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Shaw-like rat brain potassium channel cDNA's with divergent 3' ends

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The complete amino acid sequence of a potassium channel protein of rat brain, Kv3.2b, plus a partial sequence of a related channel, Kv3.2c, are deduced from molecular cloning of the respective cDNA's. Kv3.2b and Kv3.2c share extensive amino acid sequence identity with a previously identified channel, RKShIIIA[1], before diverging to unique carboxy termini. Probes specific for Kv3.2b and RKShIIIA detect similarly sized mRNA's on Northern blots. These two proteins are encoded by a single gene based on genomic Southern blotting, and therefore arise by alternative splicing. In vitro transcribed mRNA for Kv3.2b induces the expression of outward K^+ currents in *Xenopus* oocytes under voltage-clamp conditions.

Potassium ion channel; cDNA cloning; cDNA expression; Alternative splicing; Nothern blotting

1. INTRODUCTION

In *Drosophila*, both gene duplication and alternative splicing mechanisms have generated diversity in K^+ channel function [2]. In mammals, the existence of a family of homologs to the *Drosophila* Shaker K^+ channels [3,4] suggests previous gene duplication events. Alternative splicing is also observed, as demonstrated by the recent cloning of K_v4 [5], a rat homolog of the *Drosophila* Shaw K^+ channel [6]. We report here the molecular cloning of rat brain cDNA's containing one partial and one full length K^+ channel coding sequence, and present evidence demonstrating that they arise by alternative splicing of a transcript from the gene that also encodes a previously identified K^+ channel, RKShIIIA [1].

2. MATERIALS AND METHODS

Isolation of total and the poly-A^+ fraction of brain RNA from Sprague–Dawley rats was by standard techniques [7]. cDNA was synthesized (You-prime kit, Pharmacia), ligated into λgt10 arms (Promega) and packaged for plating (Gigapack Gold, Stratagene). RNA blots were prepared, and oligonucleotide probes synthesized and radiolabelled, as described [5]. Genomic DNA blots were prepared as described [5] and probed with restriction fragment DNA radiolabelled by the random priming method [8]. Hybridization was in 20% (RNA blots) or 50% (genomic DNA blots) formamide, 5 × SSPE, 5 × Denhardt's, 0.5% SDS, 50 μg/ml salmon sperm DNA, at 42°C overnight; filters were washed to a final stringency of 0.1 × SSC at 65°C. First strand cDNA was amplified by PCR as described by the manufacturer (Perkin-Elmer-Cetus); amplifications were run for 40 cycles of 40 s at 94°C, 1 min at 55°C, and 3–5 min at 72°C. The products were subcloned into $pKSII^+$ (Stratagene) and sequenced by the chain termination method (Sequenase kit, USB). Oligonucleotides were synthesized on a commercial DNA synthesizer (Applied Biosystems). The actin probe was used as described [3]. Sequences were analyzed with Genetics Computer Group, Inc. and Intelligenetics PC/Gene software.

For the construction of a full-length coding template for Kv3.2b, a short cDNA 87-6, extending from bp −54 through bp +318 of RKShIIIA[1], was generated by PCR and its sequence verified as above. Downstream sequence was contained in overlapping cDNA's L7-2 and L1-14 previously isolated from a rat brain λgt10 library. These clones were ligated together, truncated at a *Kpn*I site 485 bases into the 3' untranslated sequence, and cloned into pGEM-A [3]. The resulting clone, Kv3.2b, has a sequence identical to RKShIIIA from bp −55 through +1781 [1], then diverges to the unique 3' end of L1-14; this cDNA sequence was previously demonstrated by PCR to exist in cDNA synthesized from total rat brain mRNA (see section 3). For expression of Kv3.2b, the plasmid was linearized with *Not*I, and RNA was transcribed with T7 RNA polymerase. *Xenopus* oocytes were isolated, injected with approximately 50 nl of 1 mg/ml RNA, and voltage-clamped, as described previously [3].

Kv3.2b and Kv3.2c sequences have been submitted to the Genbank/EMBL database under accession numbers M59211 and M59313.

