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Molecular cloning of a member of a third class of Shaker-family K^+ channel genes in mammals

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ABSTRACT **We report the cloning of RKShIIIA, a cDNA encoding a K^+ channel sequence expressed in rat brain. This cDNA encodes K^+ channel subunits that express in *Xenopus* oocytes a slow, 4-aminopyridine- and tetraethylammonium-sensitive, delayed rectifier-type K^+ channel activated by large membrane depolarizations. This gene belongs to the Shaker (Sh) family of K^+ channel genes, since the predicted protein has the same overall structure and shows significant homology to other members of this family. However, RKShIIIA cannot be assigned to either of the two known classes of Sh-family genes in mammals based on sequence analysis. Notable features of the RKShIIIA protein product include a probable cytoplasmic loop rich in prolines and a stretch very homologous to the *Drosophila* Shaw protein, both near the amino terminus.**

Ion channels are ubiquitous membrane proteins with important and multiple functions in both excitable and nonexcitable cells (1). Potassium (K^+) channels are particularly diverse and are present in all eukaryotic cells (1, 2). In neurons specific combinations of various ion channels underlie the generation of many different signal waveforms and firing patterns and thus contribute to the complexity of neuronal information coding and integration (3).

Two unrelated sequences encoding voltage-dependent K^+ channels have been characterized, clearly representing two distinct families of genes. The first family, the Shaker (Sh) family, encodes proteins of several hundred amino acids that are similar to those encoded in the Shaker gene in *Drosophila*, a gene that generates several products by alternative splicing (4–6), and includes several members in mammals (7–14) and in *Drosophila* (15). A gene encoding a voltage-dependent K^+ channel with extremely slow kinetics cloned originally from kidney (16) defines the second K^+ channel gene family. The products of the Sh-family genes consist of a core region with six hydrophobic sequences of which probably five are membrane-spanning domains. Between the third and fourth hydrophobic segments there is a sequence, thought to be membrane-spanning (17, 18), consisting of an arginine (sometimes lysine) at every third position and hydrophobic amino acids in the other positions. This motif, believed to be responsible for voltage sensing, is known as S4 and is also present in voltage-dependent Na^+ and Ca^{2+} channels (17, 18). In contrast, the gene cloned from kidney encodes a protein with a single membrane-spanning domain and no real S4 sequence.

A comparison of the known sequences in the Sh family suggests the presence of several groups or classes (13). We now report on the cloning of a K^+ channel cDNA from rat brain (RKShIIIA) that clearly belongs to the Sh family.§ A comparison with other members of this family suggests that this cDNA is a member of a third class of K^+ channel genes in the Sh family in mammals.

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MATERIALS AND METHODS

DNA Sequencing. Sequences were obtained by the dideoxynucleotide chain-termination method using Sequenase (United States Biochemical) and plasmid DNA as template.

RNA Expression. The recombinant pBluescript (Stratagene) plasmid containing the RKShIIIA insert was linearized by digestion with *Apa* I or *Xho* I, and full-length capped RNA transcripts were synthesized with T3 polymerase as described (19). Standard methods were used to inject RNA into *Xenopus laevis* oocytes and to record ionic currents (19, 20), except that often the oocytes were not defolliculated until use. All electrophysiological recordings were carried out at 21–22°C in ND96 solution (96 mM NaCl/2 mM KCl/1.8 mM $CaCl_2$/1 mM $MgCl_2$/5 mM Hepes, pH 7.5).

Southern Blot Analysis. Digested rat genomic DNA was electrophoresed into 0.8% agarose in 1× TBE buffer (21). Southern blot transfers into Hybond (Amersham) were prepared essentially as described (21). The blots were hybridized with ^{32}P-labeled DNA probes synthesized by the random hexamer primer method (22).

