Distinct Spatial and Temporal Expression Patterns of K+ Channel mRNAs from Different Subfamilies

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Different types of K+ channels play important roles in many aspects of excitability. The isolation of cDNA clones from *Drosophila, Aplysia, Xenopus,* and mammals points to a large multigene family with several distinct members encoding K+ channels with unique electrophysiological and pharmacological properties. Given the pivotal role K+ channels play in the fine tuning of electrical properties of excitable tissues, we studied the spatial and temporal basis of K+ channel diversity. We report the isolation of two putative K+ channels that define two new subfamilies based upon amino acid sequence similarities with other known K+ channels. Northern blot and *in situ* hybridization studies revealed differences in the spatial and temporal expression patterns for these two new clones along with mRNAs from other K+ channel subfamilies. Two of the K+ channels studied are predominantly expressed in the brain. One of the "brain-specific" K+ channels is first expressed after about 2 weeks of postnatal cerebellar development and remains at levels about 10-fold higher in the cerebellum than in the rest of the brain.

Potassium (K+) channels are plasma membrane proteins that are ubiquitous in both excitable and nonexcitable cells. Major functions of the voltage-gated K+ channels include determination of the resting membrane potential, shaping of the action potential, modulation of transmitter release, and involvement in rhythmic firing patterns (Hille, 1984; Catterall, 1988; Jan and Jan, 1989). Many cell types express subpopulations of K+ channels that are characterized by different functional and pharmacological properties (Rudy, 1988). Given the wide diversity of K+ channels and the crucial role they play in controlling the proper functioning of excitable tissues, it is important to elucidate the spatial and temporal basis of channel diversity at the molecular level.

Several Shaker cDNA clones coding for one particular K+ channel type, the A-channel, had been isolated from *Drosophila* (Baumann et al., 1987, 1988; Kamb et al., 1987, 1988; Papazian

et al., 1987; Tempel et al., 1987; Iverson et al., 1988; Pongs et al., 1988; Schwarz et al., 1988; Timpe et al., 1988; Butler et al., 1989; Wei et al., 1990). *In vitro* synthesized RNA of these clones, when microinjected into *Xenopus* oocytes, gave rise to voltage-dependent, fast-transient outward K+ currents (I_A), characteristic of A-type channels. Using a Shaker clone, members of three new K+ channel subfamilies, Shab, Shaw, and Shal, were isolated (Butler et al., 1989; Wei et al., 1990). The *Drosophila* clones were also used to isolate rat, mouse, frog, *Aplysia,* and human cDNA clones (Stühmer et al., 1988, 1989; Tempel et al., 1988; Christie et al., 1989; McKinnon, 1989; Yokoyama et al., 1989; Chandy et al., 1990; Grupe et al., 1990; Koren et al., 1990; McCormack et al., 1990; Ribera, 1990; Swanson et al., 1990; Kirsch et al., 1991; Luneau et al., 1991; Pfaffinger et al., 1991; Philipson et al., 1991; Roberds and Tamkun, 1991; Tamkun et al., 1991). Our laboratory used the approach of expression cloning in *Xenopus* oocytes to isolate the rat brain K+ channel DRK1 (Frech et al., 1989). This isolate expressed a delayed rectifier-type current, and the amino acid sequence showed a high degree of similarity to Shab.

Based upon similarities throughout the core region, all of the mammalian K+ channel clones isolated belong to one of the four subfamilies, originally defined in *Drosophila* as Shaker, Shab, Shaw, and Shal. The only exception is a very slowly activating K+ channel (I_{sk}), which is completely unrelated (Takumi et al., 1988). Members within the same subfamily are approximately 70% identical at the amino acid sequence level, whereas members among different subfamilies show 40–50% sequence identity (Wei et al., 1990). The Shaker subfamily includes several *Drosophila* splice variants and the rat K+ channels RCK1–5. However, in contrast to the fast transient I_A currents elicited by the Shaker transcripts, *in vitro* mRNA generated from most mammalian isolates yielded K+ channels in *Xenopus* oocytes that gave voltage-sensitive, sustained or slowly inactivating outward currents (I_k), characteristic of delayed-rectifier K+ channels. The second subfamily contains Shab and DRK1, both channels characterized by slowly activating, slowly inactivating, sustained outward currents (Frech et al., 1989; Wei et al., 1990). The third subfamily includes Shaw in *Drosophila,* NGK2 (from a mouse neuroblastoma–rat glioma hybrid) (Yokoyama et al., 1989), and the rat brain isolates RKShIII (McCormack et al., 1990), Raw3 (Schröter et al., 1991), and K$_v$4 (Luneau et al., 1991). So far, the only fast-inactivating mammalian K+ channels are RCK4 of the Shaker subfamily and Raw3, a rat homolog in the Shaw subfamily. The fourth subfamily, Shal, contains the *Drosophila* isolate and RK5, a rat heart K+ channel (Roberds and Tamkun, 1991).

The fact that *Drosophila* and mammalian K+ channels can be

allocated to four different subfamilies, and the evidence that K^+ channels with different functional and pharmacological properties exist, prompted a search for new members of as yet unknown K^+ channel subfamilies. Here, we report the isolation of two putative K^+ channels that define two new subfamilies based upon similarities of the amino acid sequences in the core region. So far, attempts to express cRNA in *Xenopus* oocytes have not been successful. Northern blot and *in situ* hybridization studies reveal differences in the spatial distribution and temporal expression patterns for these two new types along with other K^+ channel mRNAs.

Materials and Methods

Recombinant DNA techniques. Unless otherwise stated, standard recombinant DNA procedures were used for handling of RNA and DNA, subcloning, DNA sequencing, and radiolabeling (Berger and Kimmel, 1987; Sambrook et al., 1989).

cDNA libraries and cloning. Three different cDNA libraries made from rat brain polyA$^+$ RNA in λZAP were used (Frech and Joho, 1989). The libraries were made from size-fractionated mRNA between 2.6–3.3 kilobases (kb), 3.3–4.2 kb, and 3.3–5.3 kb. The cDNAs from the first two size cuts were used to construct directional libraries with mean insert sizes of 2.4 and 3.9 kb. The cDNA from the largest size cut was used to generate a nondirectional library with a mean insert size of 4.2 kb. The libraries were screened at low stringency either with a ^{32}P-labeled oligonucleotide corresponding to the conserved amino acid sequence Asn-Glu-Tyr-Phe-Phe-Asp-Arg (positions 77–83 in DRK1) for RCK1, RCK2, and K$_v$4, or with a nick-translated DRK1 probe for IK8 and K13. All isolates encompassed the entire coding region, except for K13, which was truncated at the 5' end. After a second round of screening, a full-length clone for K13 was isolated.

Northern analysis. Total RNA was isolated by homogenizing tissues in 4 M guanidinium isothiocyanate and centrifugation through a cushion of 6.2 M CsCl (Chirgwin et al., 1978). PolyA$^+$ was prepared by chromatography on oligo-dT–cellulose (Collaborative Research). Samples (3 μg except where noted) were run on 1.0% agarose-formaldehyde denaturing gels. The RNA samples were transferred to Hybond-N nylon membranes (Amersham). Before and after transfer the gels were stained with ethidium bromide to assess the amount of RNA loaded and the extent of transfer. Prehybridization (1–4 hr) and hybridization (16–24 hr) were done at 42°C for randomly primed ^{32}P-labeled DNA probes (K$_v$4 and K13) or at 68°C in the case of ^{32}P-labeled RNA probes (DRK1, RCK2 and IK8) in 50% formamide, 5 × 150 mM NaCl, 10 mM NaH$_2$PO$_4$ (pH, 7.0), 1 mM EDTA (SSPE), 5 × Denhardt's solution, and 0.5% SDS. Membranes were washed in 0.1 × SSPE, 0.1% SDS at 68°C for at least 1 hr and subsequently exposed at −80°C on Fuji RX film for 1–6 d with an intensifying screen.

