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Kv8.1, a new neuronal potassium channel subunit with specific inhibitory properties towards *Shab* and *Shaw* channels

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Outward rectifier K^+ channels have a characteristic structure with six transmembrane segments and one pore region. A new member of this family of transmembrane proteins has been cloned and called Kv8.1. Kv8.1 is essentially present in the brain where it is located mainly in layers II, IV and VI of the cerebral cortex, in hippocampus, in CA1–CA4 pyramidal cell layer as well in granule cells of the dentate gyrus, in the granule cell layer and in the Purkinje cell layer of the cerebellum. The Kv8.1 gene is in the 8q22.3–8q24.1 region of the human genome. Although Kv8.1 has the hallmarks of functional subunits of outward rectifier K^+ channels, injection of its cRNA in *Xenopus* oocytes does not produce K^+ currents. However Kv8.1 abolishes the functional expression of members of the Kv2 and Kv3 subfamilies, suggesting that the functional role of Kv8.1 might be to inhibit the function of a particular class of outward rectifier K^+ channel types. Immunoprecipitation studies have demonstrated that inhibition occurs by formation of heteropolymeric channels, and results obtained with Kv8.1 chimeras have indicated that association of Kv8.1 with other types of subunits is via its N-terminal domain.

Keywords: brain/chromosomal mapping/inhibition/K^+ channel/*Xenopus* oocytes

Introduction

There is a great diversity of voltage-gated K^+ channels in neuronal and muscular cells which have different biophysical, regulational and pharmacological properties. These channels contribute to the control of resting potential, membrane excitability, shape and frequency of action potential (Rudy, 1988; Hille and Catterall, 1994). Since the first K^+ channel was cloned in *Drosophila* (Kamb *et al.*, 1987; Papazian *et al.*, 1987; Pongs *et al.*, 1988), molecular biology has provided a wealth of results which explain many of the mechanisms involved in the generation of the diversity of recorded K^+ currents (Betz, 1990).

Fifteen homologous genes encoding the α-subunit of outward rectifier K^+ channels have now been cloned in mammals. They belong to four different subfamilies designated Kv1 (*Shaker*), Kv2 (*Shab*), Kv3 (*Shaw*) and Kv4 (*Shal*). Within the same family, the K^+ channel α-subunits share a large percentage of sequence identity (>70%), while this percentage falls to ~40% between α-subunits from different subfamilies (Pongs, 1992; Chandy and Gutman, 1995). Some of the K^+ channel genes give rise to multiple protein products through alternative splicing, thereby increasing the variety of K^+ channel subunits (see, for example, Luneau *et al.*, 1991; Attali *et al.*, 1993b). A new subunit of voltage-gated K^+ channel, sharing ~35% identity with other cloned subunits, has been cloned recently from *Aplysia*. This new protein, which was called aKv5.1 (Zhao *et al.*, 1994) may be the precursor of a new subfamily of subunits. Additional proteins with the characteristic structure of the α-subunits of K^+ channels (six transmembrane segments) have been isolated recently (Drewe *et al.*, 1992). Because they have only ~40% structural identity with other K^+ channels α-subunits, they cannot be classified in the *Shaker*, *Shal*, *Shaw* or *Shab* families and should be called Kv6.1 and Kv7.1. Their cRNA does not elicit K^+ current when injected in *Xenopus* oocytes.

Outward rectifier K^+ channels are tetramers of α-subunits. Therefore, another factor of functional diversity can then be achieved by the formation of heteromultimeric channels with properties distinct from those of their parent homomultimers (Christie *et al.*, 1990; Isacoff *et al.*, 1990; Ruppersberg *et al.*, 1990; Sheng *et al.*, 1993; Wang *et al.*, 1993). However the diversity seems to be limited by the fact that only α-subunits from the same family (*Shaker*, *Shal*, *Shab* or *Shaw*) can apparently assemble to form heterotetramers (Christie *et al.*, 1990; Covarrubias *et al.*, 1991). An additional level of diversity is due to the fact that α-subunits of K^+ channels are tightly associated with β-subunits, which modify their kinetics as well as their regulation (Rehm and Lazdunski, 1988; Rettig *et al.*, 1994).

Here we present a novel member of this complex family of K^+ channel subunits. We describe the structural and functional properties of a new cDNA coding for a protein designated Kv8.1 which shares 40% identity with others K^+ channel α-subunits, but which has no K^+ channel activity by itself and instead inhibits expression of *Shab* and *Shaw* channels.