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3. RESULTS AND DISCUSSION

An adult rat brain cDNA library was screened with a probe, O123 [5], based on a conserved region of two previously identified K^+ channel cDNA's, NGK2[9] and K_v4 [5], and two clones, L1-6 and L1-14, were isolated. The L1-14 cDNA sequence is identical to bp +604 through +1781 of a previously identified K^+ channel, RKShIIIA[1] (see Fig. 1, legend), then diverges to a unique 3' end. L1-6 identical to bp +1280 through +1781 of RKShIIIA, and also diverges to another unique 3' end. L1-14 and L1-6 contain 871 and 652 bp of sequence below their respective first in-frame STOP codons. Each clone contains an almost identical

insertion of approximately 100 bases in the region of overlap with RKShIIIA (at thymidine-1616 [1]), generating a frameshift which results in premature termination of the ORF. Comparison of these sequences to RKShIIIA and subsequently isolated PCR clones suggests that L1-6 and L1-14 represent incompletely spliced mRNA's, as was observed with another rat brain K^+ channel cDNA [5].

L1-14 formed the basis for generation of a full length coding sequence for a channel we have termed Kv3.2b, in keeping with a proposed nomenclature for K^+ channel clones (K. Chandy, et al., personal communication). Rat brain cDNA was used as a template for amplification by PCR, with the 5' primer based on an 18 base region of the 5' end of RKShIIIA (nt −54 through −37[1]) and the 3' primer based on an 18 base region within the unique 3' end of L1-14 (the sequence 28 through 45 bases 3' to the STOP codon). The

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Kv3.2b  MGKIE.NNE.RVILNVGGTRHETYRSTLKTLPGTRLALLASSEPQGDCLTAAGDKLQPLPPPLSPPPRPPPLSPVPSGCF   78
  Kv4   MGQGD.ESE.RIVINVGGTRHQTYRSTLRTLPGTRLAWLAEPDAHSHFDYDPPA.....                        52
 Shaw   MNLINMDSENRVVLNVGGIRHETYKATLKKIPATRLSRLTEALAN....YDPIL.....                        50

Kv3.2b  EGGAGNCSSHGGNGSDHPGGGREFFFDRHPGVFAYVLNYYRTGKLHCPADVCGPLFEEELAFWGIDETDVEPCCWMTYRQ  158
  Kv4   .....DEFFFDRHPGVFAHILNYYRTGKLHCPADVCGPLYEEELAFWGIDETDVEPCCWMTYRQ  111
 Shaw   .....NEYFFDRHPGVFAQVLNYYRTGKLHYPTDVCGPLFEEELEFWGLDSNQVEPCCWMTYTQ  109

Kv3.2b  HRDAEEALDIFETPDLIGGDPGDDEDLGGKRLGIEDAAGLGG.......PDGK.SGRWRKLQPRMWALFEDPYSSRAARF  230
  Kv4   HRDAEEALDSFGGAPLDNSADDADADGPGDSGDGEDELEMTKRLALSDSPDGRPGGFWRRWQPRIWALFEDPYSSRYARY  191
 Shaw   HRDTQETLAVLDRLDLDTEKPSEEELARKFGFEEDYYKGTISWWQ.....EMK.........PRIWSLFDEPYSSNAAKT  175

                S1                                                          S2
Kv3.2b  IAFASLFFILVSITTFCLETHEAFN..IVKNKTEP..VINGTSAVLQYEIETD..PALTYVEGVCVVWFTFEFLVRIVFS  304
  Kv4   VAFASLFFILVSITTFCLETHERFN.PIV.NKTEIENVRNGTQVRYYREAETE..AFLTYIEGVCVVWFTFEFLMRVVFC  267
 Shaw   IGVVSVFFICISILSFCLRTHPDMRVPIVRNITVKTA..NGSNGWFLDKTQTNAHIAFFYIECVCNAWFTFEILVRFISS  253

