Positive numbers are assigned to nucleotides 3' to the A of the initiation codon, which is assigned as +1. Nucleotides 5' to this codon are assigned negative numbers. The periods at the ends of the sequence indicate that the cDNA is incomplete. Although there is a stretch of A residues at the 3' end of the clone it is unlikely to represent a poly(A) tract, since there is no signal for adenylation upstream of these residues. There is one potential CAMP- and CGMP-dependent protein kinase phosphorylation site at amino acid T-36. There are nine potential protein kinase C phosphorylation sites at S-70, T-101, T-166, T-291, T-316, S-447, S-531, S-537, and S-548, and there are twelve potential casein kinase II phosphorylation sites at T-54, S-70, S-113, S-263, T-280, S-459, S-460, S-472, T-489, S-502, S-552, and T-606 and one tyrosine kinase phosphorylation site at Y-592 (Aitken, 1990).

The amino acids of the rat Shal and Drosophila (fly) Shal were aligned as described by Myers and Miller (1988). A colon indicates amino acid identity; a period indicates amino acid similarity where amino acids within each of the following groups are classified as similar: A, S, and T; D and E; N and Q; R and K; I, L, M, and V; F, Y, and W. Dashes indicate gaps introduced into the sequence to improve alignment. The proposed transmembrane regions H1, H2, H3, H4, and H6, the S4 sequence, and the hydrophobic sequence H5 are identified as boxed sequences. They were deduced from comparing models predicted by the algorithms of Eisenberg et al. (1984), Rao and Argos (1986), Klein et al. (1985), and Kyte and Doolittle (1982) and by alignment to sequences as described by Jan and Jan (1990).

in Drosophila Shal (Figure 2). The regions of strong sequence similarity are unevenly distributed in the protein and include the amino terminus. In contrast, sequence similarity between members of the Shaker/RCK family of K⁺ channels does not extend to the termini of the protein. The most conserved regions between rat and Drosophila Shal are the proposed membrane-spanning regions and H5, each of which demonstrates between 86%-100% amino acid identity, much higher than that between rat Shal and the Shaker/RCK family of K⁺ channels (73% identity over 54, 74% identity over H6, and only 5%~68% identity over the other hydrophobic sequences) (Table 1). However, this sequence analysis also suggests that the Shal family of K⁺ channels is more closely related to the Shaker/RCK family than the Shab/drkl or Shaw family of K⁺ channels.

Rat Shal Is Encoded by a 6 kb mRNA Expressed in All Major Regions of the Rat Brain and Heart