Results

Isolation of a new putative voltage-gated K^+ channel α-subunit

The project was started with the purpose of isolating new sequences coding for ATP-regulated K^+ channels in HIT-T15 insulinoma cells. Two nested couples of degenerating primers were used in a polymerase chain reaction performed on reverse transcripted mRNA (RT–PCR) reaction.

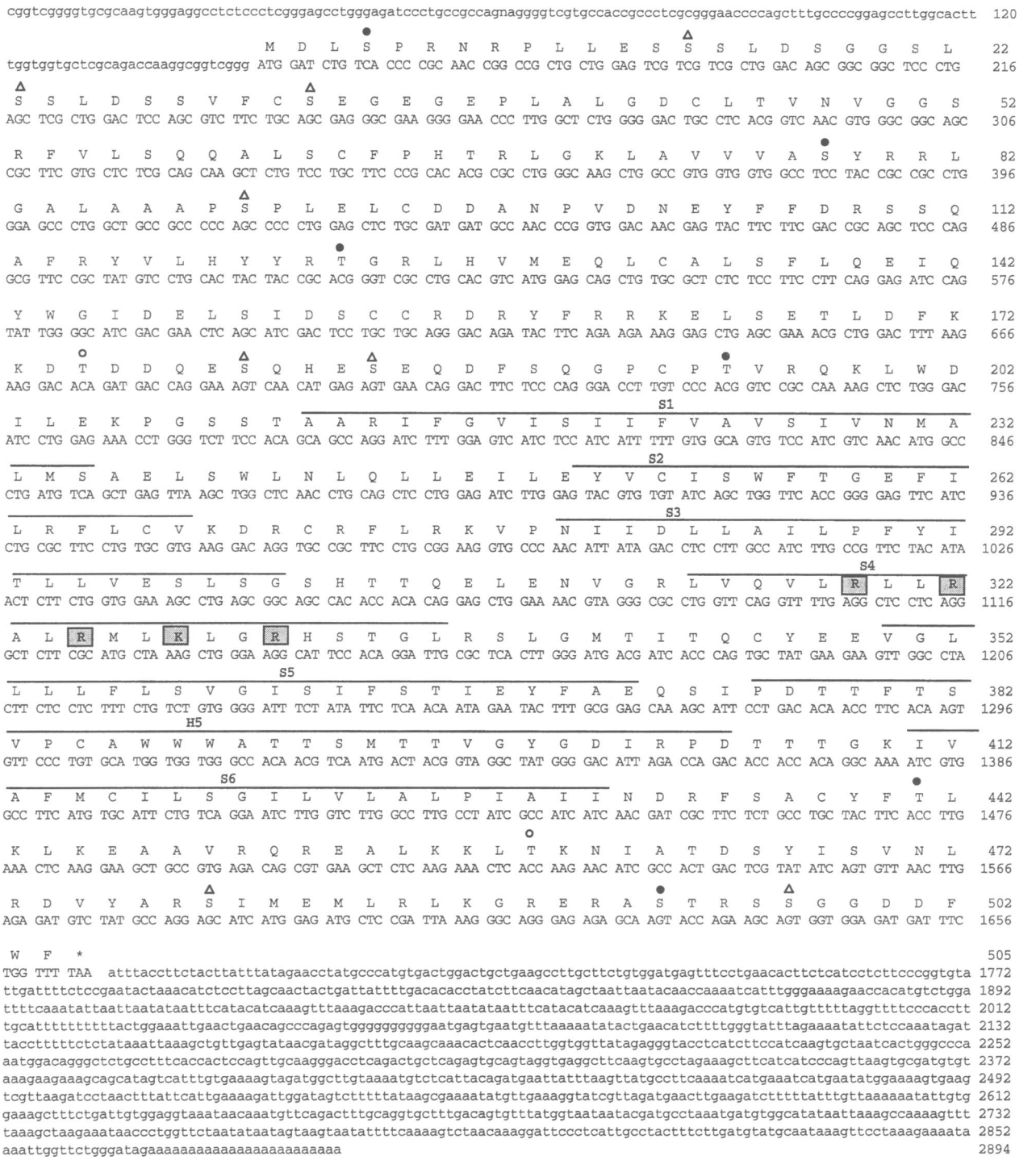
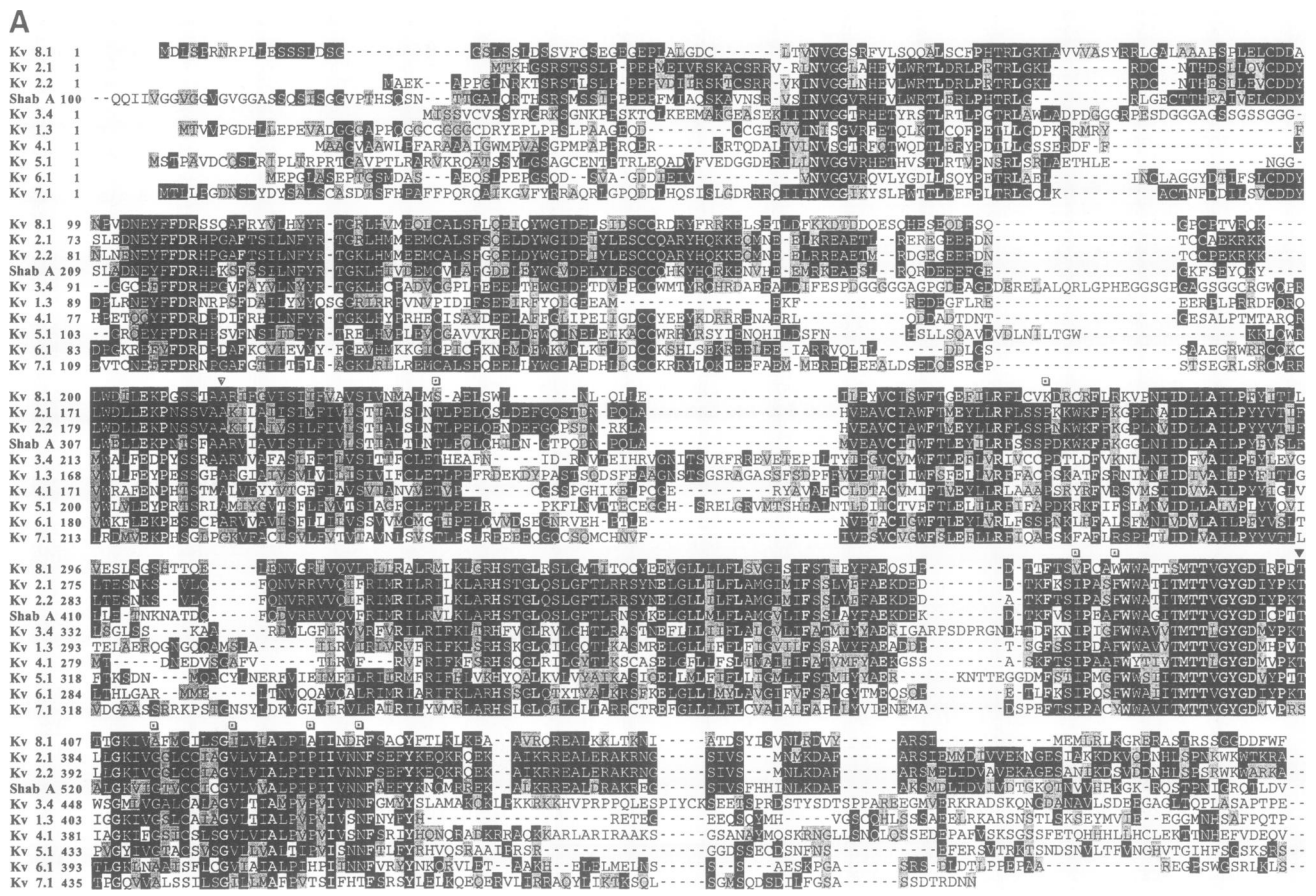


