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Electrophysiological characterization of a new member of the RCK family of rat brain K⁺ channels

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Received 23 May 1990; revised version received 16 August 1990*

A novel member of the RCK family of rat brain K⁺ channels, called RCK2, has been sequenced and expressed in *Xenopus* oocytes. The K⁺ currents were voltage-dependent, activated within 20 ms (at 0 mV), did not inactivate in 5 s, and had a single channel conductance in frog Ringers of 8.2 pS. Compared to other members of the RCK family the pharmacological profile of RCK2 was unique in that the channel was resistant to block (IC₅₀ = 3.3 μM) by charybdotoxin [(1988) Proc. Natl. Acad. Sci. USA 85, 3329–3333] but relatively sensitive to 4-aminopyridine (0.3 mM), tetraethylammonium (1.7 mM), α-dendrotoxin (25 nM), noxiustoxin (200 nM), and mast cell degranulating peptide (200 nM). Thus, RCK2 is a non-inactivating delayed rectifier K⁺ channel with interesting pharmacological properties.

Potassium channel; cDNA cloning; cDNA expression; Delayed rectifier; *Xenopus* oocyte

1. INTRODUCTION

Voltage-activated K⁺ channels are integral membrane proteins which regulate the transmembrane diffusion of K⁺ ions. Channel activation controls neuronal excitability through repolarization of the action potential and modulation of the frequency of repetitive firing [5]. From whole-cell and single channel electrophysiological measurements [25,27], neurons are thought to express heterogeneous populations of K⁺ channels which differ in their biophysical and pharmacological properties. Until recently the structural basis of K⁺ channel diversity was not known; however, molecular cloning methods have now shown that mammalian brain mRNA encodes several distinct voltage-activated K⁺ channels [2,8,15,22,24,25]. Expression of cloned rat brain K⁺ channels provides a new basis for understanding the relationships between primary structure and channel function.

Using an oligonucleotide probe encoding a strictly conserved sequence located upstream of the membrane spanning core region of several known K⁺ channels, we have isolated a cDNA clone which encodes a new member of the RCK family of K⁺ channels [2,10]. When expressed in *Xenopus* oocytes this clone produces non-inactivating delayed rectifier K⁺ current. Its sen-

sitivity to blockers such as 4-aminopyridine, dendrotoxin and mast cell degranulating protein suggests that it may be related to the K⁺ channels which affect neurotransmitter release in rat brain synaptosomes [1].

RCK2, a cDNA encoding a rat brain K⁺ channel has recently been described by Grupe et al. [10]. The cDNA clone reported here encodes a polypeptide which differs from RCK2 at only one residue and hence will be referred to as RCK2, also. Our electrophysiological results are closely comparable to those obtained by Grupe et al. [10], except that in our experiments charybdotoxin (ChTX) was 1000-fold less potent in blocking K⁺ current than was reported by Grupe et al. [10].

2. MATERIALS AND METHODS

2.1. Isolation and sequencing of cDNA

Rat brain cDNA libraries enriched for full-length inserts [7] were screened at low stringency using an oligonucleotide probe encoding the amino acid sequence Asn-Glu-Tyr-Phe-Phe-Asp-Arg (position 82–88 of RCK2) which is conserved in most of the known voltage-activated K⁺ channels. Run-off RNA transcripts of positive clones were made as described previously [12] and stage V–VI *Xenopus* oocytes were injected with 10 ng of RNA in 75 nl 0.1 M KCl and screened for K⁺ current expression. RNA from one clone (RCK2) bearing an approximately 5 kb insert, gave large K⁺ currents, and had a DNA sequence which was different from all known K⁺ channels.

2.2. Electrophysiological recording

Oocytes were incubated at 19°C in modified Barth's solution for 2–7 days and then tested for expression using a two-microelectrode voltage clamp [12]. Manually defolliculated oocytes were placed in a recording chamber continuously superfused at 3 ml/min with a test solution consisting of (mM): 120 NaOH, 120 methanesulfonic acid, 2.5 KCl, and 10 Hepes, adjusted to pH 7.3 with NaOH. Oocytes were impaled with 3 M KCl-filled micropipettes (resistance 1–2 MΩ).