Figure 2. Alignment of the Amino Acid Sequences of Rat and Drosophila (Fly) Shal

The amino acids of the rat Shal and Drosophila (fly) Shal were aligned as described by Myers and Miller (1988). A colon indicates amino acid identity; a period indicates amino acid similarity where amino acids within each of the following groups are classified as similar: A, S, and T; D and E; N and Q; R and K; I, L, M, and V; F, Y, and W. Dashes indicate gaps introduced into the sequence to improve alignment. The proposed transmembrane regions H1, H2, H3, H4, and H6, the S4 sequence, and the hydrophobic sequence H5 are identified as boxed sequences. They were deduced from comparing models predicted by the algorithms of Eisenberg et al. (1984), Rao and Argos (1986), Klein et al. (1985), and Kyte and Doolittle (1982) and by alignment to sequences as described by Jan and Jan (1990),
and the heart, a 6 kb mRNA band hybridized to the full-length rat Shal cDNA probe (Figure 3A). In addition, a number of generally weaker bands of 1–3 kb hybridized with the rat Shal cDNA probe. Hybridization of the same filter with a-tubulin sequences revealed no extensive degradation of the RNA during isolation (Figure 3B). Furthermore, the 6 kb band and the 1–3 kb smear were also recognized by a probe hybridized with the rat Shall cDNA probe. Hybridization, a number of generally weaker bands of 1–3 kb resulted in the expression of voltage-gated, rapidly inactivating K⁺ channels. Activation of these channels was evident at membrane potentials above -40 mV (Figures 4A and 4B). The reversal potential of tail currents is a function of external K⁺ concentration, which approximates the Nernst equation expected for a channel selective for K⁺ (Hille, 1984); the reversal potential shifted by 52 mV per 10-fold change in extracellular K⁺ concentration (Figures 4E and 4F). This K⁺ current showed moderately rapid inactivation: if the membrane potential was raised from a holding potential of -100 mV to +60 mV or more depolarized potentials, the current inactivated fairly rapidly and completely (the major time constant was 155 ms at 11°C) (Figure 4C; Table 2). It took a few seconds at -100 mV (time constant for recovery was approximately 1 s) for the channels to recover from inactivation (Figure 4D). Holding the membrane potential at levels more depolarized than -100 mV led to various degrees of inactivation; all of the channels were inactivated by holding the membrane potential at or above 0 mV (Figure 4B). The midpoints for activation and inactivation were -4.0 mV and -48 mV, respectively (Figures 4A and 4B). These kinetic and voltage-dependent properties were similar to the properties of A-channels recorded from neurons (Rogawski, 1985) or from Xenopus oocytes injected with rat brain mRNA (Rudy et al., 1988). This similarity also extends to the pharmacological properties; the rat Shal K⁺ channels were sensitive to 4-AP (46% ± 8% block; mean ± standard deviation; N = 9, at 5 mM 4-AP) (Figure 4C). Note, however, that 4-AP was more effective in blocking the component with the fastest rate of inactivation, even though a single type of cDNA was responsible for generating the K⁺ current with

### Table 1. Extents of Amino Acid Identity over Proposed Transmembrane Regions and H5 of Different K⁺ Channels

<table>
<thead>
<tr>
<th>K⁺ Channels</th>
<th>H1</th>
<th>H2</th>
<th>H3</th>
<th>S4</th>
<th>H4</th>
<th>H5</th>
<th>H6</th>
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<tr>
<td>Rat Shal/RKShl11A</td>
<td>5</td>
<td>31</td>
<td>36</td>
<td>36</td>
<td>57</td>
<td>36</td>
<td>60</td>
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<tr>
<td>Rat Shal-Shaw</td>
<td>0</td>
<td>36</td>
<td>36</td>
<td>47</td>
<td>45</td>
<td>60</td>
<td>70</td>
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<tr>
<td>Rat Shal-NCK2</td>
<td>10</td>
<td>41</td>
<td>40</td>
<td>57</td>
<td>31</td>
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<td>60</td>
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<td>Rat Shal-RCK4</td>
<td>10</td>
<td>40</td>
<td>36</td>
<td>73</td>
<td>36</td>
<td>64</td>
<td>74</td>
</tr>
<tr>
<td>Rat Shal-RCK3</td>
<td>10</td>
<td>40</td>
<td>45</td>
<td>73</td>
<td>36</td>
<td>64</td>
<td>74</td>
</tr>
<tr>
<td>Rat Shal-BKURCK5</td>
<td>5</td>
<td>40</td>
<td>50</td>
<td>73</td>
<td>36</td>
<td>68</td>
<td>74</td>
</tr>
<tr>
<td>Rat Shal-MBK1/RCK1</td>
<td>10</td>
<td>36</td>
<td>45</td>
<td>73</td>
<td>36</td>
<td>64</td>
<td>74</td>
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<tr>
<td>Rat Shal/Shaker</td>
<td>15</td>
<td>40</td>
<td>45</td>
<td>73</td>
<td>36</td>
<td>60</td>
<td>74</td>
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<tr>
<td>Rat Shal/Shal-fly Shal</td>
<td>94</td>
<td>95</td>
<td>96</td>
<td>100</td>
<td>90</td>
<td>95</td>
<td>96</td>
</tr>
</tbody>
</table>

The proposed transmembrane regions of rat Shal were aligned to those of Shaker B sequences (Schwarz et al., 1988), Drosophila Shal (Wei et al., 1990), MBK1 (Tempel et al., 1988), RCK1-5 (Baumann et al., 1988; Stühmer et al., 1989), Shal, Shaw (Butler et al., 1989), dkl1 (Frech et al., 1989), Kvl1 (Swanson et al., 1990), NCK2 (Yokoyama et al., 1990), and RKShl11A (McCormack et al., 1990) as described by Jan and Jan (1990). Alignment of S4 sequences was made from the first Arg of the S4 of rat Shal and the second Arg of the S4 sequences of other K⁺ channels.