Fig. 1. Nucleotide and deduced amino acid sequence of Kv8.1 cDNA. The 5′- and 3′-non-coding region of cDNA are represented by lower case letters. The putative transmembrane segments S1–S6 and the pore-forming region H5 are underlined. The basic amino acids within the S4 segment are indicated by shaded boxes. The stop codon is marked by an asterisk. Potential cytoplasmic sites for protein kinase C (●), cAMP-dependent kinase (○) and Ca^{2+}-calmodulin kinase (△) are shown.

Forward and reverse primers were designed to hybridize respectively to the H5 pore region of K^+ channel subunits and to a putative region encoding a Walker A ATP binding site (Walker *et al.*, 1982). Subsequent cloning and sequencing of the polymerase chain reaction (PCR) products allowed the isolation of a fragment sharing homology with classical voltage-gated K^+ channel α-subunits. We then screened a cDNA library prepared with HIT mRNA, and four independent cDNAs (2.9 kb) were isolated.

The nucleotide sequence (Figure 1) indicates that the first ATG codon initiates an open reading frame of 504 amino acids. This codon is probably the initiating codon since (i) the four independent cDNAs are 2.9 kb long and thus are likely to represent full-length reverse transcription of the 2.9 kb messengers expressed in HIT cells (see



B

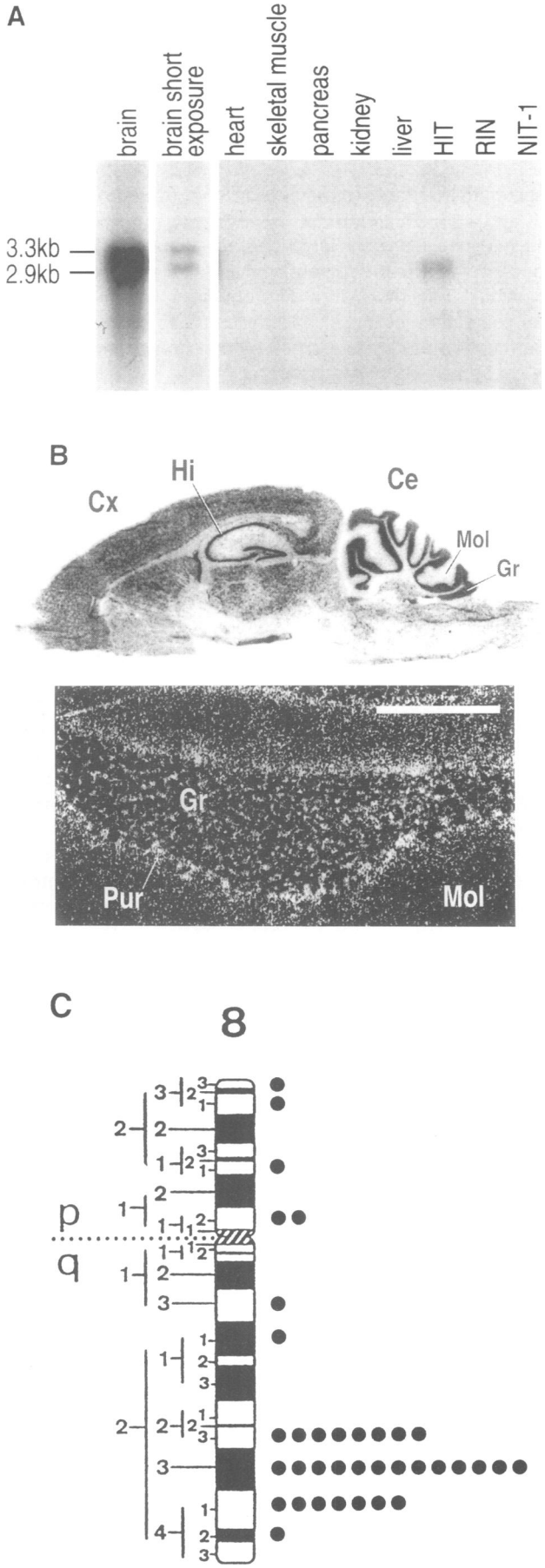
	Kv 1.3	Kv 2.1	Kv 3.4	Kv 4.1	Kv 5.1	Kv 6.1	Kv 7.1
Kv 8.1: % of homology	62.4	71.4	62.7	63.9	59.8	64.4	66.9
Kv 8.1: % of identity	37	43.6	37.6	39	33.2	39.4	42.2