                        S3                                  S4
Kv3.2b  PNKLEFIKNLLNIIDFVAILPFYLEVGLSGLSSKAAKDVLGFLRVVRFVRILRIFKLTRHFVGLRVLGHTLRASTNEFLL  384
  Kv4   PNKVEFIKNSLNIIDFVAILPFYLEVGLSGLSSKAAKDVLGFLRVVRFVRILRIFKLTRHFVGLRVLGHTLRASTNEFLL  347
 Shaw   PNKWEFIKSSVNIIDYIATLSFYIDLVLQRFASH..LENADILEFFSIIRIMRLFKLTRHSSGLKILIQTFRASAKELTL  331

          S5                                                          S6
Kv3.2b  LIIFLALGVLIFATMIYYAERVGAQPNDPSASEHTQFKNIPIGFWWAVVTMTTLGYGDMYPQTWSGMLVGALCALAGVLT  464
  Kv4   LIIFLALGVLIFATMIYYAERIGAQPNDPSASEHTHFKNIPIGFWWAVVTMTTLGYGDMYPQTWSGMLVGALCALAGVLT  427
 Shaw   LVFFLVLGIVIFASLVYYAERI..QPN.P....HNDFNSIPLGLWWALVTMTTVGYGDMAPKTYIGMFVGALCALAGVLT  404

Kv3.2b  IAMPVPVIVNNFGMYYSLAMAKQKLPRKRKKHIPPAPLASSPTFCKTELNMACNSTQSDTCLGKENRLLEHNRSV.....  539
  Kv4   IAMPVPVIVNNFGMYYSLAMAKQKLPKKKKKHIPRPPQLGSPNYCKSVVNSPHHSTQSDTCPLAQEEILEINRADSKLNG  507
 Shaw   IALPVPVIVSNFAMFYSHTQARAKLPKKRRRVLPVEQPRQPRLPGAPGGVSGCGTPGSGPHSGPMGSGGTGPRRMNNKTK  484

                                                                X
Kv3.2c                                                  ASTLEPMESTSQTKGDTRPE  ---
RkShIIIA                                                DNCKDV.VITGYTQAEARSL  612
Kv3.2b  ......LSGDDSTGSEPPLSPPERLPIRRSSTRDKNRRGETCFLLTTGDYTCASDGGIRKGYEKSRSLNNIAGLAGNALR  613
  Kv4   EVAKAALANEDCPHIDQALTPDEGLPFTRSGTRERY..G.PCFLLSTGEYACPPGGGMRKDLCKESPVIAKYMPTEAVRV  584
 Shaw   DLVSPKSDMAFSFD                                                               498

Kv3.2c  AHWNCAHLLNFGCPTGSSFPTL                                                         ---
RkShIIIA T                                                                            613
Kv3.2b  LSPVTSPYNSPCPLRRSRSPIPSIL                                                     638
  Kv4   T                                                                             585
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Fig. 1. Deduced amino acid sequences for Shaw [6] and four rat Shaw homologs, Kv3.2b, Kv3.2c, RKShIIIA[1], K,4 [5]. The RKShIIIA sequence is based on a cDNA sequence identical to that published, except that the thymidine at nt 1663 [1] has been removed. (We isolated a partial RKShIIIA cDNA by PCR spanning bp 1259 through 1863 (extending 82 bp into the unique 3' end) of the published sequence. Neither this clone, nor the Kv3.2b or Kv3.2c cDNA's contain this extra T. Comparison of the translation of RkShIIIA, without the one base insertion, with that of K,4 reveals significant homology below the frameshift; 29/50 amino acids are identical). Amino acid sequence for RKShIIIA, N-terminal to that shown, is identical to Kv3.2b. Kv3.2c sequence is based on clone L1-6 and PCR clone B3-1, with sequence identity to Kv3.2b N-terminal to that shown through Phe-428 (see text). Putative transmembrane regions S1-S6 [2,10] are boxed. (●) leucine repeats (see text); (▼) potential sites for N-linked glycosylation; (*) potential cAMP-dependent kinase recognition site; (arrow) point of divergence of Kv3.2 family. Potential sites in the unique C-terminal ends for casein kinase II are also shown for Kv3.2c (+) and RKShIIIA (×).

resulting clone has 36 bp of 5' untranslated and 1780 bp of coding sequence identical to RKShIIIA before diverging to the unique 3' end of L1-14, and did not include the insertion present in L1-14 (above). Including L1-14 sequence, this Kv3.2b cDNA spans 2605 bp, with an open reading frame that could encode a protein of 638 amino acids, of which the N-terminal 593 are identical to RKShIIIA (Fig. 1). The Kv3.2b protein sequence contains six hydrophobic regions, suggesting six membrane spanning domains, S1-S6, as observed in other cloned K^+ channels [2,10]. The S4 region contains a series of six positive charges at every third position, forming a possible voltage sensor for the channel [2,10].