The "families" of K⁺ channels are identified by underlining, and the numbers associated with them represent amino acid identities between all members of the family over the conserved hydrophobic regions.
three time constants of inactivation. Compared with other K+ channel cDNAs that give rise to inactivating K+ currents when expressed in Xenopus oocytes, the fastest component of inactivation for rat Shal is approximately 3 times faster than for Drosophila Shal (480 ms at 11°C; Wei et al., 1990), but considerably slower than those reported for Shaker K+ channels (Timpe et al., 1988b; Iverson et al., 1988; Isacoff et al., 1990) and comparable with the fastest component of inactivation for RCK4 and a RCK4-like clone, RHK1, from heart (65 ms at 20°C-23°C; Stühmer et al., 1989; W. Stühmer, personal communication of recording temperature for RCK4; Tseng-Crank et al., 1990).

Single-K+ channel activities were detected in patch-clamp recordings of Xenopus oocytes expressing rat Shal (Figure 5). The amplitude of the single-channel current was approximately half that seen with Shaker B (Isacoff et al., 1990). Furthermore, the outward and inward currents evoked during depolarizing and hyperpolarizing phases of the voltage step were approximately equal in amplitude. Thus, unlike Shaker B (MacKinnon and Yellen, 1989) rat Shal does not demonstrate obvious open-channel inward rectification.

The single-channel records and the ensemble averages of the records (obtained at 22°C ± 2°C) confirm the observation made from the whole-cell clamp recordings (obtained at 11°C) that rat Shal encodes an inactivating A-type K+ channel, except that the inactivation rates are much greater at higher temperatures.

Because rat Shal encodes a K+ channel with mod-
Figure 4. Expression of Transient Outward Currents in Xenopus Oocytes Injected with Rat Shal mRNA

(A) Transient outward currents recorded in oocytes at 11°C. The traces show the membrane currents evoked by depolarizing voltage steps from -100 mV to levels between -80 and +60 mV in 20 mV increments and applied every 30 s. Records were leak subtracted assuming a linear leak current using a 20 mV hyperpolarizing test pulse (p/4 method; Bezanilla and Armstrong, 1977). The capacitive current is not shown.

(B) Conductance-voltage relations (closed circles) and steady-state inactivation properties (closed squares) of rat Shal. To determine the conductance, the amplitude of the leak-subtracted peak current was divided by the driving force across the membrane using a reversal potential of -100 mV obtained by extrapolation from measured reversal potential at 20, 40, and 89 mM external K+. The conductance values were normalized to the maximal conductance and plotted against the membrane potential. The symbols represent data from a single representative
erately rapid inactivation, we examined its amino-terminal region to determine whether there were sequences that might correspond to a region in the amino-terminal region of the Shaker K⁺ channel that is necessary for fast inactivation of those channels (Zagotta et al., 1990; Hoshi et al., 1990; Isacoff et al., 1990). In both the rat and Drosophila Shal K⁺ channel the amino terminus is hydrophobic and is followed by a cluster of positively charged amino acid residues (35-37 of rat Shal; 37-39 of Drosophila Shal). The latter is analogous to amino acids 17-19 of Shaker B, which also follow a hydrophobic amino terminus (Schwarz et al., 1988) and are involved in fast inactivation (Isacoff et al., 1990; Hoshi et al., 1990). Similar to what has been found for the Shaker K⁺ channel, the error bars indicate the standard deviation. The slope of the fit is 52 mV per 10 fold change in extracellular K⁺, which approximates the theoretical Nernst relationship for a K⁺-selective channel.