In situ hybridization. The protocol of Simmons et al. (1989) was followed. Briefly, rats were perfused with 4% paraformaldehyde. Brains were removed and postfixed overnight in 10% sucrose in 4% paraformaldehyde solution. Coronal sections (25 μm thick) were cut using a sliding microtome, mounted on subbed and poly-L-lysine–coated slides, and treated with 10 μg/ml proteinase K at 37°C for 30 min. Hybridization was done in 50% formamide, 0.3 M NaCl 10% dextran sulfate, 1 × Denhardt's, 2 mM Tris HCl (pH 8.0), 5 mM EDTA, 10 mM dithiothreitol, and 0.5 mg/ml tRNA. ^{35}S-labeled antisense or sense RNA transcripts (labeled with 1200 Ci/mmol ^{35}S-UTP; 10,000 cpm/μl) were used as probes for positive signals and nonspecific background, respectively. The antisense probes were derived from the 3' untranslated regions to maximize K^+ channel subtype specificity. Following hybridization, the sections were treated with 10 μg/ml RNAase A at 37°C for 30 min. Finally, sections were washed at 47–57°C in 15 mM NaCl, 1.5 mM Na-citrate (pH, 7.0) and dehydrated in ethanol. Sections were exposed on

Fuji RX film for 4–5 d. Brain structures were identified according to Paxinos and Watson (1986).

Amino acid sequence alignments. K^+ channel amino acid sequences were broken down into smaller regions of known similarity (in Fig. 1, bracketed by ⟨ ⟩) and aligned using EUGENE software (Molecular Biology Information Resources, Department of Cell Biology, Baylor College of Medicine). The nonaligned regions were typed in manually. Gaps in the final alignment were centered in the linker regions by visual inspection. To generate a phylogenetic tree, only aligned channel sequences starting with the conserved Asn at the beginning of region A through the end of S6 (up to the conserved Tyr) were used, and gaps were not considered in the analysis.

Results

Subfamilies of K^+ channels

In an attempt to obtain more cDNAs encoding different K^+ channels and, perhaps, define new K^+ channel subfamilies, we screened several rat brain cDNA libraries enriched for long inserts. In addition to two isolates with longer 5' untranslated regions than our original DRK1 clone, we obtained full-length cDNAs for RCK1, RCK2, K$_v$4, and two novel isolates, IK8 and K13. The insert of our original DRK1 clone carried only 21 nucleotides of noncoding region upstream of the assigned methionine initiation codon, and the first eight nucleotides were linker derived (Frech et al., 1989). Upon inspection of the longer 5' untranslated regions (474 and 201 nucleotides), it became clear that the open reading frame of the original DRK1 started with methionine at amino acid position 5 (Fig. 1). The new isolates, if the first AUG is utilized, would encode an identical open reading frame with four extra N-terminal amino acids (Met-Pro-Ala-Gly). There are several in frame stop codons upstream of the first ATG starting the longer open reading frame. Therefore, this increases the length of DRK1 from 853 to 857 amino acids. The same four amino acids are present in the mouse and human homologs of DRK1 (Pak et al., 1991; F. Soler and R. H. Joho, unpublished observations).

K13 and IK8 are two new putative K^+ channel clones whose amino acid sequences are shown in Figure 1. K13 contains a 2.8 kb insert with a open reading frame of 1542 nucleotides encoding a protein of 514 amino acid residues. The methionine initiation codon for the longest open reading frame is preceded by 245 nucleotides of 5' untranslated region carrying one ATG codon immediately followed by the termination triplet TGA, and several more in-frame upstream termination triplets. The TGA termination codon for the longest open reading frame is followed by approximately 1000 nucleotides of 3' untranslated region. IK8 contains a 5.0 kb insert of which the first 2478 nucleotides have been sequenced. The 5' untranslated region is 505 nucleotides in length and contains two ATG codons followed by termination triplets before the ATG assigned as the start of an open reading frame of 1515 nucleotides encoding a protein of 505 amino acids. Following the TGA termination triplet, 458 nucleotides of 3' untranslated region have been sequenced. Hydropathy analyses of the cDNA-derived amino acid sequences of IK8 and K13 showed profiles similar to the ones found for other K^+ channels suggesting six α-helical transmem-

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Figure 1. Amino acid sequence alignment of representative members of six subfamilies of voltage-gated K^+ channels. The single letter code for amino acids is used; *hyphens* indicate identity to the topmost sequence (DRK1); *dots* indicate gaps introduced to maintain alignment; *numbers* show the positions in the respective sequences. *Stippled bars* delineate the extent of the conserved regions *A* and *B*, and the pore region. The putative transmembrane segments *S1–S6* are shown by a *line*. *Letters above* the DRK1 sequence show positions conserved in at least 12 of 15 K^+ channels compared. *Boldface letters* indicate absolutely conserved residues. *Shab, Shaw, ShB1,* and *Shal* are *Drosophila* sequences. *DRK1, K$_v$4, RKShIII, Raw3, RCK1, RCK2, RK5, IK8,* and *K13* are rat sequences. *XSha2* and *AK01* are from *Xenopus* and *Aplysia*, respectively.

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A
                                                                    <NV G R          TL   P TL
DRK1     MPAGMTKHGSRSTSSLPPEMEIVRSKACSRRVRLNVGGLAHEVLWRTLDRLPRTRLGKLRDCNTH......DSLLQVCDDYSLE    75
Shab     TSQQIVGGVGVGVGGASSQSISGGVPTHSQSNTTG-LQRT-SRSMS-IP----FM-AQ---VNS--SI----E--H----R-GE-T---......EAIVEL-----A  325
Kv4                                         MGQGDE-E-IVI----TR-QTYRS--RT--G--AW-AEPDA......SHF--DPR    51
RKShIII                                       MGKIENNE-I------TR--TYRS--KT--G--AL-ASSEPQ...... 55 aa insert   98
Raw3     MISSVCVSSYRGRK-GNKPPSKTCLK-EMAKGEA-EKIII----TR--TYRS--RT--G--AW-A-PDGGRPESDGGGAGSSGSGGGG    91
Shaw                          MNLINMDSEN--V------IR--TYKA--KKI-A--SR-TEALAN......-DPI    49
RCK1     MTVMSGEN-DEASAAPGHPQDGSYPRQADHDDHE--CE---VI--IS--RF-TQLK--AQF-N-L--NPKKRMRY.......-FDPL    76
RCK2     MRSEKSLTLAAPGEVRGPEGEQODAGEFQEA-GGGGCCS-E-LVI-IS--RY-TQL--SLF-D-L--DPGRRVRF.......-FDPL    80
XSha2    MTVATGDLTD--VGFAGH-QDSYDPEPDHE--CE---VI--IS--RF-TQLK--SQF-E-L--DPKKRMRY.......-FDPL    72
AKO1     MEVAMAGIENGGPAGYRDSYHSSQRPLLRSSNLPNSRSFP-LSEEDNANENGMVPGSDYDCSCE--VI---RF-TQLK--NQF-D-L--NPQKR-RY......-DPL   105
ShB1     QKEEQKKIAERKLQLREQQLQRNSLDGYGSLPKLSSQDEEGGAGHGFGGG-QHFEP-PHDHDFCE--VI---S--RF-TQL--NQF-D-L--DPARRLRY......-FDPL  136
RK5      MAAGVAAWLPFAR-AAIGWMPVASGPM-AP-RQERKR.TQDALIV---S-TRFQTWQD--E-Y-D-L--SSERDFF......-HP-    79
Shal     MASVAAWLPFAR-AAIGWPIA--HP----P--PKD-R-TDDEKLLI--S-RRF-TWRN--EKY-D-L--SNEREFF......-DED    79
IK8      MEPGLASEP-GSMDA-AEQSL---GSQDSVAGDDIEIVV---VRQVLYGDL-SQY-E---AE-IN-LAGGY......-TIFSL---DPG    85
K13      MTLLPGNSDYDYSALSCASDTSFHPAFFPQRQAIKGVFYRRAQRLGPQDDLHQSISLGDRR-QIII---EF-L----Q-KA-TNF.......-DI--S-----DVT  111