Fig. 2. (**A**) The amino acid alignments of Kv8.1 protein with mouse Kv2.1, rat Kv2.2, *Drosophila Shab* (*SHAB* A), mouse Kv3.4, rat Kv4.1, *Aplysia* aKv5.1, rat Kv6.1 (IK8) and rat Kv7.1 (K13). Identical amino acids are indicated by white on black print and homologous residues are shaded. Squares indicate amino acids in Kv8.1 which diverge from conserved residues in functional Kv channel subunits. The C-terminal extremities of sequences are cut after alignment with Kv8.1. The first 213 amino acids of the *Shab Drosophila* channel do not align with the N-terminus of Kv8.1 and thus are not represented. The borders of the chimeric subunits are indicated by open triangles for (N_t Kv1)–Kv8 and (N_t Kv 8)–Kv1 and filled triangles for (N_tH5 Kv8)–Kv1 and (N_tH5 Kv1)–Kv8. (**B**) Relationships between Kv8.1 and the different subfamilies of potassium channel subunits. The percentages of identical and homologous amino acids are calculated for the conserved regions between amino acid 48 of Kv8.1 and the end of the S6 transmembrane segment (NVGG....NDRF).

below) and (ii) the sequence surrounding the ATG codon (GTCGGG**ATG**G) is in good agreement with Kozak consensus sequences [GCC(A/G)CC**ATG**G] (Kozak, 1987). The deduced protein sequence shows the hallmarks of outward rectifier voltage-gated K^+ channel α-subunits (Kv), i.e. six putative transmembrane segments, a transmembrane region (S4) showing five positively charged amino acids and a conserved pore-forming region (H5). It has a number of putative phosphorylation sites located in the cytoplasmic regions. Two sites for protein kinase A are at positions 175 and 459, six sites for protein kinase C are at positions 4, 78, 123, 195, 441 and 493 and eight sites for Ca^{2+}-calmodulin protein kinase II are at positions 14, 23, 32, 90, 180, 184, 179 and 497. No *N*-glycosylation sequence was detected. Sequence comparisons indicate that the new Kv protein shares no more than 33–43% identity and 60–72% homology with other classes of Kv subunits belonging to the four classical subfamilies Kv1, Kv2, Kv3 and Kv4 and with Kv5.1, Kv6.1 and Kv7.1 proteins (Figure 2B). Therefore, this new Kv protein was designated Kv8.1. The best homology is with subunits of the Kv2 subfamily (*Shab*), and sequence alignments (Figure 2A) indicate the presence of '*Shab*-specific' amino acids in Kv8.1.

Tissue distribution of the Kv8.1 subunit and chromosomal localization of the Kv8.1 gene

Northern blots of mRNA extracted from various hamster tissues were probed with the Kv8.1 cDNA (Figure 3A). Two transcripts of 3.3 and 2.9 kb were detected, but only in the brain. Similar results were obtained with mouse and rat tissues (data not shown). Using mRNA isolated from various insulin-secreting cell lines, HIT-T15 (Santerre *et al.*, 1981), RINm5F (Gazdar *et al.*, 1980) and NIT-1



(Hamaguchi *et al.*, 1991), the Kv8.1 transcript could only be detected in HIT cells as a 2.9 kb messenger. The Kv8.1 mRNA was not detected in pancreas slices by *in situ* hybridization (data not shown).

Figure 3B shows a characteristic autoradiogram illustrating the distribution of Kv8.1 mRNAs in sagittal sections of adult hamster brains. The Kv8.1 transcript was heterogeneously expressed. The highest levels of expression were in the neo- and allocortical regions, hippocampus, habenula of the epithalamus and cerebellum. The expression of the Kv8.1 gene was observed throughout the cell layers of the cerebral cortex, especially in layers II, IV and VI. A uniformly high hybridization signal was found in all the fields of the hippocampal formation. The Kv8.1 transcripts were located in the CA1–CA4 pyramidal cell layer as well as in the granule cells of the dentate gyrus. In the cerebellum, the Kv8.1 mRNA was uniformly and highly detected in the granule cell layer and in the Purkinje cell layer, whereas the molecular layer remained unstained. No labeling was seen in the deep cerebellar nuclei. Moderate hybridization signals were present over most other regions, including the olfactory bulb, amygdaloid complex, thalamus, hypothalamus, midbrain and brainstem (including pons and medulla). A weak hybridization signal was detected in the globus pallidus of the basal ganglia.

In situ hybridization was also carried out to determine the chromosomal localization of the Kv8.1 gene. In the 150 metaphase cells examined there were 277 silver grains associated with chromosomes, and 36 of these (12.9%) were located on chromosome 8; the distribution of grains was not random: 28/36 (77%) mapped to the q22.3–q24.1 region of the chromosome 8 long arm with a maximum in the q23 band. These results allow us to map the Kv8.1 gene to the 8q22.3–8q24.1 region of the human genome.