Discussion

By use of degenerate oligonucleotide primers that code for highly conserved amino acid sequences in voltage-dependent K⁺ channels we have identified a mammalian K⁺ channel gene that gives rise to rapidly inactivating voltage-sensitive K⁺ channels when expressed in Xenopus oocytes. This rat K⁺ channel has strong amino acid sequence identity to the Drosophila K⁺ channel named Shal and shows similar electrophysiological properties when expressed in Xenopus oocytes. Thus this channel has been named rat Shal. Northern analysis revealed that the mRNA encoding rat Shal is expressed at a similar level in the rat brain and is also expressed at a similar abundance in rat heart. Higher resolution of the distribution of transcripts is achieved by in situ hybridization, which reveals a concentration of the rat Shal mRNA in the hippocampus, dentate gyrus, and habenular nucleus of the brain. In the hippocampus rat Shal mRNA appears expressed in a gradient where CA1 demonstrates higher levels of expression than CA3. The distribution pattern throughout the brain and in the hippocampus observed with rat Shal is distinct from those observed with other K⁺ channel probes (M.-L. T., unpublished data). In contrast, in the heart, rat Shal RNA appears evenly distributed throughout the cardiac tissue. In addition to rat Shal, two K⁺ channel genes of the Shaker/RCK family are expressed in the heart as well as the brain (Swanson et al., 1990; Tseng-Crank et al., 1990).

Previous studies of rapidly inactivating K⁺ channels suggest that inactivation usually follows activation and is relatively insensitive to voltage (Zagotta et al., 1989; Zagotta and Aldrich, 1990). Moreover, cytoplasmic application of proteases can remove the fast component of inactivation, suggesting that inactivation involves a structural element on the cytoplasmic side of the membrane (Matteson and Carmeliet, 1988; Hoshi et al., 1990). Studies of splice variants of Shaker
Table 2. Macroscopic Inactivation Kinetics of Rat Shall and Mutants

<table>
<thead>
<tr>
<th></th>
<th>( \tau_1 ) (ms)</th>
<th>( \Lambda_1 )</th>
<th>( \tau_2 ) (ms)</th>
<th>( \Lambda_2 )</th>
<th>( \tau_3 ) (ms)</th>
<th>( \Lambda_3 )</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shall</td>
<td>155 ± 8</td>
<td>0.41 ± 0.03</td>
<td>688 ± 33</td>
<td>0.27 ± 0.01</td>
<td>5550 ± 409</td>
<td>0.32 ± 0.02</td>
<td>9</td>
</tr>
<tr>
<td>Shall A35-37</td>
<td>223 ± 11</td>
<td>0.19 ± 0.02</td>
<td>1144 ± 55</td>
<td>0.24 ± 0.01</td>
<td>4139 ± 177</td>
<td>0.57 ± 0.03</td>
<td>5</td>
</tr>
<tr>
<td>R35Q</td>
<td>153 ± 16</td>
<td>0.49 ± 0.05</td>
<td>796 ± 79</td>
<td>0.19 ± 0.04</td>
<td>5832 ± 840</td>
<td>0.32 ± 0.03</td>
<td>4</td>
</tr>
<tr>
<td>K36Q</td>
<td>149 ± 16</td>
<td>0.47 ± 0.06</td>
<td>717 ± 82</td>
<td>0.22 ± 0.04</td>
<td>5837 ± 715</td>
<td>0.32 ± 0.04</td>
<td>6</td>
</tr>
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</table>

Values were measured from experimental fits to the onset of inactivation of currents recorded from oocytes injected with wild-type or mutant mRNA. Currents were evoked by a 10 s depolarizing voltage pulse from a holding potential of -100 mV to + 60 mV. Exponential fits were done using pCLAMP's least squares minimization procedure. The minimum number of exponential required for an adequate fit of the currents was 3. The time constants \( \tau_1, \tau_2, \) and \( \tau_3 \) (in ms) and amplitudes \( \Lambda_1, \Lambda_2, \) and \( \Lambda_3 \) represent the relative contribution of each component to the peak, so that the current as a function of time \( t \) corresponds to the equation \( \Lambda_1 \exp(-t/\tau_1) + \Lambda_2 \exp(-t/\tau_2) + \Lambda_3 \exp(-t/\tau_3) \). The means, standard deviation, and number of individual oocytes tested are given. Recording temperature was 11°C ± 1°C.