RESULTS AND DISCUSSION

Primary Structure of RKShIIIA. The nucleotide and deduced amino acid sequence of clone RKShIIIA is shown in Fig. 1. The predicted product of the cDNA is a protein of 563 amino acids with a calculated relative molecular mass of 61,894. The conceptual translation of RKShIIIA (Fig. 1), a hydropathy analysis (not shown), and a comparison with the amino acid sequence of three classes of Sh-family genes (Fig. 2) indicate that our sequence is similar in overall structure to other Sh-family genes. Like all Sh-family gene products, RKShIIIA contains six hydrophobic regions (H1–H6) and a well conserved S4 domain.

A special feature of RKShIIIA is a 44-amino acid sequence inserted in the amino end region of the protein (enclosed in a box in Fig. 2). The sequence contains several stretches of consecutive prolines, a rare feature found in some other proteins such as the hinge region of some IgGs (25), myelin A1 basic protein (26), and synapsin (27). In addition, two serines in this sequence are surrounded by amino acids similar to those that surround serines or threonines that undergo O-glycosylation (26). According to the proposed topology of this channel (Fig. 1), these putative O-glycosylation sites are on the cytoplasmic side and thus might be involved in the regulation of phosphorylation and protein assembly and targeting (28).

We find that the fifth hydrophobic domain (H5) is consistently one of the most conserved regions among known Sh K^+ channel sequences. This sequence is probably not hydrophobic enough to constitute a membrane-spanning domain (6, 18). Furthermore, if one assumes that H5 is not membrane-spanning, the predicted topology of Sh K^+ chan-

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M34052).