B
         E FFDR       F   L   Y   G   L            E> F
DRK1     DNEYFFDRHPGAFTSILNFYRTG.RLHMMEEMCALSFSQELDYWGIDEIYLESCCQARYHQKKEQMNEELKREAETLR.......ERGEEFDNTCCA  165
Shab     ----------KS-S---------K--IVD---V-A-GDD-E---V--L-------HK---NVH--MRK---S---......Q-DE---GEGKFS  415
Kv4      AD-F---------------V-AH--Y-----K--CPADV-GPLYEE--AF-----TDV-P--WMT-R-HRDAEEALDSFGGAP-DNSADDADADGPGDAGDGEDELEMTKRLALSDSDGRPGGFWR  170
RKShIII  GR-F---------------V-AYV--Y-----K--CPADV-GPL-EE--AF-----TDV-P--WMT-R-HRDAEEALDIF-TPD-IGGDPGDDEDLGGKRLGIED----AAGLG-PDGKSGRWR  209
Raw3     GC-F---------------V-AYV--Y-----K--CPADV-GPL-EE--TF-----TDV-P--WMT-R-HRDAEEALDIF-SPDGGGGAGPGDEAGDDERELALQRLGP...HEGGS-PGAGSGG-R  207
Shaw     L------------------V-AQV--Y-----K--YPTDV-GPL-EE--EF--L-SNQV-P--WMT-T-HRDTQETLAVLDRLD-DTEKPSEEELARKFGFEEDY......YKGTISWWQ  154
RCK1     R----------NRPS-DA--YY-QS-G--RRPVNVPLDM--E-IKFYELG-EAM-KFREDEGFI-.......-ERPLPEK  147
RCK2     R----------NRPS-DA--YY-QS-G--RRPVNVPLDI-ME-IRFYQLGDEA-AAFREDEGCLPEGG.......-DEKPLPSQ  154
XSha2    R----------NRPS-DA---Y--QS-G--RRPVNVPLDI---E-IRFYELG-EAM-IFREDEGFI-......-ERPLPDN  143
AKO1     R----------NRPS-DA--Y--QS-G--RRPVNVPLDI---E-IKFYELG-NAF-RYREDEGFI-......-EKPLPQN  176
ShB1     R----------SRPS-DA--YY-QS-G--RRPVNVPLDV---E-IKFYELGDQAINKFREDEGFI-......-ERPLPDN  207
RK5      TQQ--------D-DI-RH------K--YPRHE-ISAYDE--AFF-LIPEIIGD--YEE-KDRRRENA-R-QDD-......DTDNTGESALPTM  165
Shal     CK---------D-DI-RH---Y-----K--YPKHE-LT-YDE--AFF--MPDVIGD--YED-RDR-RENA-R-MDDK.......LS-NGDQNLQQLT  165
IK8      KR-FY------D-D--KCVIEV-YF-.EV--KKGI-PIC-KN-M-F-KV-LKF-DD--KSHLSE-R-ELE-IAR-VQLI-......DDL-SSAAEGRWR  174
K13      C--F-------N----GT--T-L-A--K-RLLR------QB--L----A-DH-DG--KR--L---I-EFA-MME--D-EEALDSEDQ......-S--PSTSEGRLS  207


S1                                                                               S2
         W   EP SS  A    A   S     I    S               T P                        E C
DRK1     EKRKKLWDLLEKPNSSVAAKILAIISIMFIVLSTIALSLNTLPELQSLD.......EFGQSTDNPQLAHVEAVCIAW  235
Shab     -YQ-Y--E-----T-F--RVI-V---L-------T----Q--HI-.........N-TPQ-------M-----T---  484
Kv4      RWQPRI-A-F-D-Y--RY-RYV-FA-LF--LV-ITTFC-E-HERFNPIVNKTEIENV.......RNGTQVRYREAETEAF-TYI--G---VV-  255
RKShIII  KLQPRM-A-F-D-Y--R--RFT-LF--LV-ITTFC-E-HEAFNIVKNKTEPVI.......NGTSAVLQYEIETD-A-TYI--G---VV-  292
Raw3     GWQPRM-A-F-D-Y--R--RVV-FA-LF--LV-ITTFC-E-HEAFNIDRNVTEIHRV......GNITSVRFRREVETE-I-TYI--VM-  292
Shaw     -MKPRI-S-FDE-Y--N---TIGVV-VF---CI-ILSFC-K-H-DMRVPIVRNITVKTA......NGSNGWFLDKT-TNAHIAFFYI--C--N--  241
RCK1     -YQRQV-L-F-Y-E---GP-RVI--V-V-LI-IVIFC-E-----KDDKDFTGTIH.......RIDNTTVYTSNIFTDPFFI--TL--I-  231
RCK2     PFQRQV-L-F-Y-E---GP-RGI--V-V-LI-IVIFC-E-----QFRADGRGSNEGSGTRMSPASRGSHEEEDEDEDSYAFPGSIPLGGLGTGTSSFSTL-G-FFTDPFFL--TL--V-  274
XSha2    -FQ-QV-L-F-Y-E---GP-R-I----VTV-LI-IVSFC-E---VFRDENENMHGSG......DSKESIE-DDIPKF-EPFFII--TC--I-  232
AKO1     -FQRRV-L-F-Y-E--A--RLC--F-VVI-L---VFV-L--IVIFC-E---QFKHYRVVNSTAN......NGTKIE-DEVPDITDPFLI--TL--I-  260
ShB1     -QR-V-L-F-Y-E--Q--RVV-VL-FLL-V-SVVMCMG-I----VV-S-......PCGERYAVAFFCLDTA-VMI  289
RK5      TA-QRV-RAF-N-HT-TM-LVFYYVTGF--AV-VM-NVVE-V-CGHRPGRAGTL......PCGERYKIVFFCLDTA-VMI  239
Shal     NM-Q-M-RAF-N-HT-TS-LVFYYVTGF--AV-VM-NVVE-V-CGHRPGRAGTL......E-NRVEH-T-EN--TA--G-  239
IK8      RCQ-CV-KF----E--CP-RVV-VL-FLL-V-SVVMCMG-I----VV-S-......QGQC-QMCHNVFI--S--VG-  244
K13      RCMRR-R-MV---H-GLPG-VF-CL-VL-VTVTAVN--VS----S-REBEE......  278
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S3   **S4**   **S5**

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                                                                              R R  RI KL RHS GL  LG TL  S  E  LL FL G
        FT E L R    P K F    N  ID AI P                        R  R  RI QIFRIMRILRILKLARHSTGLQSLGFTLRRSYNELGLLILFLAMGIMI
DRK1    FTMEYLLRFLSSPKKWKFFKGPLNAIDLLAILPYYVTIFLTESNKSVLQ.........  FQNVRRVVQIFRIMRILRILKLARHSTGLQSLGFTLRRSYNELGLLILFLAMGIMI   340
Shab    --L----S---D------G--I------------FL----L-T--NATDQ.........  ----D------V----------------------------------M---K---VL-  590
Kv4     --F-F-M-VVFC-N-VE-I-NS--I--FV-----F-LEVG-SGLSSKAA.........  KD-LGFLRVV-FV----F--T--FV--RV--H---A-T--FL----I--L-VL--  358
RKShIII --F-F-V-IVF--N-LE-I-NL--I--FV-----F-LEVG-SGLSSKAA.........  KD-LGFLRVV-FV----F--T--FV--RV--H---A-T--FL----I--L-VL--  395
Raw3    --L-F-V-IVCC-DTLD-V-NL--I--FV-----F-LEVG-SGLSSKAA.........  RD-LGFLRVV-FV----F--T--FV--RV--H---A-T--FL----I--L-VL--  395
Shaw    --F-I-V--I---N-EI-SSV-I--YI-T-SF-IDLV-ORFASHI...........  ...ENADILEF-s--I--M-LF-VT---S---KI-IQ-F-A-AK--T--VF-VL--V--  342
RCK1    -SF-LVV--FAC-S-TD---NIM-F--IV--I--FI-LGTEIAEQEGNQK.......  .GEQATSLAILRVI-LV-VF---S---K---I---Q--KA-MR------F-FI-VIL  339
RCK2    --L-V---SAC-S-AA--RNIM---V--F--FI-LGTELVQRHEQQPVSGG....  SGONGOOAMSLAILRVI-LV-VF---F---S---K---I---Q--KA-MR-----F-FI-VIL  342
XSha2   -SF-F-VLV-AC-S-AV--TNLM--I--I--FI-LGTVVADQ-KSN.........  GOQGOOAMSLAILRVI-LV-VF---F---S---K---I---Q--NA-MR------F-FI-VIL  341
AK01    --F-L-V--AC-E-LG---NIM-C--IV--I--FI-LGTVADQ-KSN.......  .NQAMSLAILRVI-LV-VF---F---S---K---I---R--KA-MR------F-FI-VIL  390
ShB1    --F-LTV---AC-N-LN-CRDVM-V--II--I--FI-LATVAEEDTLNLPKAPVSPQDKSSNQAMSLAILRVI-LV-VF---F---S---Q--RI--Y--KSCAS---F-LFS-T--A-I--  366
RK5     --V-----LAAA-SRYR-VRSVMSI--VV-----IGLVM-DNED...........  .....SGAFVTL-VF-VF---F-FS----Q--RI--Y--KSCAS---F-VFS---A-I--  409
Shal    --A-----LFAA-DRC--VRSVMSI--VV--M--IGLGI-DNDD...........  .....SGAFVTL-VF-VF---F-FS----Q--RI--Y--KSCAS---F-VFS---A-I--  337
IK8     --L---V-LF---N-LH-ALSFM-TV-V-----F--SLT--HLGARME........  .....LT--QA--AL------A--F------S---T--TYA-K--FK----LMY--V--FV  337
K13     -SL-F----IQA-S-FA-LRS--TL--V----------LIVDGAAS--RRKPST.....  GNSYLDK-GL-LRVL-AL--YVMR------T--L-A--CTR-F--L---CVA-AL  349
                                                                                                                          390
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S6