K⁺ channels suggest that both the amino and carboxyl termini are likely to be involved in governing inactivation kinetics (Timpe et al., 1988a, 1988b). In particular, a group of basic residues that follow the hydrophobic amino terminus appears to play an important role in inactivation of the Shaker K⁺ channel (Hoshi et al., 1990). We have obtained preliminary evidence suggesting that a group of basic residues at an analogous location in rat Shall is also necessary for rapid inactivation of this mammalian K⁺ channel. Given that only moderate levels of sequence identity are found between Shaker and rat Shall, the involvement of basic residues near the amino terminus in inactivation may turn out to be a general phenomenon. Further support for this notion is provided by the fact that the rat K⁺ channel RCK4, which appears to inactivate over a similar time course to rat Shall, also has multiple basic residues in the amino-terminal region (Stühmer et al., 1989; Tseng-Crank et al., 1990).

Rudy et al. (1988) showed that injection of rat brain mRNA into oocytes gives rise to fast transient K⁺ channels (A-channels). Fractionation of brain RNA separated mRNAs of 5-7 kb encoding A-channels from mRNAs that produce other voltage-dependent K⁺ channels. However, A-channels induced by the fractionated mRNA have different pharmacological and kinetic properties than unfractionated brain mRNA. The kinetic properties of A-channels induced by un-

Figure 5. Patch-Clamp Recordings of Rat Shall Channels in Xenopus Oocytes

Inside-out patch recordings were done in symmetric K⁺ concentration solutions. The internal (bath) solution contained 98 mM KCl, 0.5 mM MgCl₂, 1 mM EGTA, and 10 mM HEPES-KOH (pH 7.1), and the external (pipette) solution contained 98 mM KCl, 1.5 mM MgCl₂, 0.3 mM CaCl₂, and 10 mM HEPES-KOH (pH 7.1) (22°C ± 2°C). Records were leak subtracted using averages of 5-10 pulses, which evoked no channel openings. Single-channel traces, which show openings during the depolarizing (outward currents) and hyperpolarizing phases (inward currents) of the voltage step, were selected to show both outward and inward currents, though reopenings at -100 mV were seen infrequently. The lower trace shows an ensemble average of 50 traces, which reveals inactivation at the macroscopic level. Steps were made at 4 s intervals to indicated potentials from a holding potential of -100 mV. Data were sampled at 5 kHz and low pass-filtered at 1 kHz.

Figure 6. Inactivation Is Reduced in the Mutant A35-37 Shall

Normalized currents from wild type and the mutant in which amino acids 35-37 were deleted from rat Shall (A35-37 Shall) elicited by a 400 ms (A) or 2000 ms (B) depolarizing voltage pulse to +60 mV from a holding potential of -100 mV. Both of the fast time constants \( \tau_1 \) and \( \tau_2 \) were decreased by the deletion mutation (Table 2). It also took longer to reach the peak current amplitude in the deletion mutant. Whether this was a consequence of the mutant effects on inactivation, or a separate effect on channel activation, remains to be determined by single-channel analysis. Currents were leak subtracted assuming a linear leak current using a 20 mV hyperpolarizing test pulse (p4 method; Bezanilla and Armstrong, 1977). The capacitive current is not shown. Recordings were done at 11°C ± 1°C.
fractionated mRNA could be reconstituted by injecting the 5–7 kb fraction together with a subfraction of mRNA in the range of 2–4 kb, which on its own did not produce an A-current. Both kinetic and pharmacological properties of the rat Shal K⁺ channel are similar to those of the A-current induced by the 5–7 kb fraction of rat brain mRNA. Furthermore, since rat Shal hybridizes to a 6 kb mRNA it may correspond to a major mRNA species encoding rapidly inactivating K⁺ channels in the rat brain. It is important to note that the ability of K⁺ channels to assemble as heteromultimers with different properties (Christie et al., 1990; Issacoff et al., 1990; Ruppersberg et al., 1990) provides an additional level of diversity that may alter the kinetic and pharmacological properties of K⁺ channels formed by rat Shal. The preferential block by 4-AP of the component of rat Shal-induced currents in Xenopus oocytes that has the fastest rate of inactivation (Figure 4G), on the other hand, may serve as a cautionary note for the use of pharmacological criteria alone in the dissection of different K⁺ currents recorded from a cell.