An analogous PCR was attempted with a 3' primer based on the unique sequence of L1-6, without success, suggesting that this cDNA may not be co-linear with the 5' sequence of Kv3.2b and RKShIIIA. PCR amplification using the same 3' primer with a 5' primer located between S5 and S6 produced a clone, B3-1, that did not have the insertion mentioned above, but was otherwise identical to L1-6. Therefore the deduced partial amino acid sequence for Kv3.2c, presented in Fig. 1, is based on the L1-6 sequence, without this insertion. None of the C-termini shown are homologous to each other or any other known ion channels or sequences in the combined Genbank/EMBL database. Also, none of the three isotypes, Kv3.2b, Kv3.2c, and RKShIIIA, share any sequence in the 3' untranslated regions.

Kv3.2b, Kv3.2c, and RKShIIIA share a consensus site for phosphorylation by cAMP-dependent protein kinase (Ser-564 in Kv3.2b)[11], absent in the homologous K^+ channel proteins K_v4, NGK2, and Shaw. Shaker and mammalian Shaker homologs also conserve such a site in their C-terminal regions [3,4]. Kv3.2b contains several consensus sites for other kinases throughout the sequence shared with RKShIIIA (not shown). Within their unique carboxy terminal domains, however, Kv3.2c has three potential casein kinase II recognition sites [12], RKShIIIA, one, and Kv3.2b, none (Fig. 1), suggesting the possibility of differential phosphorylation of these three channels. Kv3.2b contains two consensus sites for N-linked glycosylation[13], Asn-259 and Asn-266, in the S1-S2 loop, as observed in most other K^+ channel proteins [3,4]. Two other N-glycosylation sites, Asn-84 and Asn-91, exist in the proline-rich region of the N-terminus [1], and two in the C-terminus, Asn-518 and Asn-536. Kv3.2b shares conserved charges in the putative S2 and S3 transmembrane regions with K_v4 and Shaw, similar to those observed in the rat Shaker homologs [3,4]. Kv3.2b, K_v4, and Shaw also share imperfect heptad repeats of leucine overlapping the S4 region, the putative 'leucine zipper' [14], each substituting phenylalanine for a leucine at one position. The functional roles of two interesting regions of Kv3.2b, a proline-rich segment in the N-terminus [1],