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                          <F SIP FWWA   TMTT GYGD  P T  G   VG  C    GVL IA PVP  IV NF       Y>
        F             AE            >   FKSIPASFWWATIMTTVGYGDIYPKTLLGKIVGGLCCIAGVLVIALPIPIIVNNESEFYKEQKRQEKAIKRREALERAKRNGSIVSMNMKDAFA  451
DRK1    FSSLVFFAEKDEDDTK............  ---LAY...-----K-------V--EA----G--------C-T-A---VI-TV--IC-----V-----------A---N-M-R---L--HSIVPSGR-QHCLLPSYQSER  701
Shab    ...-LAY..........-----K-------V--EA----G---------C-T-A---VI-TV--IC-----V-----------A---N-M-R---L--HSIVPSGR-QHCLLPSYQSER  701
Kv4     -ATMIYY--RIGAQPNDPSASEHTH----  ---N--IG-------VV----L---M--Q-WS--ML-A-AL------T--M-V-------GMY--SLAMAKQ-LP--KKKKHIPRPPQLGSPNYCKSVVNS  478
RKShIII -ATMIYY--RVGAQPNDPSASEHTQ----  ---N--IG-------VV----L---M--T-WS--ML-A-AL------T--M-V-------GMY--SLAMAKQ-LPRK-KKHIPPAPLA-SPTFCKTELNM  515
Raw3    -ATMIYY--RIGARPSDPRGNDHTD----  ---N--IG-------VV----L---M--T-WS--ML-A-AL------T--M-V-------GMY--SLAMAKQ-LP-K-KKHVPRPPQLESPIYCKSEETS  515
Shaw    -A----YY--RIQPNFHND.........  ---N----LGL----LV------MA---YI-MF--A-AL------T-----V-S------AMY--SHTQARA-LP-K-RRVLPVEQPRQPRLPGAPGGVS  455
RCK1    ---A--Y----AE-AESH.........  ---A----DA----VVS------M--M-V-IG---------S-A------T-------V-S------NY--HRETEG-EQAQLLHVSSPNLASD-DL-RRSSSTIS  450
RCK2    ---A--Y----A-DV-SL.........  ---P----DA----VV------M--M-VG--------S-A------T-------V-VG-----NY--HRETE--EQQYTHVTCGQPTPDLKATD-GLGKPD  501
XSha2   ---A------A-R-SO...........  ---P----DA----VVS------M--T-IG--------S-A------T-------V-IG-----NY--HREGESTDKGQYKHVQSCPNYPEKKD-LDSECGSD  452
AK01    ---A--Y----A-Q-H...........  ---A----DA----VV------MR--IGVW--L---S-A------T-------V-V------NY--HREGESTDKGQYKHVQSCPNYPEKKD-LDSECGSD  477
ShB1    ---A--Y----AGSENF.........  ---A----DA----VV------MT-VGVW----S-A------V--------V-------NY--EMQSQNFHVTSCPYLPGTLGQHMKKSS  520
RK5     -ATVM-Y---GSSAS............  ---T-----A----YTIV----L---MV--IA---F-SI-SLS--------V--S---RI-HQNQ-AD-RRAQKK-RLARI-AAKSG-A-AYMQSK  448
Shal    -ATVM-Y---NVNG-N..........  ---T-----A----YTIV----L---MV--IA---F-SI-SLS--------V--S---RI-HQNQ-AD-RKAQ-K-RLARI-IAKAS-GAAFVSKK  448
IK8     -A-GYTM-QSQPE-L...........  -----------Q----I------T--V---------I-------V-S---HP--I--VRY-NK-RVL-T-A-HELE-MELNSSAEKPGASRSDL  460
K13     -AP-LYVI-NEMA-SPE..........  ----T-----CY---V-------MV-RSTP-QV-ALSSILS-I-LM-F-VTS-FHT--RS-L--QEQERVLI-R-QYLI-TKSQLSG-SQDSDIL  502
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DRK1    RSIEMMDIVVEKNGESIAKKDKVQDNHLSPNKWKWTKRALSETSSKSFETKEQGSPEKARSSSSPQHLNVQQLEDMYSKMAKTQSQPILNTKEMAPQSKPPEELEMSSMPSPVAPLPAR  571
Shab    SFAKS--LIDVIVDTGKQTNVVHPKGKRQSTPNIGRQTLDVQSAPGHNLSQTDGN-T-GEST-GRNPATTGTGCYKN-DHV-NLRNSNLH-RRGSSSEQDAVPPYSFDNPNARQTSMMAM  821
Kv4     PHHSTQSDTCPLAQ-E-LEINRADSKLNGEVAKAALANEDCPHIQQALTPDEGLPFTRSGTRERYGPCFLLSTG-YACPPGGMRKDLCKESPVI-KYMPTEAVRVT  511
        NGK2 splice variant            :GRKP-RGMSI  585
RKShIII ACNSTQSDTCLGKENRLLEHNRSVLSGDDSTGSEPPLSPSGKAPHQTL  585
Raw3    PRDSTYSDTSPPAR-EGMVER-RA-SKQNGDANAVLSDEEGAGLTQPLASAPTPEERRAL-R-GTRDRNKAAAACFLL-AGDYACADGSVQKEGSVEPKACVPVSHTCAL  625
Shaw    GCGTPGSGPHSGPMG-GGTGPRRMN-KTKDLIVSPKSDM-F-FD  498
RCK1    K-EY-EIEEDMN-SIAHYRQANIRTGNCTATDQNCVNKSLLTDV  495
RCK2    FAEASRERRSSYLPTPHRAYAEKRMLTEV  530
XSha2   L-KSDYMEIQ-GVNH-NEDFREKNLKTANCTLGNTNYVNITKMLTDV  499
AK01    IMEMEEGNHSTPLT-KVKENHAIKA-NPGSDYGLE-DV  515
ShB1    L-ESSS-MMDLDD-VESTPGLTETHPGR-AVAPFLGAQQQOQOQOPVA-SLSMSIDKQLQHPLQHVT-TQLY--QQQOQOQOQNGFK--QQQQ-QQQLQ-QQSHTINASAAAATSGSGSSGL  640
RK5     -NGLLSNQLQSSED-PAFVSKSGSSFETT-PPASLPGENHEP  490
Shal    KAA-ARWAAQ-SGI-LDDNYRDEDIFE-QHHHLLRCLEKTTM  490
IK8     DTLPPEPAAR-GPSWGSRL-LSHS-TFIPLLTEEKHH-TRLQSCK  505
K13     FGSASS-TRDNN  514
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brane regions (data not shown). Amino acid sequence alignment with members of the four known K⁺ channel subfamilies revealed that K13 and IK8 are not additional members of one of these subfamilies but rather represent prototypes of two new subfamilies (see Figs. 1, 5). The highest degree of conservation is in the putative transmembrane segments S1–S6, particularly in the linker between S5 and S6. This region shows a preponderance of conserved amino acid positions, and recently it has been shown to be involved in ion permeation and block by internal and external tetraethylammonium all the characteristics expected of a K⁺ channel pore region (MacKinnon and Yellen, 1990; Hartmann et al., 1991; Yellen et al., 1991; Yool and Schwarz, 1991). The K13 and IK8 cDNA clones have relatively long 5' untranslated regions with upstream ATG initiation codons that might interfere with proper initiation of translation (Kozak, 1989). In the light of the fact that the DRK1 transcript, which has a very short 5' untranslated region without any additional AUG triplets, expressed extremely well in oocytes, we generated variant clones of K13 and IK8 where most of the 5' untranslated regions had been removed. The new cDNA constructs of K13 and IK8 carry 4 and 25 nucleotides of 5' untranslated regions, respectively. In neither construct were upstream ATG codons present. Microinjection of cRNA transcripts (up to 20 ng/oocyte) with and without long 5' untranslated regions did not lead to any measurable voltage-dependent currents (J. A. Drewe, S. Verma, G. Frech, and R. H. Joho, unpublished observations).