Phosphorylation of channels may regulate channel function (Levitan, 1985). For example, Strong (1984) and Kaczmarek and Strumwasser (1984) reported that the inactivation kinetics of a transient outward K⁺ current (A-current) in Aplysia bag cell neurons could be modified in a cAMP-dependent manner. Similarly, the modulation by norepinephrine of inactivation kinetics of the transient outward current of Purkinje cells of the canine heart can be mimicked by increasing intracellular cAMP concentration (Nakayama and Fozzard, 1988). It is intriguing that a potential cAMP- and cGMP-dependent protein kinase phosphorylation site (Thr-38) is next to the 3 basic residues (35–37), which when deleted led to a slowing of fast inactivation. It remains to be determined whether Thr-38 can be phosphorylated and, if so, whether the introduction of negative charges via a phosphate group next to these positively charged residues modulates inactivation of this mammalian K⁺ channel. In any case, our observation that fast inactivation of rat Shal was slowed by deletion of the 3 basic residues, but not neutralization of residue 35 or 36, indicates that the effect of the deletion mutation on fast inactivation rate was due to the removal of multiple basic residues rather than the removal of the consensus sequence for phosphorylation. This is because all three mutations should have rendered Thr-38 no longer a potential site for cAMP- and cGMP-dependent protein kinases.

During the preparation of this manuscript we became aware of reports in press describing another rat and a mouse homolog of Drosophila Shal (Roberds and Tamkun, 1991; Pak et al., 1991). The mouse homolog of Shal encodes an inactivating K⁺ channel with properties similar to rat Shal reported here. However, there is only 77% amino acid identity between rat Shal and this mouse Shal. In contrast, the mouse and rat Shaker homologs MBK1 and RCK1 are virtually identical at the nucleotide and amino acid level. This level of identity also extends to other members of this Shaker subfamily; MBK2 and MBK3 (Chandy et al., 1990) are virtually identical to RCK2 and RCK3, respectively (Stuhmer et al., 1989). Therefore rat Shal and mouse Shal are likely to represent distinct members of the mammalian Shal family. Roberds and Tamkun (1991) describe the cloning of a rat Shal homolog, called RK5, from rat heart. The nucleotide and deduced amino acid sequences of rat Shal and RK5 are virtually identical except for a distinct carboxyl terminus of RK5 at the amino acid level. However, the nucleotide sequence of rat Shal and RK5 remain almost identical beyond the point of divergence in the amino acid sequence in the carboxyl terminus. With PCR using primers made to nucleotides 1318–1338 (sense) and 1555–1573 (antisense) of rat Shal and thus correspondingly nucleotides 1318–1338 and 1553–1571 of RK5 (Roberds and Tamkun, 1991), we have amplified the intervening sequences from first-strand cDNA made from adult rat brain and heart poly(A)+ RNA that covers the divergence in nucleotide sequence in the open reading frame between rat Shal and RK5. Determination of the sequence of these amplified DNAs reveals only the nucleotide sequence corresponding to rat Shal but not RK5. Furthermore, diagnostic restriction enzyme mapping of the PCR product suggests only one sequence is obtained with these primers (data not shown). In the case of mouse Shal, Pak et al. (1991) reported that deletion of 31 amino acids (residues 2–32) had relatively mild effects on the inactivation kinetics as compared with amino-terminal deletion mutants of Shaker B (Hoshi et al., 1990). One possible explanation is that the 31 residues at the amino-terminus of mouse Shal contain only 1 basic residue and are largely hydrophobic. To compare with mutations that delete the cluster of positively charged residues present in Shaker B (residues 17–19) or rat Shal (residues 35–37), it would be of interest to determine whether deletion mutations in mouse Shal, which remove positively charged amino acids that are 3' to this hydrophobic amino terminus, produce a considerable slowing of fast inactivation.