Expression of Kv8.1 subunit in Xenopus laevis oocytes

Injections of *Xenopus* oocytes with the Kv8.1 cRNA were used to test if the Kv8.1 protein is able to give rise to K^+ currents. Following test pulses from −130 to +60 V, neither outward nor inward currents could be detected. Modification of the external pH as well as of external ionic concentrations, addition of oxidative (H_2O_2) or reducing agents (dithiothreitol) and activation of different protein kinases (kinase A, kinase C) were assayed to reveal an expression of K^+ channels, but none of these treatments led to current detection. Also, attempts to detect K^+ currents from CHO and COS-7 cells after transfection with a Kv8.1-expressing vector were unsuccessful.

Fig. 3. Expression pattern and chromosomal localization of the Kv8.1 gene. (**A**) Northern blot analysis of Kv8.1 in hamster adult tissues and in the insulin-secreting cell lines HIT-T15, RINmF5 and NIT-1. The standard exposure time was 24 h at −70°C. For the brain, the short exposure was 4 h. (**B**) *In situ* hybridization of the Kv8.1 transcript in hamster brain. Upper panel: X-ray film autoradiographs illustrating the expression patterns of Kv8.1 mRNAs in sagittal hamster brain sections following *in situ* hybridization with a specific oligonucleotidic probe. Lower panel: dark field photomicrographs of emulsion autoradiograms illustrating expression of the Kv8.1 transcript in Purkinje and granular cells of cerebellum. Scale bar 400 μm. Abbreviations: Cx, cortex cerebral; Ce, cerebellum; Gr, granular layer; Mol, molecular layer; Hi, hippocampus; Pur, Purkinje cells. (**C**) Chromosomal localization of the Kv8.1 gene. Idiogram of the human G-banded chromosome 8 illustrating the distribution of labeled sites with the Kv8.1 cDNA probe.