One of our isolates is virtually identical to the recently isolated K⁺ channel cDNA K_v4 (Luneau et al., 1991). Over the 1755 nucleotides of coding region, there are eight differences between our sequence and K_v4, leading to one amino acid change: a serine at position 142 of K_v4 is replaced with an alanine.

In vitro RNA transcripts of RCK1, RCK2, and K_v4 express functional K⁺ channels after microinjection of Xenopus oocytes (Taglialatela et al., 1991). The electrophysiological characteristics of the DRK1 isolates with an open reading frame encoding four additional N-terminal amino acids were not measurably different from the original DRK1 clone (Drewe, Verma, Frech, and Joho, unpublished observations).

Tissue distribution of mRNAs encoding K⁺ channels from different subfamilies

Because K⁺ channels play important roles in regulating excitability, their presence in part determines the electrophysiological properties of different tissues and the neuronal physiology in different parts of the brain. For this reason, we studied the expression patterns of mRNAs encoding K⁺ channel subunits from different subfamilies by Northern blot analysis and their distribution in the brain by in situ hybridization. Samples of polyA⁺ RNA from whole embryo (day 15), neonatal (1–3 d of age), and adult (2 months or older) rat tissues were analyzed with radiolabeled probes specific for DRK1, RCK2, K_v4, K13, and IK8. Figures 2 and 3 show Northern blots using RNA from brain, heart, kidney, liver, lung, and skeletal muscle. The spatial distribution and temporal expression pattern are distinct for different K⁺ channel mRNAs. A K_v4-specific probe reveals the most restricted expression pattern (Fig. 3A). RNA from adult brain shows a signal corresponding to an mRNA of approximately 4.5 kb. In all other tissues, including neonatal brain and whole embryo, no signal could be detected. High levels of RCK2 as an approximately 6 kb band are found in neonatal and adult brain. A very faint RCK2 signal can be visualized in embryonal

RNA and neonatal heart RNA, whereas adult heart RNA does not show a signal. In addition, short RNA species of approximately 0.6 and 0.8 kb can be seen in brain and adult skeletal muscle, respectively. The sequences and functional importance of these short RNA species are not known (when a probe derived from the coding region of RCK2 was used, no short RNA species and no trailing were discernible). In contrast to the restricted expression of RCK2 and K_v4, there is a wider tissue distribution of DRK1, K13, and IK8. The expression level of DRK1 is higher in heart and skeletal muscle than in brain or any other tissue. A prominent band at 4.3 kb is visible in RNA from neonatal and adult cardiac tissues, and the same size band of somewhat lower intensity is detected in adult skeletal muscle. Although DRK1 had originally been isolated from a brain cDNA library, its level of expression is lower in brain where several bands between 4 and 6 kb can be detected. IK8 shows expression of mRNA of two different sizes, approximately 5.3 and 5.0 kb. The highest levels of expression are in neonatal (5.3 kb) and adult brain (5.0 kb). Neonatal and adult heart and neonatal kidney and skeletal muscle show fainter bands. An approximately 4.7 kb RNA detected with a K13-specific probe is very abundant in adult lung and liver, in contrast to embryonic day 15 and neonatal brain and heart, and adult brain and kidney, where fainter bands are detected. Also, faint bands of higher molecular weights can be seen in adult liver, lung, and skeletal muscle.

Different K⁺ channel subunits show a distinct pattern of distribution throughout the rat brain

To study the contribution of individual channel mRNAs to K⁺ channel diversity in the rat brain, we studied the expression patterns by in situ hybridization. Coronal sections were hybridized with probes specific for particular K⁺ channels. The results shown in Figure 4 are summarized below.

DRK1. For the most part, mRNA is present homogeneously throughout the brain. However, higher expression levels appear in the piriform cortex, the olfactory tubercle, hippocampal regions CA1 through CA3, the dentate gyrus, and the medial habenular nucleus. DRK1 is expressed at low levels in cerebellum and even at lower levels in the brainstem.

K_v4. RNA encoding this channel is predominantly, yet not exclusively, expressed in the adult cerebellum. Both Purkinje and granule cells give intense signals, in contrast to the molecular layer and white matter where levels are low. Signals can also be seen in the cochlear nuclear complex and in the trigeminal nucleus. In contrast to the other K⁺ channels studied, K_v4 has a punctate appearance, particularly in the hippocampal formation and in cortical layers. This punctate pattern appears because individual K_v4-expressing cells are scattered through various cortical layers, and through the pyramidal and granule cell layers of the hippocampus (data not shown). Also, there is a low level of expression in the medial septal nucleus, the reticular thalamic nucleus, the ventral posteriomedial thalamic nucleus, and the zona incerta. In contrast to other K⁺ channels examined, K_v4 mRNA levels are relatively low in piriform cortex.

RCK2. This member of the RCK family shows homogeneous expression levels through most parts of the brain. This apparent homogeneity may correlate with the fact that an RCK2 probe derived from the 3' untranslated region lights up many RNA species of differing sizes on a Northern blot (Fig. 2). However, somewhat higher levels can be detected in the piriform cortex,
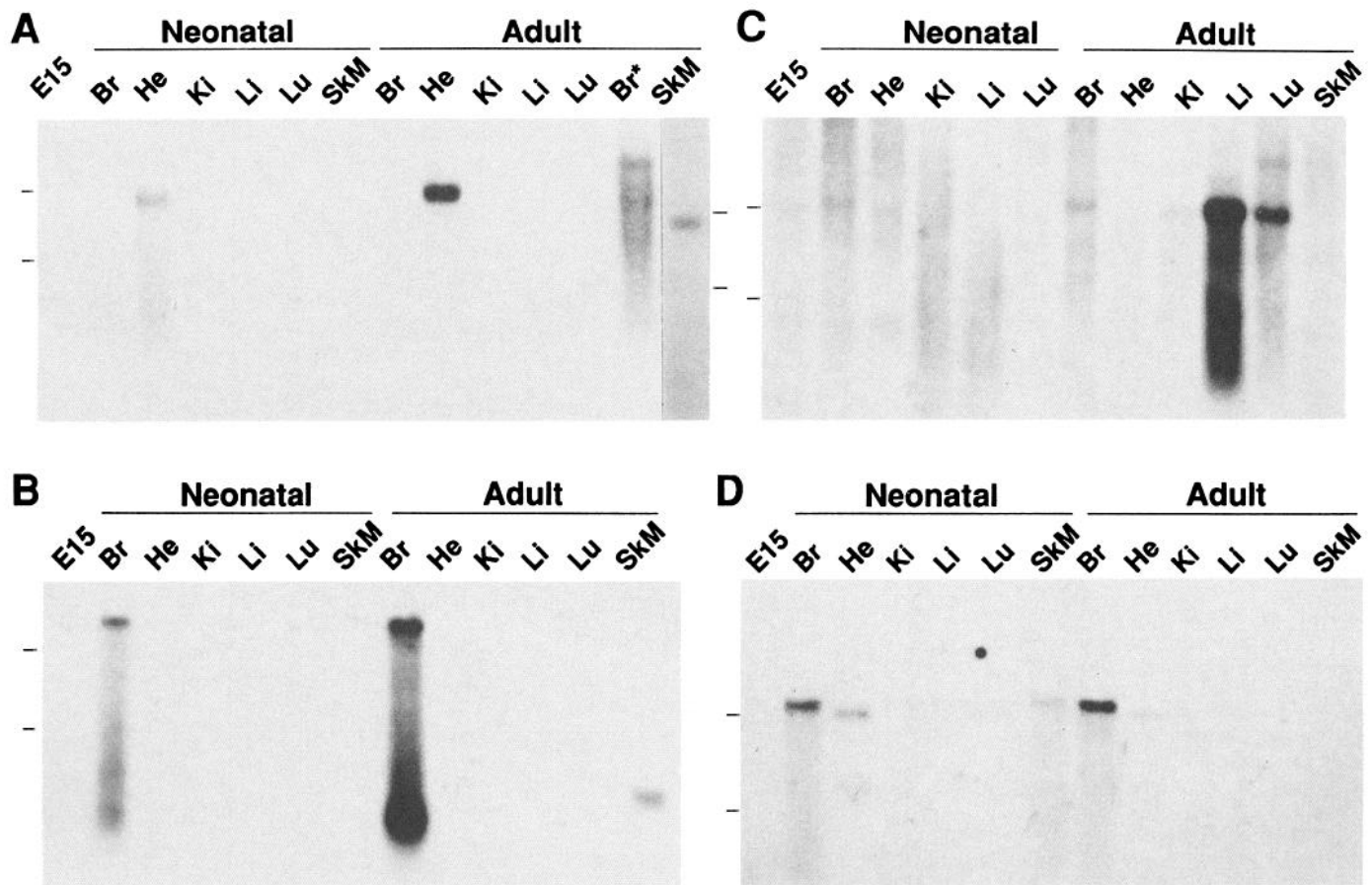
Figure 2. Tissue distribution of different K⁺ channel mRNAs (*A*, DRK1; *B*, RCK2; *C*, K13; *D*, IK8). Probes for the 3′ untranslated regions for various K⁺ channel subunits were used on polyA⁺ RNA from different tissues: *E15*, 15-d-old whole embryo; *Br*, brain; *He*, heart; *Ki*, kidney; *Li*, liver; *Lu*, lung; *SkM*, skeletal muscle. Ten micrograms of polyA⁺ RNA per lane were used with the K13 probe; all other blots contained 3 µg per lane, except the lane labeled *Br**, which contained 10 µg. *Tick marks* indicate position of 28S and 18S RNA.