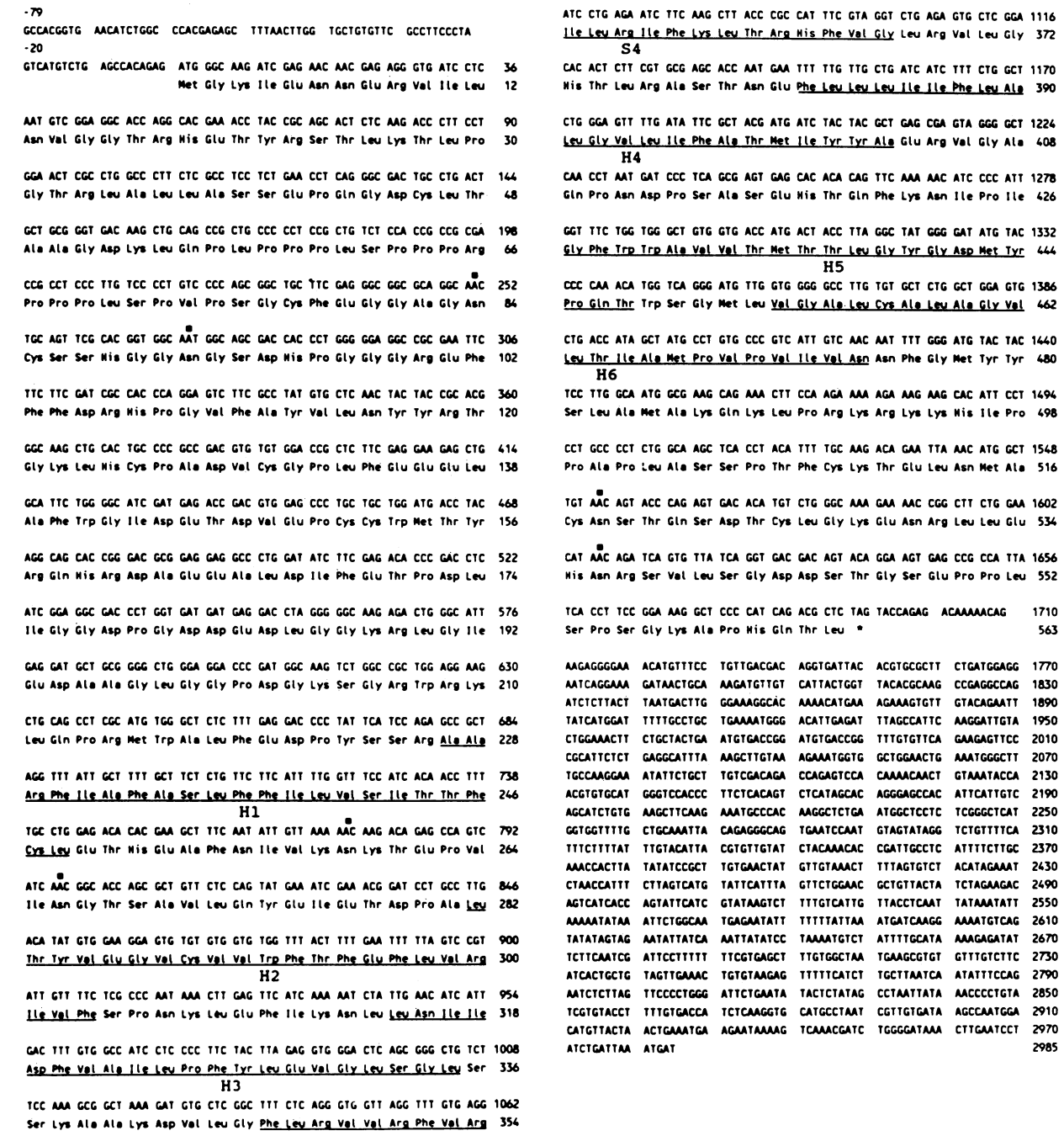

FIG. 1. Nucleotide and deduced amino acid sequence of RKShIIIA. The name given to this clone attempts to indicate its relationship to other K^+ channel sequences. The R indicates the clone is from rat, K that it encodes a K^+ channel, Sh that it belongs to the Sh family of genes, III that it is a member of the third known class of genes in this family, and A that it is the first known member of this class in rat. We have not indicated tissue of origin in the name, because other sequences have been found in more than one tissue (e.g., RCK4 and RHK1, found in brain cortex and heart, respectively, are the same sequence). The numbers indicate the nucleotide and amino acid positions with the chosen initiation codon as 1. The ATG triplet chosen as the initiation codon is the second found in the sequence, but it produces the longest possible open reading frame and predicts a protein that is highly homologous to other Sh K^+ channel sequences. The first stop codon in frame is shown by an asterisk. Hydrophobic domains (H1–H6) and the S4 motif are underlined. Consensus sequences for N-glycosylation (23) are indicated with filled squares. Only two of these (Asn-259 and -266) are extracellular in the expected topology of the protein assuming that the amino terminus is intracellular (because of a lack of signal-peptide sequence) and that H1–H4, S4, and H6 are membrane-spanning domains (6, 18). The clone containing the RKShIIIA insert was obtained by screening (21) a rat brain cDNA library in λZAP (Stratagene) with DNA probes derived from the amino-terminal end of a partial clone obtained previously. Hybridization to nitrocellulose replicas of cultures was carried out in 50% (vol/vol) formamide/5× SSPE at 42°C (21). Another isolate was partially sequenced. It is identical to RKShIIIA except that in the 3′ end instead of a T as the last nucleotide it contains a stretch of 10 A residues. The initial partial clone was obtained by screening, at low stringency [20% formamide/5× SSPE at 30°C], an oligo(dT)-primed rat brain λgt11 cDNA library with an oligonucleotide probe corresponding to the first 60 bases of the (H5) domain of the *Drosophila* Shaker H4 cDNA (4). The recombinant pBluescript plasmid containing the RKShIIIA insert was obtained from the purified λZAP clone by *in vivo* excision using Stratagene protocols.

nel subunits will be similar to that of the homologous repeats in voltage-dependent Na^+ and Ca^{2+} channels (18). We propose that H5 enters the membrane partially and helps to form the K^+ selectivity filter. If a K^+ channel is a tetramer of Sh-family gene products (18), the center of the H5 sequences could form a ring containing 12 hydroxyl groups. These