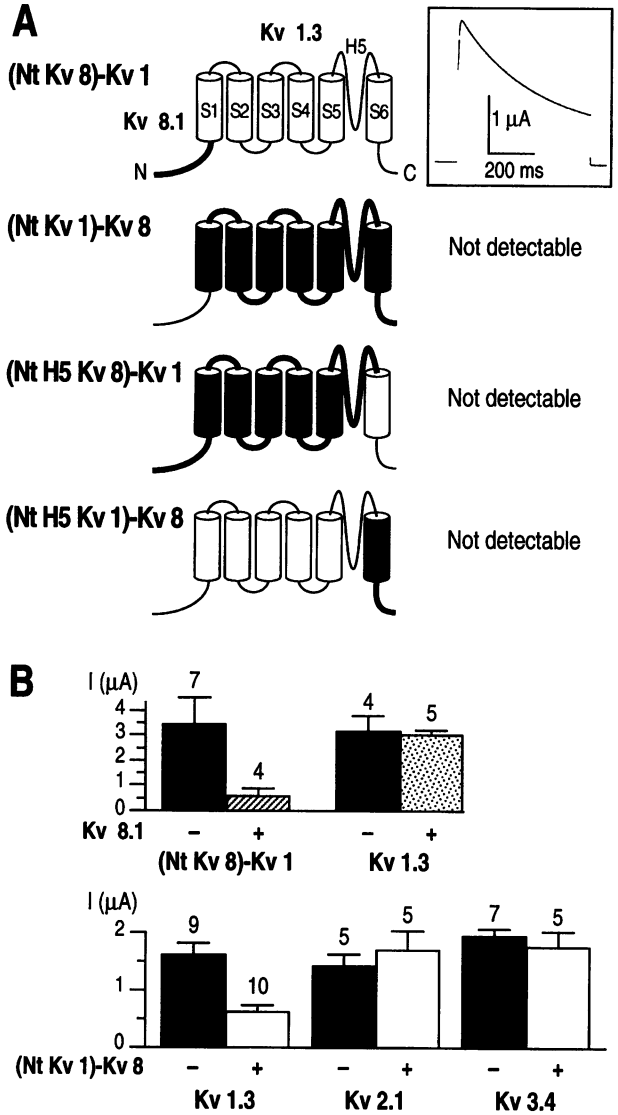


Fig. 4. Expression of Kv1.3–Kv8.1 chimera subunits in *Xenopus* oocytes. (**A**) Schematic representation of chimera subunits and the oocyte currents recorded 2 days after injection of corresponding cRNAs are indicated. (**B**) Current measurements elicited by injection of the indicated cRNAs with (+) or without (–) Kv8.1 and (N_t Kv1)–Kv8 cRNA co-injection. The amount of transcript injected per oocyte is 10–50 ng for chimeric subunits. For co-expression experiments, 10 ng of Kv1.3 and (N_t Kv 8)–Kv1 were co-injected with three times the amount (in moles) of Kv8.1 cRNAs and 10–20 ng of Kv1.3, Kv2.1 and Kv3.4 were co-injected with three times the amount (in moles) of (N_t Kv1)–Kv8 cRNAs. The holding potential is –90 mV. Outward currents are recorded after a depolarizing step to +40 mV. The peak current values are the average of currents recorded from the indicated number of oocytes.

In order to identify structural elements in Kv8.1 which might be responsible for this absence of current, chimeric subunits were created between Kv8.1 and Kv1.3, a well studied functional α-subunit belonging to the *Shaker* family (Attali *et al.*, 1992) (Figure 4). Replacing the first 181 amino acids of Kv1.3, corresponding to the cytoplasmic N-terminal part of the subunit up to the beginning of the first transmembrane domain (Figures 2A and 4A), with the first 213 amino acids of Kv8.1, generated a functional K^+ channel called (N_t Kv8)–Kv1 with electrophysiological characteristics similar to those of Kv1.3, i.e. a slow inactivation (Figure 4A). Interestingly, this current was totally abolished on co-injection with the Kv8.1 cRNA, while co-expression of Kv8.1 with Kv1.3 did not affect the Kv1.3 current (Figure 4B). Another chimeric protein called (N_t Kv1)–Kv8 was made with the first 181 N-terminal amino acids of Kv1.3 followed by the rest of the structure of Kv8.1 from the first transmembrane segment S1 to the C-terminal end (S1–ter region) (Figures 2A and 4A). This chimera was expressed in oocytes but did not give any detectable current (Figure 4A). It thus appears that it is the region from the first transmembrane segment S1 to the C-terminus of Kv8.1 which prevents the generation of detectable currents. A more detailed examination of the properties of this domain was made by creating two new chimeras. (N_tH5 Kv1)–Kv8 is a chimera constituted by the Kv1.3 protein from the N-terminus to the end of the H5 pore region followed by the S6 and C-terminal regions of Kv8.1 (Figures 2A and 4A). (N_tH5 Kv8)–Kv1 is a chimera containing the Kv8.1 protein sequence from the N-terminus to the end of the H5 pore region followed by the S6 and C-terminal regions of Kv1.3 (Figures 2A and 4A). Neither (N_tH5 Kv8)–Kv1 nor (N_tH5 Kv1)–Kv8 were able to generate detectable K^+ currents in *Xenopus* oocytes (Figure 4A), although *in vitro* translation of the chimeric cRNAs indicated that all constructs gave rise to proteins of the predicted size (data not shown).

As described before, an interesting property of Kv8.1 is that it abolishes the K^+ current elicited by the (N_t Kv8)–Kv1 chimera (Figure 4B). For that reason, we decided to examine the effects of the co-expression of Kv8.1 cRNA on currents elicited by different K^+ channel subunits in the Kv1, Kv2, Kv3 and Kv4 subfamilies. In order to calibrate for a possible variation of expression levels for the same cRNA in different oocytes, a control was introduced in all these experiments by co-injecting the same amount of cRNA corresponding to the inward rectifier K^+ channel subunit IRK1 (Kubo *et al.*, 1993) with all the cRNA combinations. A hyperpolarization step to –120 mV then allowed the recording of the IRK1 current, and only oocytes expressing inward currents of similar intensities were retained for evaluation of their outward current. Following co-injection with Kv8.1 cRNA, the amplitude and shape of the current elicited by Kv1.3, Kv1.5 and Kv4.1 subunits were hardly modified (Figure 5A and B). Conversely, currents elicited by Kv2.1 and Kv3.4 subunits were totally abolished. The same result was observed with the Kv2.2 subunit (Hwang *et al.*, 1992), the other member of the *Shab* subfamily (Figure 5B). This inhibitory behavior of Kv8.1 was also observed with a Kv8.1 cDNA cloned from rat brain (data not shown).