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* Due to unfortunate circumstances the publication of this paper was delayed. Under normal editorial handling the paper should have been published in 1990 – The Accepting Editor

RCK4	MEVAMVSAESSGCNSHMPYGYAAQARARERERLAHSRAAAAAVAAATAAVE	52
RCK4	GTGGSGGGPHHHHQTRGAYSSHDPQGSRGSRREEATRTEKKKKLHHRQSSFPHCSDLMP	112
RCK1	MTVMSENADESAAPGHPQDGSYPRQADHD	31
RCK3	MTVVPGDHLLEPEAAAGGGGGDPQGGCVSGGGCDRYEPLPPALP-AGE	48
RCK4	GSEKILRELSEEEEEDEEEEEEEEEEGRFYYSEEDHGDGCSYTDLLPQD--GGGGYSSVR	172
RCK5	---AT-DFV---A-L-----TYD-EA....	27
RCK6	MRSEKSLTLAAPGEVRGPEGEQQDA-EFQEAEGGG	35
RCK1	DHECCERVVINISGLRFETQLKTLAQFPNTLLGNPKKRMRYFDPLRNEYFFDRNRPSFDA	91
RCK3	QDC-G-----C---E---D---R-----L--	108
RCK4	YSD-----V-----M-----E---D-E---TQ-----	232
RCK5	-----E---D-----	87
RCK6	GCCSS--L-----Y---R--SL--D---D-GR-V-F-----	95
RCK1	ILYYYQSGGRILRRPVNVLDMFSEEIKFYELGEEAMEKFREDEGFIKE...EERPLPEKE	148
RCK3	-----I-----I-I---R---Q-----LR...--RRD	165
RCK4	-----K-----F-I-T-V---Q-----LL-----VR-.E-D-A---N-	290
RCK5	-----I-----R-----M-----Y-----N-	144
RCK6	-----I-M---R---Q---D---LAA-----CLP-GGED-K---SQP	155
S1		
RCK1	YQRQVWLLFEYPSSGPARVIAIVSVMLISIVIFCLETLPKDDKDFTG.....	200
RCK3	F-----G-----L-----FR-EK-YPASPSQ....	221
RCK4	FKK-I-----S---G---L-----FR--R-LIMALSAGGH.	349
RCK5	F-----I-----S-----IFR-ENEDMHGG.....	198
RCK6	F-----G-----L-----QFRADGRGGSNEGSGTRM	215
S2		
RCK1TIHRIDNTTVIYTSNIFTDPFFIVETLCIIWF	232
RCK3DVFEAANNSTSGASSGASS-S---V-----	256
RCK4SRLNDTSAPHLENSGHT--N-----V--V--	384
RCK5GVTFHTYSNS-IGYQQ-TS-----	233
RCK6	SPASRGSHHEEDEDSDSYAFPGSIPLGGLTGTTGSSSTLGGSF---L-----V--	275
S3		
RCK1	SFELVVRFFACPSKTDFFKNIMNFIDIVAIIPYFITLGTETAEQEGNQ.....KGEQ	284
RCK3	---L-----AT-SR---L-----L-RQ-----Q-	306
RCK4	---F---C---QAL-----I---S-L-----DL-Q-Q-GGN.....GQQQ-	438
RCK5	---FL-----SC---T---I---T-----L-KPEDA.....QHTN	285
RCK6	T---L---S---AA--R---I---L---F-----LVQRHEQ-PVSGGSGQNGQQ	335
S4		
RCK1	ATSLAILRVIRLVRFRIKLSRHSKGLQILGQTLKASMRELGLLIFFLFIGVILFSSAV	344
RCK3	-M-----	366
RCK4	-M-F---I-----H-R-----	498
RCK5	RM---T-----	345
RCK6	-M-----K-Q-----	395
S5		
S6		
RCK1	YFAEAEAEASHFSSIPDAFWWAVVSMTTVGYGDMYPVTIGGKIVGSLCAIAGVLTIALPV	404
RCK3	---DDPS-G-N-----T-----H-----	426
RCK4	---D-PTT-Q-----T-----K-I-V-----	558
RCK5	---D-RD-Q-P-----V-T-----	405
RCK6	---DDVD-L-P-----T-----M-V-----	455
RCK1	PVIVSNFNFYHRETEGEEQAQLLVSSPNLASDSDLR....RSSSTISKSEYMEIEE	459
RCK3	---YM--G-CQHL-S-AEEL..RKA--N--L-----V--	484
RCK4	---N---T---TQNAVSCPYP-L-N-LKKFRSST---LGD---L-M--	618
RCK5	---Y-Q-T-CPKIPS-PDLKK...S-A-----D---Q-	462
RCK6	---Q---G-YT---TCGQPTP-LKATDN.....	495
RCK1	DMNNSIAHYRQANIRTNCTATDQN....CVNKSLLTDV	495
RCK3	GGM-HS-FPQTPFKTGNSTATCTT-NNPNS---IK-IF---	525
RCK4	GVKE-LCGKEEKQCGK-DDSE--K-N....-S-AKAVE---	655
RCK5	GV---NEDF-EE-LK-A---LANT-....Y--IT-M---	498
RCK6GLGKPDFAEASRRSSYLPTPHRAYAEKRM--E-	530