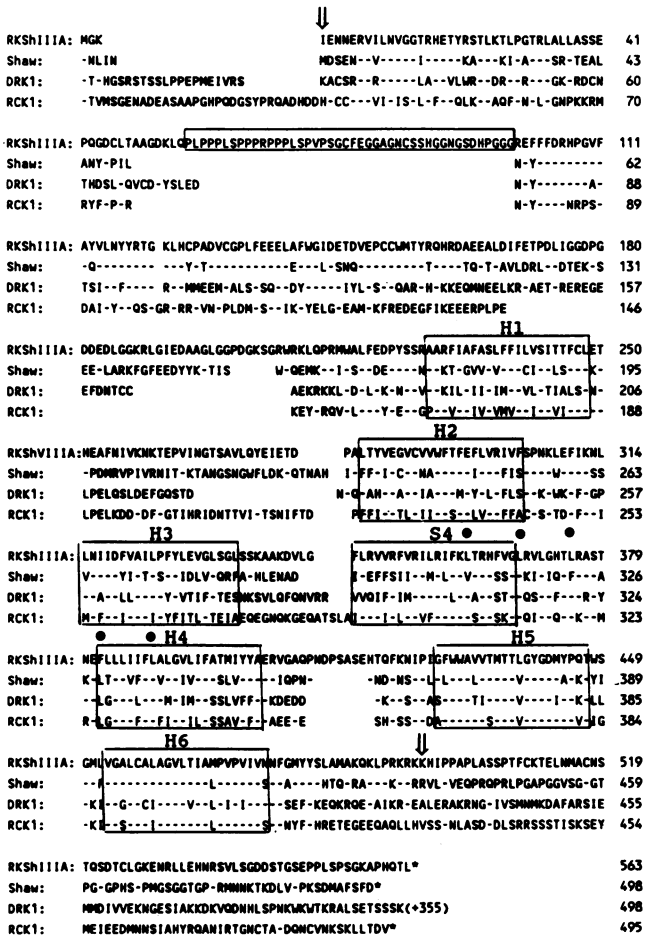

FIG. 2. Comparison of predicted amino acid sequences of RKShIIIA and other Sh K^+ channel genes. The complete sequences of RKShIIIA, Shaw, and RCK1 are shown. DRK1 extends 355 amino acids further at the carboxyl end. Identical amino acids are shown with dashes. The sequences were aligned to maximize long stretches of homology. Gaps in the sequence are shown as blanks. The beginning and the end of the central core region used for calculations of identity are marked by arrows. The H1–H6 and S4 sequences and a 44-amino acid insert peculiar to RKShIIIA are boxed. The leucines in heptad repeat ("leucine zipper" motif) adjacent to the S4 domain are indicated with filled circles (24). In RKShIIIA the fourth leucine is replaced by phenylalanine.

hydroxyls could provide the water-like environment which dehydrates K^+ ions selectively (1, 29).

Functional Properties of RKShIIIA Channels. *In vitro* transcribed RNA from RKShIIIA expresses K^+ channels when injected into *Xenopus* oocytes. Large currents absent in uninjected oocytes are observed in oocytes injected with RKShIIIA transcripts (Fig. 3 *A–C*). The reversal potential of these currents, determined from tail-current analysis (19), depends on external K^+ concentration as expected for a K^+-selective channel. A reversal potential of −85 mV is obtained in 2 mM external K^+ and −55 mV in 10 mM (data not shown). These values are close to the expected equilibrium potential for K^+ (−95 mV and −55 mV, respectively), assuming an internal K^+ concentration of 90 mM, and indicate a very high selectivity for K^+ ions (1). The currents expressed by RKShIIIA activate slowly, starting at about −10 mV, and their rise time decreases with increasing depolarization (Fig. 3 *A–C*). These currents inactivate very slowly (Fig. 3*B*) and activate with a delay (Fig. 3*C*). All these are features of delayed rectifier-type K^+ channels (1, 2).