the olfactory tubercle, and the dorsal endopiriform nucleus. Low uniform expression is seen in the hippocampus, the central medial thalamic nucleus, the zona incerta, the medial amygdaloid nuclei, and lateral amygdaloid area. Expression in the cerebellum is low overall but significant in the Purkinje cell layer (data not shown).

K13 and IK8. K13 RNA is widely expressed throughout the brain. It is more abundant in the superficial cortical layers in contrast to IK8, which is expressed in deeper cortical layers. Both mRNAs are present in the piriform cortex, olfactory tubercle, and medial habenular nucleus. For the limbic system, K13 is expressed at relatively high levels in hippocampus and dentate gyrus. It can also be detected in zona incerta. These latter areas show very low expression of IK8. However, IK8 is expressed in the medial amygdaloid nuclei and the lateral amygdaloid area. IK8 is low or undetectable in the cerebellum and brainstem, whereas K13 is found at high levels both in the Purkinje and granular layers of the cerebellum and also in the brainstem.

Temporal expression pattern of K_v4

Northern blot analysis indicated that K_v4 was only expressed in adult brain (Fig. 3*A*). No signal could be detected for RNA isolated from neonatal brain, whole embryo, or any of the other tissues tested. From *in situ* hybridization studies, it had become apparent that RNA levels for this K⁺ channel subunit were particularly high in the cerebellum (Fig. 4). Therefore, we investigated the temporal expression pattern during cerebellar development, which takes place mainly postnatally. PolyA⁺ RNA samples isolated at different time points after birth were used to determine the expression levels of K_v4 in the cerebellum and in the rest of the brain (Fig. 3*B*). During the first 10 d of postnatal development, no mRNA could be detected. A very faint signal of approximately 4.5 kb can be seen in the cerebellum at day 11, and the level continues to increase up to and past day 20. By day 20 the expression level has reached approximately one-fifth the level found in the adult cerebellum. At all time points, the expression levels in the rest of the brain are too low to be detected. In adult brain, the expression level in the cerebellum is still at least an order of magnitude higher than in the rest of the brain. Also, in addition to the major RNA species at 4.5 kb, adult brain shows three transcripts of substantially larger sizes.

Discussion

K^+ channels are members of a large multigene family

We have isolated several rat brain cDNAs encoding voltage-gated K⁺ channels. The isolation and expression of DRK1, RCK1, RCK2, and K_v4 have been described previously (Frech et al., 1989; Kirsch et al., 1991; Taglialatela et al., 1991). Our K_v4 isolate is basically identical to an independent rat brain clone
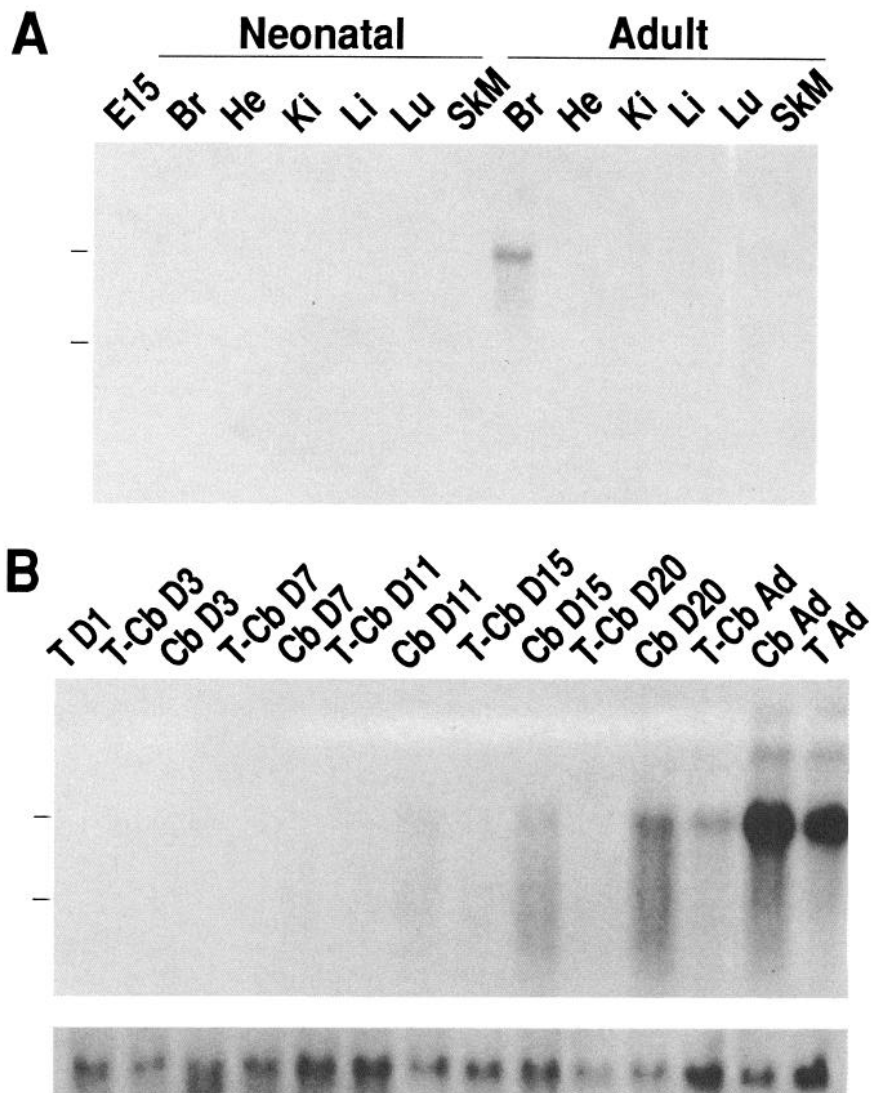
Figure 3. Brain-specific expression (*A*) and temporal expression pattern (*B*) of K$_v$4 during cerebellar development. In *A*, 3 μg polyA+ RNA samples were analyzed with a DNA probe derived from the 3' untranslated region of K$_v$4. For abbreviations, see Figure 2. In *B*, 10 μg of polyA+ RNA from total brain (*T*), cerebellum (*Cb*), and brain after removal of cerebellum (*T-Cb*), collected at different days after birth (*D1–D20*) and adult rat brain (*Ad*), were probed with a DNA probe specific for the 5' untranslated region of K$_v$4. Even transfer of RNA to the membrane was monitored by a second hybridization (*below B*) with a probe specific for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase.