Fig. 1. Amino acid sequence alignment of members of the RCK family. Identical amino acids are indicated by dashed line. Gaps (dotted lines) have been introduced to achieve maximum homology. Putative transmembrane segments (S1-S6) are indicated by solid bars. RCK-1, -3, -4 and -5 are from Baumann et al. [2] and Stühmer et al. [22]. Sequence alignment was obtained using EuGene software (Molecular Biology Information Resources, Department of Cell Biology, Baylor College of Medicine).

4-Aminopyridine (4-AP), tetraethylammonium chloride (TEA) and mast cell degranulating peptide (MCDP) were obtained from Sigma Chemical Co. (St. Louis, MO). Charybdotoxin (ChTX) was obtained from Latoxan (Rosans, France) and is prepared by the method of Gimenez-Gallego [9]. Noxiustoxin (NTX) and α -dendrotoxin (DTX) were the generous gifts of Dr L.D. Possani and Dr M.P. Blaustein, respectively.

Single channel recording was performed in oocytes dissected free of the vitelline envelope and patch clamped [12] using fire-polished, Sylgard-coated micropipettes of 2–5 M Ω resistance when filled with the test solution described above. Data acquisition and analysis was performed using pCLAMP software (Axon Instruments, Burlingame, CA). Single channel records were filtered at 500–1000 Hz and digitized at 1000–2000 Hz. Where appropriate, data are expressed as mean \pm SE.

3. RESULTS

3.1. Structural properties of RCK2

Putative K⁺ channel clones were isolated by hybridization screening of rat brain cDNA libraries with a K⁺ channel-specific oligonucleotide probe. Expression screening yielded a clone whose sequence (Fig. 1) and electrophysiological properties clearly place it in the

RCK family originally described by Baumann et al. [2] and its deduced amino acid sequence (Fig. 1) shows that it is nearly identical to RCK2, recently described by Grupe et al. [10] and KV2, recently described by Swanson et al. [23]. A single amino acid (leucine-241) is replaced by a serine in the previous reports [10,23].

Hydropathy analysis indicates six hydrophobic, putative membrane-spanning regions (S1–S6). This core region of the molecule shows strong sequence homology with all members of the RCK family [2,22]. Highest levels of sequence identity (up to 89%) were observed from the beginning of the S4 to the end of the S6 regions. Less conservation is evident in the terminal regions and the putative extracellular S1–S2 and S3–S4 linker regions which are longer in RCK2 than in the other RCK variants. These differences may account for some of the unique functional characteristics of RCK2 described below.

3.2. Electrophysiological properties of RCK2

The steady-state current/voltage (I–V) relationship for RCK2 is shown in Fig. 2. Voltage-dependent outward currents of 2–6 μ A (at a test potential of +50 mV) were recorded in oocytes injected with 10 ng RNA, whereas under the same experimental conditions, outward currents of less than 150 nA were recorded in uninjected oocytes. The expression of exogenous mRNA therefore is responsible for >90% of the outward current recorded in the injected oocytes. Panel A shows an I–V family of superimposed currents evoked by test pulses ranging from –50 to +50 mV, in 10 mV increments from a holding potential of –80 mV. The records are corrected for linear capacitive and leakage currents, and therefore represent the activation of a voltage-dependent conductance. Fig. 2A shows that RCK2 expresses an outward current which does not inactivate during 4.7 s pulses. A similar lack of inactivation is characteristic of RCK1 and RCK5; in contrast, RCK3 and RCK4 inactivate 80–100% over this time period [22]. We have not looked for ultra-slow components of inactivation which might become apparent when the holding potential is made more positive. It is clear, however, that RCK2 lacks fast inactivation.