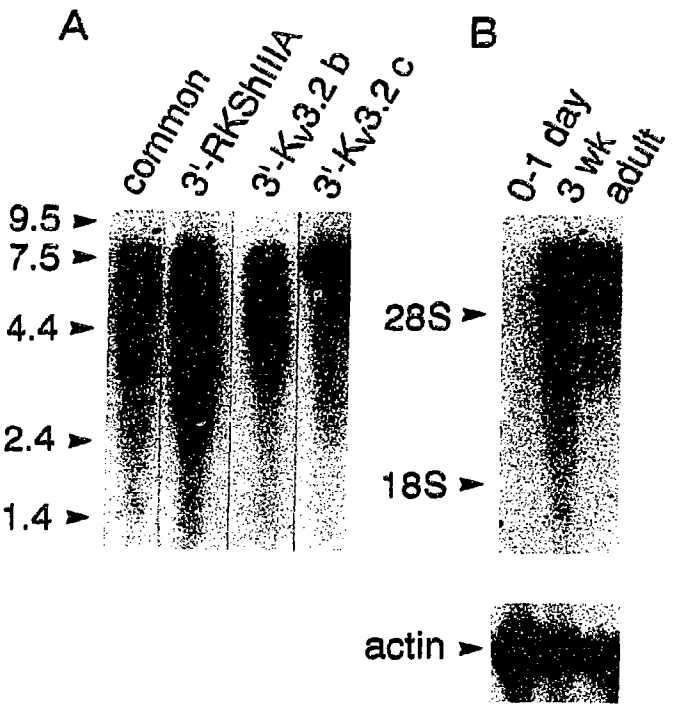

Fig. 2. (a) Northern blots of poly-A^+ brain RNA (5 μg) isolated from 3-week-old rats and hybridized with oligonucleotide probes. The common probe contained a sequence spanning 100 bp immediately 5' to the divergence of Kv3.2b and RKShIIIA; the specific probes for Kv3.2b, Kv3.2c, and RKShIIIA contained sequence spanning the first 100 bp of 3' divergence for each. (b) Northern blot of poly-A^+ brain RNA from different aged rats, probed with the common probe of panel (a). The result of reprobing this blot with an actin probe is also shown.

and a repeat of five lysines and/or arginines C-terminal to S6, also shared with NGK2, K_v4 and Shaw, are unknown.

Probes based on the unique sequence of Kv3.2b or unique sequence of RKShIIIA detect a similar pattern of RNA's on Northern blots probed under high stringency (Fig. 2), hybridizing to transcripts of 7.5 kb and 6.5 kb. This suggests the possibility of differential exon usage beyond that observed in the carboxy terminal region of these channels. In contrast, a probe specific to Kv3.2c detected only the 7.5 kb transcript, similar to the Drosophila Shaw mRNA[6]. A probe based on the shared sequence of these three clones detected no additional bands, and demonstrated a sharp increase in expression of these mRNA's between birth and three weeks of age. Another probe derived from the common region hybridized to only a single restriction fragment in Southern blots of genomic DNA (Fig. 3), demonstrating that the region encoding the sequence shared by Kv3.2b, Kv3.2c, and RKShIIIA is represented only once in the genome.

Kv3.2b cRNA was transcribed in vitro and injected into *Xenopus* oocytes, where it induced the expression of large outward K^+ currents under voltage-clamp conditions (Fig. 4). Currents were of the delayed rectifier

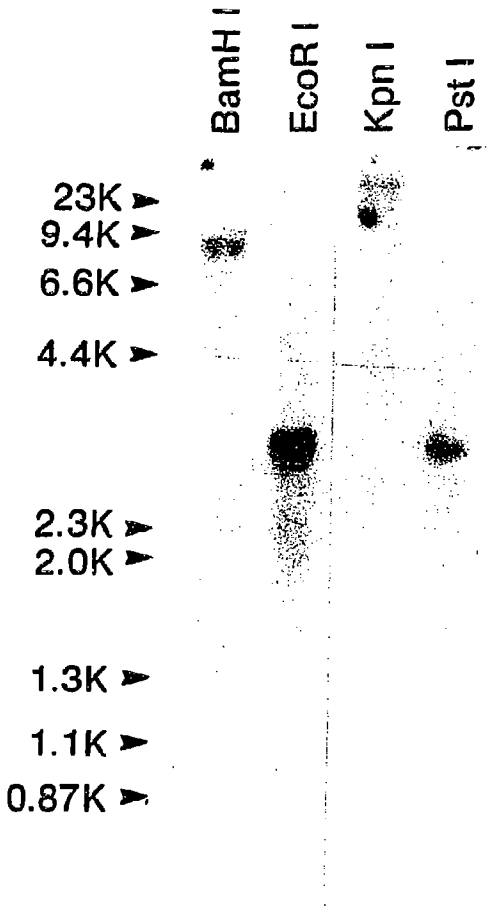

Fig. 3. Genomic Southern blot. Rat genomic DNA (20 μg) was digested to completion with the enzymes indicated and resolved on a 0.8% agarose gel. The DNA was blotted to Hybond-N and probed with a ^{32}P-labelled random-primed DNA fragment (bp 850 through 1617 of the Kv3.2b coding sequence plus 86 bp of intron) which spans a portion of the common sequence. A single restriction fragment hybridized to the probe in each digest, suggesting that the region common to Kv3.2b, Kv3.2c, and RKShIIIA is represented only once in the rat genome.