(Luneau et al., 1991) and is closely related to NGK2, a cDNA clone isolated from a mouse × rat hybrid cell line (Yokoyama et al., 1989). Most likely NGK2 is the mouse homolog of K$_v$4 since it differs in the coding region by 50 nucleotides. The derived amino acid sequences of both clones are identical, except for the C-terminal 10 amino acids of NGK2, which are absent in K$_v$4 and replaced by a stretch of 84 unrelated amino acids. It has been argued that these two cDNAs represent alternate splice products (Luneau et al., 1991). Therefore, the mechanism of alternate splicing to create K+ channel diversity, which was first described in *Drosophila,* may also be important in mammals. Also, DRK1 clones with longer 5' untranslated regions are reported here. The original DRK1 clone isolated through expression cloning was four amino acids short at its N-terminus, yet it expressed delayed-type rectifier I_k currents indistinguishable from the new longer versions containing the four extra amino acids (Met-Pro-Ala-Gly).

Apart from the K+ channels already described, we report two new cDNA clones, K13 and IK8, encoding putative K+ channels. K13 and IK8 do not show enough amino acid sequence similarities to be classified as additional new members in one of the four known subfamilies of K+ channels. However, based upon the overall relatedness throughout the core region (S1–S6), we propose that K13 and IK8 are prototype members of two new subfamilies of putative K+ channels (Table 1). This enlarges the K+ channel family, which currently contains the subfamilies exemplified by Shaker, Shab, Shaw, and Shal, by two new subfamilies each containing one member (Table 1; Figs. 1, 5). Attempts to express cRNA transcripts of K13 and IK8 in *Xenopus* oocytes were not successful. Derivatives of both cDNA clones where most of the 5' untranslated region had been removed (including upstream out-of-frame initiation and termination triplets) also failed to yield measurable voltage-dependent K+ currents after microinjection of oocytes with cRNA. A simple reason for the lack of expression may be the inability of the oocyte to process the primary translation product in a correct manner so it can be transported and incorporated in the cell membrane. In the absence of antibodies specific for K13 and IK8, it will be difficult to address this question experimentally. Translation of IK8 and K13 cRNA in a reticulocyte lysate system led to protein products of the approximate molecular weights predicted from the cDNA-derived amino acid sequence (data not shown). This indicates that in a cell-free system, the cRNAs can be translated into proteins of the appropriate sizes, and it
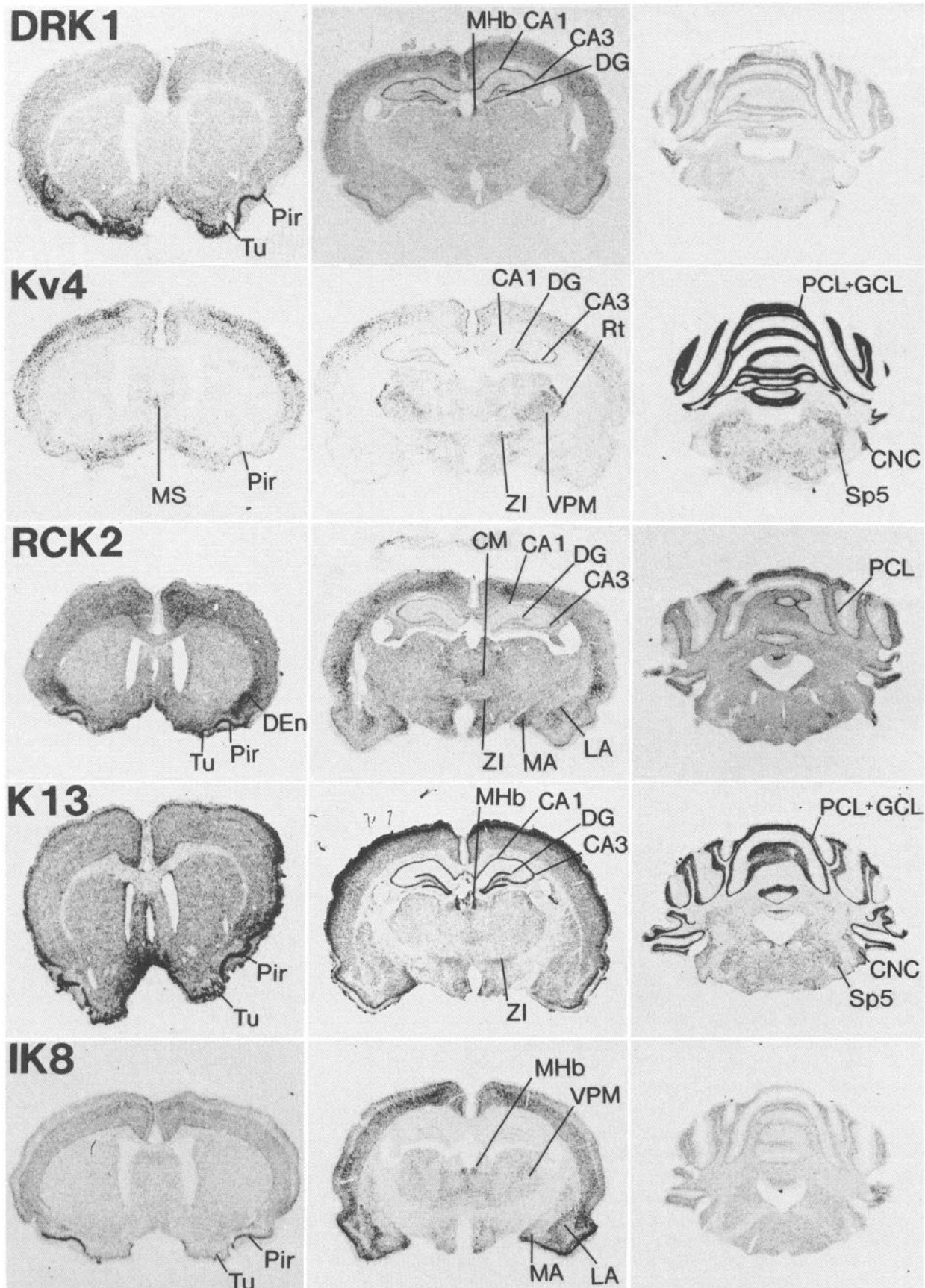
Figure 4. Distinct expression patterns of different K⁺ channel mRNAs throughout the rat brain. Coronal sections of adult rat brain were hybridized to the K⁺ channel–specific probes. *CA1* and *CA3*, CA1 and CA3 regions of hippocampus; *CM*, central medial thalamic nucleus; *CNC*, cochlear nuclear complex; *DEn*, dorsal endopiriform nucleus; *DG*, dentate gyrus; *GCL*, granule cell layer; *LA* and *MA*, lateral amygdaloid area and medial amygdaloid nucleus; *MHb*, medial habenular nucleus; *MS*, medial septum; *PCL*, Purkinje cell layer; *Pir*, piriform cortex; *Rt*, reticular thalamic nucleus; *Sp5*, spinal trigeminal nucleus; *Tu*, olfactory tubercle; *VPM*, ventral posteriomedial thalamic nucleus; *ZI*, zona incerta.

Table 1. Amino acid identity between K⁺ channels of different subfamilies

DRK1	Shab	Kv4	RKSh III	Raw3	Shaw	RCK1	RCK2	XSha2	AK01	ShB1	RK5	Shal	IK8	K13	
100	74	44	45	44	45	45	47	46	47	45	41	41	48	45	DRK1
	100	44	45	45	43	44	45	43	45	44	39	39	47	43	Shab
		100	86	86	56	46	48	46	47	46	41	42	41	38	Kv4
			100	87	56	47	49	47	49	47	41	42	42	38	RKShIII
				100	54	47	48	46	48	47	42	43	42	39	Raw3
					100	45	47	45	47	45	42	43	40	36	Shaw
						100	82	86	81	80	42	42	38	38	RCK1
							100	83	78	78	44	44	39	39	RCK2
								100	79	78	43	42	37	38	XSha2
									100	82	43	42	38	37	AK01
										100	44	43	38	36	ShB1
											100	83	37	37	RK5
												100	37	36	Shal
													100	40	IK8
														100	K13

Percentages of identical positions were calculated using the aligned regions in Figure 1. The percentages for K⁺ channels of the same subfamily are shown in boldface.

makes it unlikely that the inability to express K⁺ channels in *Xenopus* oocytes is due to the presence of an undetected cloning artifact in the cDNAs.