In order to resolve the time course of activation, we recorded single channel currents in cell-attached membrane patches. Fig. 2B shows a typical recording of the ensemble average response evoked by 100 test pulses to 0 mV in a membrane patch containing only one channel. Activation was complete within about 15 ms. The average rise time (90% of peak) was 12.4 ± 2.2 ms ($n = 8$ patches) at 0 mV, a value within the range of activation times measured in other members of the RCK family [10,22].

Fig. 2C shows the steady-state voltage dependence of activation. The conductance/voltage (G – V) relationship was fitted by a Boltzmann distribution with

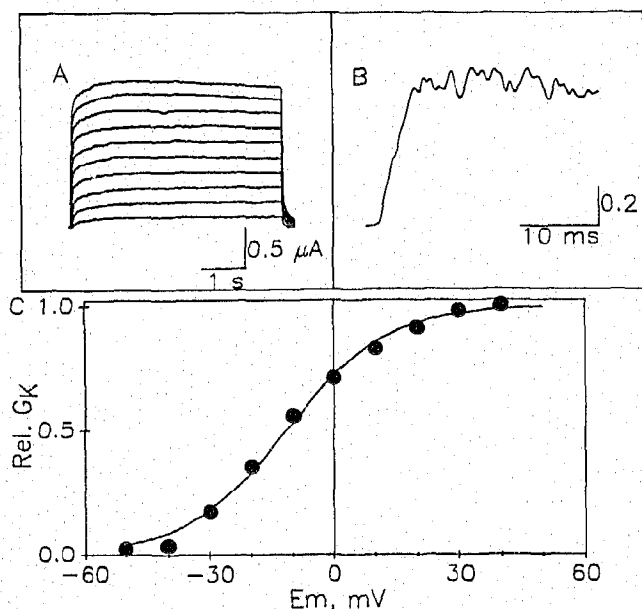


Fig. 2. Steady-state current/voltage relationship of RCK2. Oocytes injected with 10 ng mRNA transcript were voltage-clamped using two intracellular microelectrodes (A) and stimulated with a series of test pulses of –50 to +50 mV in 10 mV increments from a holding potential of –80 mV. Linear leakage and capacitive currents were subtracted digitally using a P/4 subtraction protocol. Panel (A) shows an I–V family obtained with 4.7 s pulses. Panel (B) shows the ensemble average of 100 test pulses to 0 mV in a cell-attached membrane patch which contained a single channel. The vertical calibration is units of probability of opening. In panel (C) steady-state currents from microelectrode measurements were converted to chord conductances using a reversal potential of –92 mV (measured in separate experiments), and fitted to Boltzmann distributions with midpoint of slope factor (mV): –11.5 and 12.2 for RCK2. Conductances were normalized to the maximum estimated from the Boltzmann fit.

average midpoint and slope factor of -12.3 ± 4.3 and 11.9 ± 1.7 mV ($n=6$), respectively. In the other members of the RCK family midpoints range from -22 to -34 mV and slope factors range from 5 to 17 mV [22]; RCK2 therefore has a relatively high threshold for activation compared with other members of the RCK family [10,22].

Fig. 3 demonstrates the $[K^+]_o$ -dependence of RCK2 currents. Reversal potentials were measured from tail currents evoked by a double pulse protocol shown in Fig. 3A. Conditioning pulses to 0 mV activated outward currents and test pulse potentials of -80 to $+10$