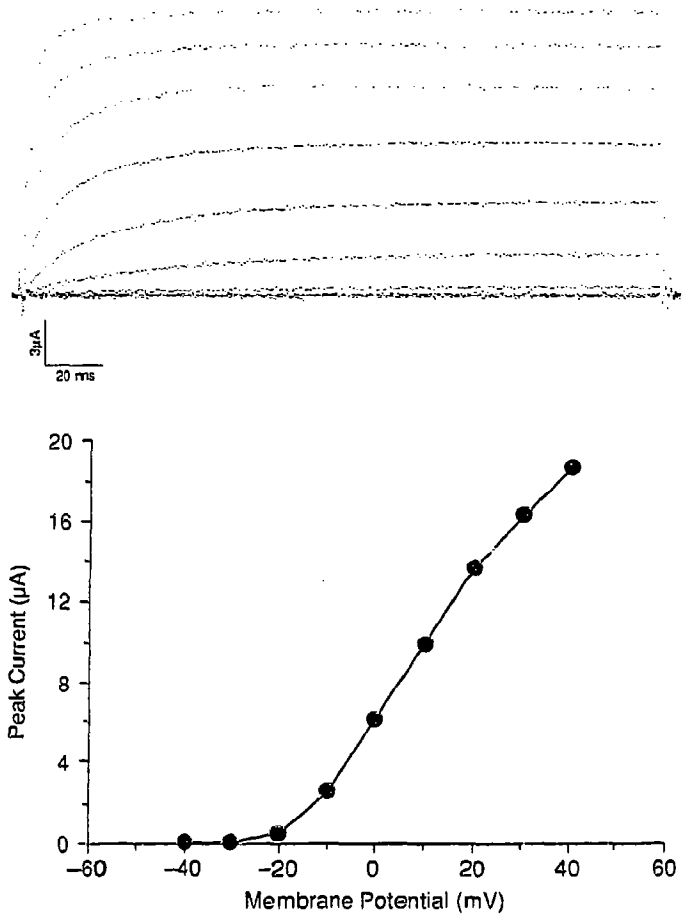

Fig. 4. Expression of Kv3.2b in *Xenopus* oocytes. Representative whole cell currents elicited under voltage clamp by injection of Kv3.2b cRNA are shown (top) along with the resulting current-voltage relationship (bottom). Oocytes were voltage-clamped at −80 mV and depolarizing voltage pulses 225 msec in duration were applied in 10 mV steps up to 40 mV. Currents were recorded in OR-2(-Ca^{2+}) buffer (82.5 mM NaCl, 2.5 mM KCl, 1.1 mM $MgCl_2$, 5 mM HEPES, pH 7.6). Data were collected at 2 kHz sampling rate, filtered at 1 kHz and analyzed using PClamp software (Axon Instruments).

type with a 10-90% risetime at 40 mV of 12 ms. No inactivation was apparent during 225 ms pulses. Half maximal inhibition ($n = 4$ eggs) was obtained at approximately 0.15 mM TEA and 0.3 mM 4-aminopyridine; charybdotoxin at 10 nM was ineffective. These data are very similar to those reported for both NGK2 and RKShIIIA [1,9]. Therefore, no effect of the different carboxy termini of Kv3.2b and RkShIIIA on their expressed currents has been detected. This suggests other roles for this domain, such as in differential post-translational modification, interaction with regulatory proteins or ligands, or subcellular localization [15]. These rat Shaw homologs share an almost identical S5-S6 region, implicated as being near to or forming the mouth of the channel's pore [1,10]; this region is 9 amino acids longer than those of rat Shaker and Shab homologs, and 7 amino acids longer than Shaw - suggesting a somewhat different structure for this domain. Selectivity to K^+, however, is comparable to other cloned rat K^+ channels (not shown), although sensitivity to TEA blockade is somewhat greater than that of K^+ channels Drk1 [16] and Kv2 [3] that also contain consensus tyrosines for TEA sensitivity[17](Tyr-444 of Kv3.2b).