A conductance–voltage curve obtained from five experiments is presented in Fig. 3*D*. The conductance begins to rise

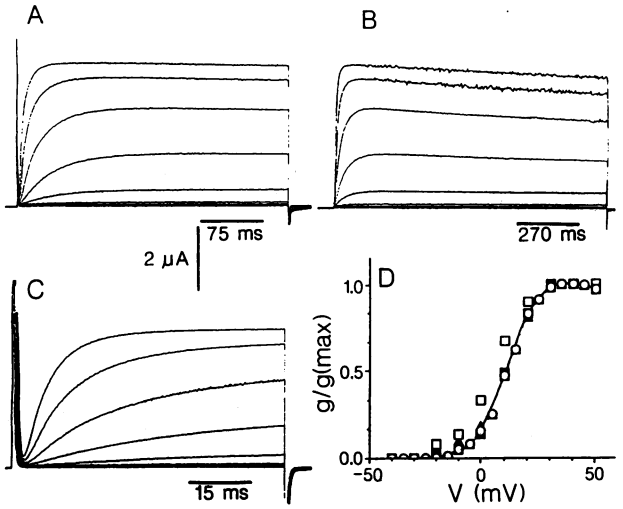

FIG. 3. Electrophysiological properties of ion currents expressed by RNA transcripts of RKShIIIA. (*A–C*) Currents recorded under voltage clamp. Depolarizing pulses, from −80 to +40 mV with 10-mV increments, were delivered at a rate of every 4 sec (*A* and *C*) or 8 sec (*B*) from a holding potential of −100 mV. The oocytes were bathed in ND96 solution and the electrodes were filled with 3 M KCl. (*D*) Plot of normalized conductance (conductance at the indicated potential divided by the maximum conductance) obtained from five oocytes and three different preparations of RNA. In one case (open circles) depolarizing pulses were given in 5-mV increments. The conductance was calculated (1) by dividing the steady-state current at the end of 500-msec depolarizing pulses by the driving force using a reversal potential of −85 mV.

between −15 and −10 mV and increases steeply to a maximum value. Sometimes a decline in conductance is observed with large depolarizations. Half-maximal conductance is obtained at around 13 mV and the limiting slope of the curve is estimated to be 7–7.5 mV for an *e*-fold change in conductance. The reduced steepness exhibited by RKShIIIA, compared to *Drosophila* Shaker channels (19), may be due to the smaller number of basic residues in the S4 domain (six vs. seven). However, amino acids in other regions of the protein may also influence the steepness of conductance–voltage relations (13).

The currents expressed by RKShIIIA are blocked 70–100% (depending on the membrane potential) by 1 mM tetraethylammonium, 5 mM Ba^{2+}, and 1 mM 4-aminopyridine, three well known K^+ channel blockers. The blockage of RKShIIIA channels by 4-aminopyridine seems to exhibit open-channel block behavior (1), contrary to what has been observed for 4-aminopyridine blockade of K^+ channels in squid giant axon (30). Specifically, the degree of blockage of RKShIIIA currents by 4-aminopyridine increases with increasing depolarization, the rise time of the currents becomes faster, and at some voltages (e.g., at +20 mV) a time-dependent inactivation is induced (Fig. 4). Furthermore, repetitive depolarization produces further blockage (see *Inset* in Fig. 4*B*), as opposed to the gradual release of block observed for the delayed rectifier of the squid giant axon (30).

K^+ Channel Classes. Based on sequence homology, and hence evolutionary relatedness, of previously known Sh-family sequences, two classes of genes (13) can be defined (classes I and II in Table 1). Members of a given class, in different species, are far more similar to each other than to members of different classes in the same species (Table 2). This suggests that these two classes originated from some ancestral sequence before the flies and mammals diverged (13–15). RKShIIIA is more similar to the *Drosophila* Shaw gene (15) than to any other known K^+ channel sequence (Table 2) or any sequence in the GenBank data base (January

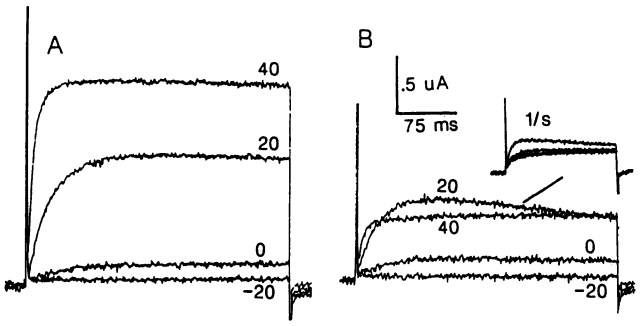

FIG. 4. 4-Aminopyridine block of RKShIIIA currents. Records of ion currents obtained as in Fig. 3 for depolarizing pulses to the indicated voltages are shown before (*A*) and after (*B*) the addition of 0.8 mM 4-aminopyridine. The *Inset* in *B* shows the currents during a depolarizing pulse to 20 mV applied at a frequency of 1/sec obtained after the experiment shown in *B* was finished.