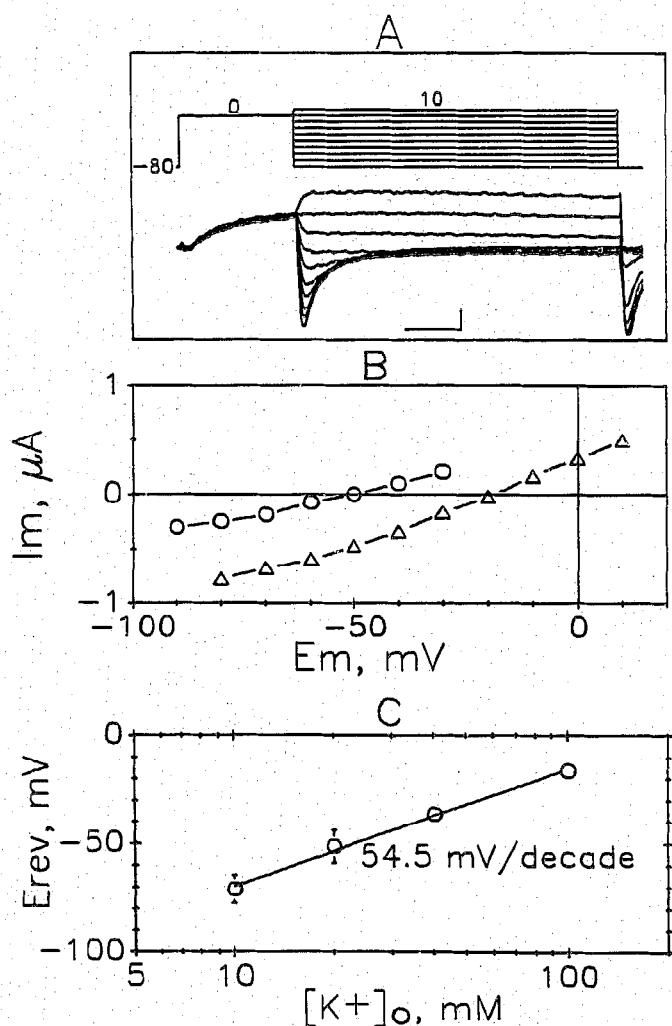


Fig. 3. Effects of changing extracellular $[K^+]_o$ on RCK2. Na^+ was replaced by the desired amount of K^+ . Tail currents were measured by the pulse protocol shown in panel (A). Lower set of superimposed traces in panel (A) show tail currents measured in 100 mM K^+ . In panel (B) peak tail currents are plotted as a function of test pulse potential, in 40 (circles) and 100 (triangles) mM K^+ . Zero current potentials were measured by interpolation of the isochronal $I-V$ curves and pooled data (linear axis) from 4 oocytes and plotted as a function of $[K^+]_o$ (logarithmic axis) in panel (C). Data are plotted as mean \pm SD. The straight line was fitted by least squares regression.