The Kv3.2 and K_v4/NGK2 families, and Shaw, are clearly related proteins. Kv3.2b and K_v4 share 50% and 47% amino acid identity with Shaw, respectively, according to the alignment of Fig. 1, or 65% and 62% similarity if conservative substitutions are allowed (ST, QNED, HKR, LIVM, FYW, AG). Kv3.2b shares 72% amino acid identity with K_v4, or 79% similarity, as above. The transmembrane and amino terminal domains are the most highly conserved. The S1-S2 loop, which displays little or no sequence homology among cloned K^+ channels, exhibits 59% amino acid identity between Kv3.2b and K_v4. Finally, various Kv3.2 cDNA

isolates (not shown) contained what appeared to be introns at three positions analogous to established splicing locations of the K_v4/NGK2 gene [5] (at the beginning of S1, at the divergence point of K_v4 and NGK2, and at Lys-564 of Kv4), suggesting a similar exon structure for the two genes.

We have presented evidence from sequence analysis, RNA blotting, and genomic DNA blotting that Kv3.2b, Kv3.2c, and RKShIIIA arise by alternative splicing. This is analogous to the transcription of the K_v4/NGK2 gene, where different 3' exons have been demonstrated to generate distinct isotypes of that channel.

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REFERENCES

[1] McCormack, T., Vega-Saenz de Miera, E.C. and Rudy, B. (1990) Proc. Natl. Acad. Sci. (USA) 87, 5227-5231.
[2] Jan, L.N. and Jan, Y.N. (1990) Trends Neurosci. 13, 415-419.
[3] Swanson, R., Marshall, J., Smith, J.S., Williams, J.B., Boyle, M.B., Folander, K., Luneau, C.J., Antanavage, J., Oliva, C., Buhrow, S.A., Bennett, C., Stein, R.B. and Kaczmarek, L.K. (1990) Neuron 4, 929-939.
[4] Stuhmer, W., Ruppersberg, J.P., Schroter, K.H., Sakmann, B., Stocker, M., Giese, K.P., Perschke, A., Baumann, A. and Pongs, O. (1989) EMBO J. 8, 3235-3244.
[5] Luneau, C.J., Williams, J.B., Marshall, J., Levitan, E.S., Oliva, C., Smith, J.S., Antanavage, J., Folander, K., Stein, R.B., Swanson, R., Kaczmarek, L.K. and Buhrow, S.A. (1990) Proc. Natl. Acad. Sci. USA, in press.
[6] Butler, A., Wei, A., Baker, K., and Salkoff, L. (1989) Science 243, 943-947.
[7] Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1987) Current Protocols in Molecular Biology, Wiley, New York.
[8] Feinberg, A.P. and Vogelstein, B. (1983) Anal. Biochem. 132, 6-13.
[9] Yokoyama, S., Imoto, K., Kawamura, T., Higashida, H., Iwabe, N., Miyata, T. and Numa, S. (1989) FEBS Lett. 259, 37-42.
[10] Catterall, W.A. (1988) Science 242, 50-61.
[11] Krebs, E.G. and Beavo, J.A. (1979) Annu. Rev. Biochem. 48, 923-959.
[12] Kuenzel, E.A., Mulligan, J.A., Sommercorn, J. and Krebs, E.G. (1987) J. Biol. Chem. 262, 9136-9140.
[13] Hubbards, S.C. and Ivatt, R.J. (1981) Annu. Rev. Biochem. 50, 555-583.
[14] McCormack, K., Campanelli, J.T., Ramaswami, M., Mathew, M.K., Tanouye, M.A., Iverson, L.E. and Rudy, B. (1989) Nature 340, 103.
[15] Smith, C.W.J., Patton, J.G. and Nadal-Ginard, B. (1989) Annu. Rev. Genet. 23, 527-577.
[16] Frech, G.C., VanDongen, A.M.J., Schuster, G., Brown, A.M. and Joho, R.H. (1989) Nature 340, 642-645.
[17] MacKinnon, R. and Yellen, G. (1990) Science 250, 276-279.

Note added in proof

McCormack et al. published the corrected sequence of RKShIIIA after this manuscript was submitted (Proc. Natl. Acad. Sci. USA 88 (1991) 4060).