Structural features common to members of all known K⁺ channel subfamilies

Although the highest degree of sequence conservation among all known K⁺ channels, including K13 and IK8, is in the putative transmembrane segments (S1–S6) and in the pore region between S5 and S6, there are stretches of conserved residues elsewhere. Near the N-terminus, members of all six subfamilies show two conserved regions (regions A and B in Fig. 1). Except for Shab and Shaker, region A is very close to the N-terminus and it contains six absolutely conserved amino acid residues. Region B, again, contains several absolutely conserved amino acids. Past S6, there is a short-stretch with similarity among members within the same subfamily; however, farther away from S6, closer to the C-termini, no sequence similarity can be detected. Although regions A and B are present in all known K⁺ channels, they are not needed for DRK1 function when expressed in *Xenopus* oocytes. N-terminal deletions of 101 and 139 amino acids that removed regions A and B changed the kinetic behavior but did not abolish K⁺ channel function (VanDongen et al., 1990). Likewise, most of the C-terminal sequence past S6 can be removed without losing K⁺ channel function (VanDongen et al., 1990; Drewe et al., 1991). Therefore, regions A and B, although as conserved as some of the transmembrane segments, are dispensible for DRK1 channel function in *Xenopus* oocytes. The conserved nature of these regions implies a conserved secondary structure and argues for an important, hitherto not understood channel property. According to the postulated K⁺ channel topography, regions A and B would face the cytoplasmic side of the membrane. This may be of crucial importance for trafficking, membrane anchoring, and/or interaction with smaller subunits such as those that have been reported to be associated with a dendrotoxin-binding protein thought to be a voltage-gated K⁺ channel (Rehm and Tempel, 1991).

Most of the conserved residues are in or near the membrane-

spanning segments. Not surprisingly, the pore region located between S5 and S6 shows the highest degree of conservation. Over a stretch of 22 amino acids there are 11 absolutely conserved positions. In membrane segments S1–S6, there are several completely conserved positions (in boldface in Fig. 1): a Cys, Phe, and Glu in S2; an Asp and Ala in S3; charge conservation in S4; two leucines and a Phe in S5; two glutamates flanking S5; a Gly, Ala, Pro, and Ile in S6; and a Phe and Tyr just past S6. Some of the differently charged conserved residues may be important for tight packing between neighboring helices.

Why do cRNAs from IK8 or K13 not yield any functional channels in *Xenopus* oocytes although IK8 and K13 "look" like bona fide K⁺ channels? Both appear to have a pore region closely related to known functional K⁺ channels. The S4 segments, proposed as the voltage sensors, show amino acid alterations that may be of functional significance. The central arginine residue, a position conserved in all other K⁺ channels, is changed to a tyrosine in K13 (Fig. 1). To the right of this central arginine, the nearest positively charged amino acid is an absolutely con-
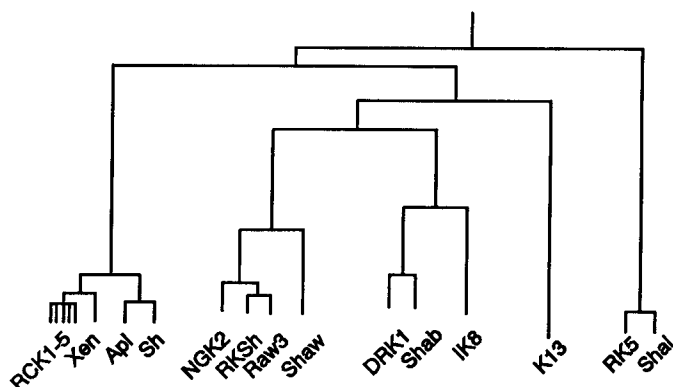

Figure 5. Phylogeny of K⁺ channels. The four subfamilies defined by *Shaker, Shaw, Shab,* and *Shal* contain members of fly and mammals. *K13* defines a new subfamily with one representative only. *IK8* may be a distant member of the Shab subfamily or a representative of a new subfamily.

served lysine in all other K⁺ channels, except in K13, where the lysine is replaced by an arginine. These changes cannot be attributed to cloning or sequencing artifacts because they have been independently found in two different cloning events (cDNAs). These differences, which lead to loss of a positive charge in the center of the putative voltage sensor and the introduction of a bulkier side chain (arginine), may be responsible for the fact that K13 cRNA did not lead to measurable voltage-dependent K⁺ currents in microinjected oocytes. Site-directed mutagenesis of the S4 region of Shaker showed that these residues are involved in voltage sensitivity of the channel (Papazian et al., 1991). The S4 region in IK8 contains five positively charged residues, but there are three alanines at positions where other known K⁺ channels carry amino acids with larger hydrophobic side chains. Besides these "subtle" changes, however, there are some substantial differences in S6 (Fig. 1). Both IK8 and K13 contain a serine at a position where all other K⁺ channels carry a conserved cysteine residue. The only other absolutely conserved cysteine is in S2. It is tempting to speculate that S2 and S6 are connected through a disulfide bridge. Therefore, it might be possible that a functionally important S–S bridge from S2 to S6 is missing in IK8 and K13. Apart from this Cys to Ser change in IK8 and K13, the beginnings of the S6 regions clearly differ from the general pattern of segment conservation. It also may be that these channels are not just voltage operated, but that their open–closed state transitions are ligand dependent, that is, through G-proteins or cyclic nucleotides. It is also possible that IK8 and K13 do not by themselves form functional tetrameric channels but work only in a heteromeric complex of unknown composition. Ongoing work in our laboratories (E. Stefani, L. Toro, and H. Sarkar, unpublished observations) showing that Xenopus oocytes contain enough intracellular Ca²⁺ for known Ca²⁺-activated K⁺ channels to be active makes it unlikely that we have isolated a functional subunit of a Ca²⁺-activated K⁺ channel, although this possibility cannot be excluded.

K13 and IK8 show high expression levels in liver and lung. Two classes of K⁺ channels have been identified in rat hepatocytes, an inwardly rectifying channel (Henderson et al., 1989) and a possible Ca²⁺-dependent K⁺ channel (Bear and Petersen, 1987). In human respiratory cells, a slightly voltage-dependent, Ca²⁺-regulated K⁺ conductance has been reported (Kunzelmann et al., 1989). It would be intriguing if K13 or IK8 belonged to these two classes of K⁺ channels.

Different K⁺ channels show distinct expression patterns in brain and other tissues

All the K⁺ channels studied reveal a distinct expression pattern of their corresponding mRNAs in different tissues and throughout the rat brain. RCK2 and K$_v$4 are restricted to brain. In other tissues they are at levels too low to be detected by Northern blot analysis. Although all the clones have been isolated from brain cDNA libraries, some are expressed at higher levels elsewhere. DRK1 is most prominent in neonatal and adult heart and skeletal muscle. K13 can be detected in brain RNA; however, its expression level is higher in liver and lung. The sizes of transcripts for some of the channels differ from one tissue to another, and even in the same tissue (i.e., brain). This may be due to alternative splicing of precursor RNA or due to transcripts encoded on closely related genes. K$_v$4 and NGK2 are probably two splice variants with an identical core region and two distinct C-termini (Luneau et al., 1991).

Using Northern blot analysis of RNA from different brain

regions, differential expression of several members of the RCK family (RCK1,3–5) has been reported (Beckh and Pongs, 1990). We have used *in situ* hybridization to study expression of K⁺ channel mRNAs throughout the rat brain. Five species of mRNAs from different channel subfamilies, including two new putative K⁺ channels, have distinct but overlapping distributions. K$_v$4 is highest in the cerebellum, where it is present in both the Purkinje and granular cell layer. In the cortex and hippocampus, K$_v$4 shows a punctate appearence due to signals from individual scattered cells. Expression of K$_v$4 is also temporally regulated. It can be first detected in the cerebellum at around 11 d after birth, and its levels increase during further development. Interestingly, this correlates with the onset of granule cell maturation that predominates the third week of postnatal development, and with synapse formation between Purkinje cells and parallel fibers (Altman, 1972). Also, maturation of the molecular layer, which includes stellate cell formation and basket cell synaptogenesis with Purkinje cells, begins around postnatal day 10, just when K$_v$4 expression can first be detected.

Recently, a series of *in vitro* studies have indicated that members of the same K⁺ channel subfamily can come together to form heteromeric channels with different properties from the two homomeric "parental-type" channels (Christie et al., 1990; Isacoff et al., 1990; Ruppersberg et al., 1990). It is, however, not known whether heteromultimeric K⁺ channels are formed *in vivo*. The fact that different K⁺ channel subunits show overlapping expression patterns fulfills one of the prerequisites of such *in vivo* heteromultimer formation.

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