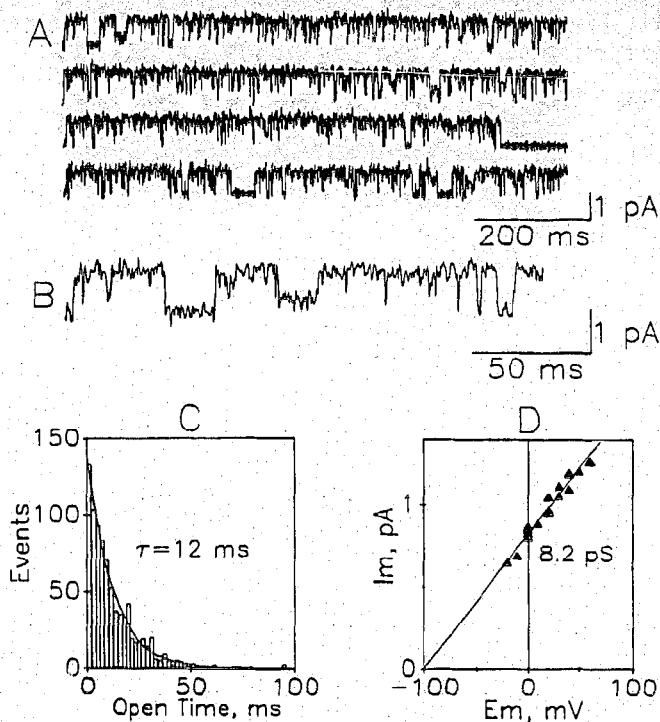


Fig. 4. Single channel RCK2 currents. Cell-attached patch clamp of RCK2-injected oocytes revealed a voltage-activated outward current which was absent in uninjected oocytes. Test pulses of varying amplitude were delivered repetitively at 0.5 Hz from a holding potential of -80 mV. Typical records of single channel activity are shown in (A) (low-pass filtered at 1000 Hz). Panel (B) shows the first 200 ms of the first trace in panel (A) at a faster time base. Records were idealized, amplitude histograms (not shown) and open time histograms (C) were constructed. Mean single channel amplitudes obtained by fitting the histograms to single Gaussian distributions and mean open times were obtained by fitting single exponential decay functions. Panel (D) shows unitary $I-V$ relationship for pooled data from 8 patches. The straight line is a least squares fit of the data.

mV were used to assess the fully activated conductance. Isochronal tail currents in 100 (triangles) and 40 (circles) mM $[K^+]_o$ are plotted as a function of test pulse potential in Fig. 3B. Reducing $[K^+]_o$ from 100 to 40 mM shifted the zero current potential by -30 mV. The pooled data from 5 experiments (Fig. 3C) show that the conductance expressed by RCK2 is highly selective for K^+ over Na^+ or Cl^- . The 54.5 mV/decade slope of the Nernst plot in Fig. 3C falls within the 61–53 mV/decade range measured in other RCK variants [22].

Single channel currents were measured in eight cell-attached patches. Representative records are shown in Fig. 4A,B. As shown in Fig. 4A channel activation evoked by test pulses to 0 mV occurred after a brief latency in long bursts which often lasted the entire duration of the test pulse. This feature can account for lack of inactivation in whole-cell RCK2 currents. At a faster time base (Fig. 4B) bursts were found to consist of long openings separated by brief (<1 ms) closed intervals. Closure of the channel to a subconductance state is also

illustrated in this record. The main conductance state had a mean open time of 12 ms (Fig. 4C) and mean amplitude of 0.89 pA. Single channel conductance estimated from pooled data in Fig. 4C was 8.2 pS (-10 to $+60$ mV), a value which is within the 4.7–10.2 pS range of conductances of other RCK variants [10,22]; and mean open time was 13.9 ± 1.3 ms ($n=8$ patches).

3.3. RCK2 pharmacology

We tested RCK2 sensitivity to K^+ channel blockers as summarized in Table I. Block was measured at one or more concentrations and the ratio of test/control currents evoked by test pulses to 10 mV, from a holding potential of -80 mV was used to estimate the IC_{50} , assuming single site binding [20]. RCK2 shows high sensitivity to all the blocking agents tested except ChTX. Our sample of ChTX blocked outward current ($IC_{50} \approx 30$ nM) in oocytes injected with another K^+ channel clone which is closely homologous to the ChTX-sensitive RCK1 channel. Thus, the ChTX insensitivity of RCK2 is a real feature of the channel rather than an artifact of inactive toxin. Interestingly, RCK2 is sensitive to noxiustoxin (NTX), a scorpion venom-derived peptide toxin which is related to ChTX by sequence homologies [26].

RCK2 is sensitive to block by α -dentrotoxin (DTX) with roughly the same potency as block of RCK1, whereas MCDP blocked RCK2 with the same potency as block of RCK5 [22]. RCK2 is sensitive to block by TEA ($IC_{50} = 1.7$ mM), consistent with the notion that delayed rectifiers are selectively blocked by this drug. However, as shown in Table I, RCK2 is also quite sensitive to 4-AP, which is often considered a selective blocker of I_A , a transient K^+ current. RCK2 appears to be slightly more sensitive to 4-AP than the other RCK variants (range 1–13 mM) [22].

4. DISCUSSION

After this paper was submitted two other groups described identical rat brain cDNA clones named RCK2 [10] and K_v2 [23], both of which encode a rat brain K^+ channel. Our results and those of Swanson et al. [23] agree quite closely, particularly with regard to ChTX insensitivity. Our electrophysiological results agree