1990), particularly in a long stretch near the amino terminus that is almost identical between these two sequences (Fig. 2). Furthermore, genomic DNA analysis suggests that several genes of the same class, with similar homologies to Shaw, are present in the rat genome (Fig. 5). However, as shown in Table 2, the overall homology between RKShIIIA and Shaw is significantly less than that among class I or class II genes (e.g., between RCK1 in rat and H4 in *Drosophila*, or between DRK1 in rat and Shab in *Drosophila*). Outside the stretch in the amino terminus, RKShIIIA is more similar to Shaw than to other Sh genes in the sixth hydrophobic domain (H6) and the sequence immediately following H6, but it is more similar to RCK1 or DRK1 in the region around the S4 domain (Fig. 2).

RKShIIIA is clearly different from class I or class II genes and thus we consider it a member of a third Sh gene class in mammals. Class I and II genes have vertebrate and invertebrate counterparts. RKShIIIA and Shaw are probably related genes; however, their evolutionary relatedness is different from that between class I and class II genes. Thus, their assignment to the same class of genes is tentative, and definite assignment must await further evolutionary analysis.

The physiological significance of these classes is not known. Inactivation is clearly not a defining parameter because, for example, class I includes channels that inactivate very fast and channels that inactivate very slowly and have delayed rectifier properties (Table 3). Differences in these properties apparently can be achieved without major changes in the core region. From the electrophysiological studies done thus far, we can distinguish two parameters of predicted physiological significance which appear to vary among classes (Table 3): class I members activate quickly and at relatively low (but not subthreshold) voltages, while

Table 1. Shaker family of K^+ channel genes

Class	Rat	Human	*Drosophila*
I	RCK1 = RBK1 (10, 8)	HuKI (12, 13)	Shaker (4–6)
	RCK5 = RBK2 (10, 9)	HuKIV (12, 13)	
	RCK4 = RHK1 (10, 11)	HuKII (12, 13)	
	RCK3 (10)	HuKIII (12, 13)	
		HuKV (12, 13)	
		HuKVI (12, 13)	
II	DRK1 (14)		Shab (15)
III	RKShIIIA (this paper)		Shaw? (15)
IV			Shal (15)
V			Shaw? (15)

This table was modified from ref. 13. A homologue of RCK1, RBK1, and HuKI is known in mouse (MBK1, ref. 7).

Table 2. Fraction of identical amino acids in analogous positions between pairs of Sh K^+ channel proteins

	Shaw	DRK1	Shab	RCK1	H4
RKShIIIA	0.52	0.42	0.41	0.41	0.40
Shaw		0.41	0.40	0.41	0.41
DRK1			0.72	0.42	0.40
Shab				0.39	0.37
RCK1					0.76

The fractions of identical amino acids in overlapping sequences in the core region were calculated using an alignment like the one shown in Fig. 3 but which included also Shab and H4. H4 is one of the products of the *Drosophila* Shaker gene (4). Shab (15) and Shaw (15) are *Drosophila* genes, and DRK1 (14) and RCK1 (10) are rat genes.

class II and class III channels (DRK1 and RKShIIIA) activate relatively slowly and require large-depolarizations. RKShIIIA currents activate at particularly high voltages. These differences might explain why there seems to be much more expression of class I genes than of class II or III genes in brain, since most neuronal K^+ channels activate in the voltage range of class I channels. The three gene classes also differ in the number of positively charged residues in the S4 domain (Table 3).