Experimental Procedures

PCR of Hippocampal mRNA

Hippocampal poly(A)+ mRNA (2 μg) was copied into first-strand cDNA using oligo(dT) priming and reverse transcriptase. Degenerate oligonucleotide primers were synthesized to correspond to sequences coding for amino acids MTLGVS and the anti-sense sequence complementary to the coding sequences for PPVIVS (amino acids 440–446 and 473–479 of the predicted sequence for the Shaker B K⁺ channel) (Schwarz et al., 1988). The oligonucleotide sequences were ATGCACAGCCTGG, where N represents A, T, G, or C. The sequences between the two primers were amplified by PCR using Ampli-Taq DNA polymerase (Perkin Elmer, Cetus) under the following conditions: 94°C for 30 s, 50°C for 30 s, and 72°C for 90 s for 40 cycles, then 72°C for 5 min (Innis et al., 1990). DNA fragments 120 bp in length were isolated from agarose gels and subcloned into the Smal site of Bluescript SKI for sequence analysis.

For PCR amplification of the carboxy-terminal sequences of
rat Shal for a probe for Northern blots, oligonucleotides CCGGCG GCCAAAGTGGAA and TGGTGAGCTGTTGGTGTGGT were synthesized and annealed to 0.1 ng of plasmid DNA and amplified over 30 cycles. Pure PCR fragments were subcloned with HindIII-digested Bluescript SK+ and analyzed by sequencing.

Isolation of Rat Brain Shal cDNA Clones

A cDNA library was prepared from adult Sprague-Dawley rat brain hippocampal poly(A)+ RNA. The RNA was copied into hemi-synthesized cDNA (Gubler and Hoffman, 1983; Sambrook et al., 1989) using a NotI adaptor-primer for priming first-strand synthesis and by adding an EcoRI adaptor after second-strand synthesis. The resulting cDNA was size selected on an agarose gel for inserts >2.0 kb and ligated with the arms of λ Zap II (Stratagene). The resultant library was composed of 4.8 × 1010 individual recombinants of which 2.2 × 1010 phage were amplified for screening. Recombinant phage (200,000) were screened with the 120 bp Shal-like PCR fragment excised from Bluescript SK+ by digestion with PstI and BamHI and labeled with ^32P to a specific activity of ~1 × 10^9 cpmpg by random priming. Duplicate filters were hybridized with ^32P-labeled probes in 50% formamide, 5 × SSC, 1 × Denhardt's solution, 20 mM sodium phosphate (pH 7.0), 1% SDS, 100 μg/ml salmon sperm DNA at 42°C for 24 hr and finally washed in 0.1 × SSC, 0.1% SDS at 65°C. Inserts harbored in positive phage were excised by plasmid rescue of Bluescript SK+ after superinfection with a R408 helper phage. For DNA sequencing single stranded DNA was isolated from bacteria containing plasmids with the insert in either Bluescript SK+, SK+, or KS+ by infection with M13KO7.

DNA Sequencing

The rat Shal cDNA was sequenced by the Sequenase (USB Corp.) modification of the chain termination technique (Sanger et al., 1977).

Isolation of RNA and Northern Blots

Poly(A)+ RNA was isolated from rat brain tissues and heart using the Fast Track mRNA isolation procedure (Invitrogen). This procedure resulted in an approximate yield of 20 μg of poly(A)+ RNA per gram of tissue. RNA was fractionated on 0.7% agarose gels containing formaldehyde (Schwarz et al., 1988) and transferred to Nytran membranes by the Southern blot technique as described by Sayers and Eckstein (1989). The wild-type strand (cDNA) was immobilized by overnight baking at 80°C, and hybridized with radioactive ^32P-labeled cDNA probes (~1 × 10^6 cpmpg) under conditions of 50% formamide, 5 × SSC, 20 mM sodium phosphate (pH 7.0), 1 × Denhardt's solution, 1% SDS, 100 μg/ml salmon sperm DNA at 42°C for 16 hr. Filters were washed with 0.1 × SSC, 0.1% SDS at 65°C. Inserts harbored in positive phage were excised by excision of labeled DNA from plasmid DNA in the cDNA library by M13KO7.