quite closely with the work of Grupe et al. [10] who first described expression of RCK2 in oocytes. Both reports show that RCK2 codes for a K^+ channel which activates slowly (tens of milliseconds), has no inactivation in the range 500–5000 ms, and has sensitivity to several K^+ -channel blockers including DTX, MCDP, TEA and 4-AP. However, in our work and that of Swanson et al. [23], RCK2 was relatively insensitive to block by ChTX. This difference cannot arise from structural differences in the expressed channels since the deduced amino acid sequences of RCK2 reported by Grupe et al. [10] and that of the ChTX-insensitive K_v2 channel [23] are identical. A plausible explanation is that different toxins were used. Our sample of ChTX was prepared by the method of Gimenez-Gallego et al. [9] whereas that used by Grupe et al. [10] was prepared by the method of Miller et al. [16]. Swanson et al. [23] obtained toxins prepared by both methods and it is unclear which toxin was tested on their RCK2 channel. When both toxin preparations were tested on delayed rectifier currents in lymphocytes [3] the blocking potency of the latter preparation was 3-fold greater than that of the former. Only the polypeptide isolated by Gimenez-Gallego et al. [9] has been sequenced and it is known that *Leirus quinquestriatus* venom contains at least two closely related ChTX isoforms [13]. We suggest, therefore, that RCK2 may be sensitive to only one of the isoforms, whereas other members of the RCK family (e.g. RCK1) may be less discriminating. The use of synthetic toxins of known amino acid sequence may be necessary to resolve this issue.

Presently there is no evidence concerning the location of the critical amino acids for binding of K^+ channel peptide toxins other than ChTX (prepared by the method of Miller et al. [16] which is thought to bind to a site located on the extracellular linker between transmembrane segments S5–S6 [14]. It is known, however, that MCDP, DTX and ChTX all bind to protein receptors that, when deglycosylated, have a molecular weight similar to that estimated from RCK clones [18], and antibodies to synthetic polypeptides deduced from the mouse homolog of RCK1 recognize the purified rat brain DTX receptor [19]. In neurons these three toxins all interact allosterically with one another, suggesting that although they do not occupy the same site on the channel, their binding sites may be close together. One possibility is that all of the toxin sites are on the S5–S6 linker. In that case the MCDP- and DTX-resistance of both RCK3 and RCK4 may be due to subtle alterations in the variable regions of the S5–S6 linker. A possible site is located at RCK1 position 353 which is occupied by a negatively charged amino acid in RCK1, RCK5 and RCK2 but is occupied by either Ser or Thr in RCK3 and RCK4. Interestingly, in the latter two clones, position 352 is occupied by a helix-distorting Pro.

Studies of the functional characteristics of muta-

Table I

Effect of K^+ channel blockers on RCK2-induced current

Drug Conc.	Rel. block ^a	Est. IC_{50}
ChTX 0.2–0.7 μ M	0.11 @ 0.7 μ M	3.3 ± 1.5 μ M (6)
NTX 20–4000 nM	0.47 @ 200 nM	205 ± 103 nM (5)
MCDP 20–920 nM	0.51 @ 230 nM	202 ± 25 nM (3)
DTX 14 nM	0.38	24.7 ± 8.6 nM (5)
4-AP 1 mM	0.79	0.3 ± 0.1 mM (6)
TEA 1 mM	0.45	1.7 ± 0.5 mM (4)

^a Rel. block = $1 - (I_{K^+, drug} / I_{K^+, control})$

genized Na⁺ [21] and K⁺ channels [11,17], and natural K⁺ channel variants [22], have helped to identify the molecular machinery responsible for voltage-dependent gating. In both K⁺ channels and Na⁺ channels rapid, voltage-dependent inactivation is thought to be localized in intracellular loops which connect the adjacent subunits or pseudosubunit repeats in Na⁺ channels [4,21]. As noted by Stühmer et al. [22], among the members of the RCK family, fast inactivation is present only in the RCK4 variant which also has a long amino-terminus. RCK2, with its relatively short N-terminus and lack of inactivation, conforms to this principle. Site-directed mutagenesis should enable us to test the structure/function correlations suggested by comparisons between different delayed rectifier K⁺ channels.

Acknowledgements: We thank Drs L.D. Possani and M.P. Blaustein for the generous gifts of NTX and DTX; and G. Schuster's expert technical assistance. This work was supported by NS0885 (J.A.D.), Advanced Technology Program of the State of Texas, (R.H.J.); HL-36930 and NS-23877 (A.M.B.); and the American Heart Association (Texas affiliate, G.E.K.).

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