Other properties such as developmental regulation, cellular localization, posttranslational modification, or participation in heteromultimer formation (31, 32) might turn out to be important properties of Sh channel classes. For example, RKShIIIA and Shaw lack a consensus sequence for cAMP-dependent phosphorylation present in most Sh K^+ channel sequences.

Functional Significance. One major task in the future of K^+ channel gene research is to understand the physiological significance of the various K^+ channel subunits that are being cloned. Because the functional properties of a given K^+ channel will depend on whether it is a homo- or heteromultimer of Sh-family gene products (19, 31, 32), on the interactions of this aggregate with smaller subunits (20, 33), and on posttranslational modifications (2), the results of the expression of a particular Sh gene will depend on the cellular context. It is interesting in this regard that the properties

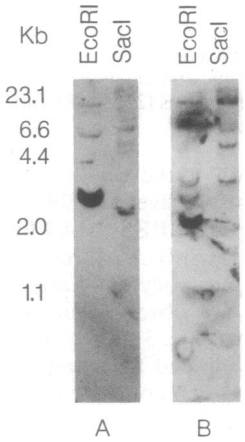

FIG. 5. Southern analysis of rat genomic DNA. DNA was digested with an excess of *Eco*RI or *Sac* I. The Southern blots were hybridized (21) under conditions of relatively low stringency (30% formamide/5× SSPE at 37°C) with one of two probes. The first probe (*A*) contains the region that is most similar between Shaw and RKShIIIA (nucleotides 301–501); the second probe (*B*) contains the sequence encoding the H3 through H4 domains (nucleotides 964–1296), where Shaw and RKShIIIA are less similar. A single strong hybridization band and several faint but discrete hybridization bands are observed with both probes, suggesting the presence of several genes homologous to both regions of RKShIIIA. *Hin*dIII-digested λ DNA was used as size markers. Kb, kilobases.

Table 3. Electrophysiological properties of three classes of Sh K^+ channels

Class	Channel (ref.)	S4 Z^*	k_{in},† msec	V_{on},‡ mV	t_{on},§ msec
I	H4 (19)	7	4	−40	5
	RCK1 (10)	7	—	−50	15
	RCK5 (10)	7	—	−55	6
	RHK1 (11)	7	30	−40	10
	RCK3 (10)	7	1000	−55	14
II	DRK1 (14)	5	—	−25	120
III	RKShIIIA	6	—	−10	80

Approximate values were determined from published data, at room temperature. All the experiments used a similar bathing solution except for those on DRK1, which used Co^{2+} instead of Ca^{2+}.

*Number of positive charge residues in S4.

†Time constant of inactivation at 0 mV. A dash implies inactivation is very slow or very small.

‡Activation voltage, the approximate voltage at which the conductance starts to rise.

§Activation rate at 0 mV determined as time to peak for rapidly inactivating currents, and time to rise between 10% and 90% for slowly inactivating and noninactivating currents.

observed here are very similar to those of one of the K^+ currents (I_{Ks}) observed in *Xenopus* oocytes injected with whole brain poly(A)$^+$ RNA (34). RKShIIIA channels activate at about the highest voltages one may expect physiologically. If RKShIIIA is expressed in the plasma membrane, and if its properties resemble those in the oocyte, we expect this channel to be particularly important in the termination of prolonged depolarizations (above 0 mV) such as plateau potentials or in the regulation of the frequency and duration of spike bursts (3). A channel such as RKShIIIA would play little role in subthreshold phenomena or in shaping the waveform of fast Na^+ spikes.

Note Added in Proof. After this paper was submitted we became aware of a sequence, obtained from NG108 cells, just published by Yokoyama *et al.* (35). Their sequence (NGK2) is similar (but not identical) to that of RKShIIIA and appears to be the same as that of a related cDNA (bn13) from PC12 pheochromocytoma cells (Vega *et al.*, ref. 36). Sequence analysis suggests that NGK2 (or bn13) and RKShIIIA are two different members of the same gene class as defined here. Interestingly, although the amino acid sequence differences between NGK2 and RKShIIIA are similar to those found among class I gene members, their electrophysiological properties are very similar.

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