In Situ Hybridization of Rat Shal to Sections of Rat Brain and Heart

Frozen sections (10 μm) were cut from rat brain and heart. The sections were air dried, fixed with 3% paraformaldehyde in PBS for 30 min, and then rinsed several times prior to hybridization. A 2.7 kb HindIII fragment from the rat Shal cDNA was subcloned into the BstXI site of the pCMVNeo vector (Invitrogen). 5% labeled mRNA was synthesized as described by Melton et al. (1984). Anti-sense RNA was synthesized with SP6 polymerase after linearization with HindIII. Sense RNA was synthesized with T7 polymerase after linearization with NotI. Before hybridization to tissue sections the radioactive RNA was hydrolyzed with base to an average length of 130 bp (Cox et al., 1994). Hybridization of the probes to sections, washing, and preparation for autoradiography were essentially as described by Cox et al. (1984). However, the hybridization was performed at 50°C and then treated with 100 U/ml RNase A and 50 μg/ml RNase A for 30 min at 37°C. Finally, the sections were washed in 2 × SSC, 10 mM sodium thiocyanate for 1 hr at 37°C followed by a wash in 0.1 × SSC, 10 mM sodium thiocyanate at 50°C for 30 min. The sections were then dehydrated through a graded ethanol series. For low resolution mapping the distribution of rat Shal mRNA levels were exposed to Kodak X-AR5 X-ray films.

In Vitro Transcription for Expression in Xenopus Oocytes

The template DNA (approximately 2 μg) was linearized with the restriction enzyme Xbal. GpppG-capped RNA was transcribed and purified as described by Timpe et al. (1988a). To confirm the size and integrity of the RNA made, denaturing gel electrophoresis was performed using one tenth of the transcription reaction, and the migration was compared with RNA size markers. The mRNA was stored in sterile water at -70°C.

Oocyte Injection and Electrophysiological Recording

Adult female Xenopus laevis were anesthetized by immersion in ice water for 1 hr followed by surgical removal of several ovary lobes. Oocytes were separated manually from the ovarian connective tissue prior to injection. Stage V and VI oocytes were injected with approximately 50 nl of RNA (2-4 mg/ml) and incubated in modified Barth's saline containing 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 10 mM HEPES (pH 7.5), 0.82 mM MgSO₄, 0.33 mM Ca(NO₃)₂, and 0.41 mM CaCl₂ (Gurdon and Wickens, 1983) at 18°C. Twenty-four hours following injection, oocytes were incubated in modified Barth's saline with 7 mg/ml collagenase (CLS 3, Worthington) for 2 hr with gentle shaking. This treatment helps to remove the overlying connective tissues and follicle cells. Oocytes were incubated in modified Barth's saline supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamicin for 4-6 days before electrophysiological recording.

Ionic currents were recorded using a standard two-microelectrode voltage clamp circuit. Data acquisition and analysis were performed on an 80386-based microcomputer using the program pCLAMP and a TL-1 A/D converter (Axon Instruments). Voltage and current microelectrodes were filled with 3 M KCl and had resistances of less than 2 MΩ. All experiments were carried out at 11°C ± 1°C. Current signals were sampled at 22°C ± 2°C using a List EPC7 and analyzed on an InDinco DPD1173. Leak subtraction was done using averages of steps during which there were no channel openings. The patch recordings were sampled and filtered as described above for the whole-cell voltage clamp.

Site-Directed Mutagenesis

Oligonucleotide site-directed mutagenesis was performed using a single-stranded rat Shal cDNA template in Bluescript SK- as described by Sayers and Eckstein (1989). The wild-type strand was removed with exonuclease III after nicking with NciI, producing an efficiency of mutagenesis greater than 90%. Mutagenized DNA was characterized by DNA sequencing. For electrophysiological analysis two independently generated mutants were analyzed. mRNA was derived from the mutagenized DNA as described above.

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References