Since the Kv8.1 cRNA used for these experiments contains β-globin sequences to enhance expression in oocytes (Guillemare *et al.*, 1992), several controls were carried out to eliminate the possibility of an artefact due to an inhibition of translation of some of the other Kv subunits when co-expressed with Kv8.1 cRNA. First, as shown in Figure 5, no reduction of inward currents was noticed following the injection of Kv8.1 cRNA in experiments carried out with the IRK1 cRNA. Second, co-expression of the Kv2.1 cRNA with a Kv8.1 transcript devoid of β-globin sequences still led to a drastic inhibition of the Kv2.1 current (Figure 5C). Third, co-injections

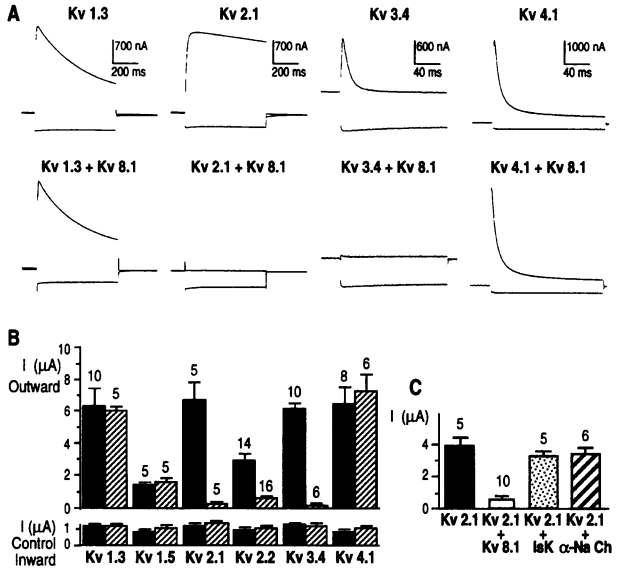


Fig. 5. Inhibitory properties of the Kv8.1 subunit. (**A**) Current traces recorded 2 days after oocytes injection of the indicated cRNAs. (**B**) Current measurements elicited by injection of the indicated cRNAs with (hatched boxes) or without (plain boxes) Kv8.1 cRNA co-injection. The amount of transcript injected per oocyte is between 1 and 10 ng. The quantity of Kv8.1 cRNA (containing β-globin sequences) injected is 2- or 3-fold (in moles) higher than that of co-expressed subunits. The same quantity (0.5 ng per oocytes) of the inward rectifier potassium channel IRK1 cRNA was co-injected in each experiment. The holding potential is –90 mV. The inward current is recorded after hyperpolarization to –120 mV. The outward current corresponds to a depolarizing step to +40 mV. (**C**) Control experiments: current measurements elicited by injection of Kv2.1 cRNA (3 ng), with Kv8.1 cRNA devoid of β-globin sequences (molecular ratio 1/3), with pBTG-IsK cRNA (molecular ratio 1/10) or with the pBTG α-subunit of the epithelial Na^+ channel (molecular ratio 1/10) cRNA. Peak outward and control inward currents are the average of currents recorded from the indicated number of oocytes.

with the Kv2.1 cRNA, of different cRNAs in a 10-fold excess coding for IsK, a protein producing a slowly activating voltage-sensitive K^+ channel (Attali *et al.*, 1993a) or coding for the α-subunit of the epithelial sodium channel (Lingueglia *et al.*, 1993) and containing β-globin sequences, led to no significant decrease in the Kv2.1 currents (Figure 5C).

The implication of the first 213 amino acids of Kv8.1 in the specificity of K^+ current inhibition by Kv8.1 is demonstrated in Figure 4B. The chimera (N_t Kv1)–Kv8 lacking the N-terminal part of Kv8.1 which has been replaced by the N-terminal part of Kv1.3 has lost the capacity to inhibit Kv2.1 and Kv3.4 currents. Conversely, following co-injection with (N_t Kv1)–Kv8, the Kv1.3 current was greatly decreased (Figure 4B).

Co-immunoprecipitation of Kv8.1 protein with Kv2.1 and Kv2.2 subunits

At this point, it was essential to test the possibility that the Kv8.1 protein can interact with other K^+ channel subunits of the outward rectifier Kv family. In order to be able to carry out immunoprecipitation experiments, an extension of eight amino acids which is recognized by a monoclonal antibody called M_2 was added at the N-terminus of Kv8.1. Upon expression in COS-7 cells, the tagged Kv8.1 protein could then be immunoprecipitated

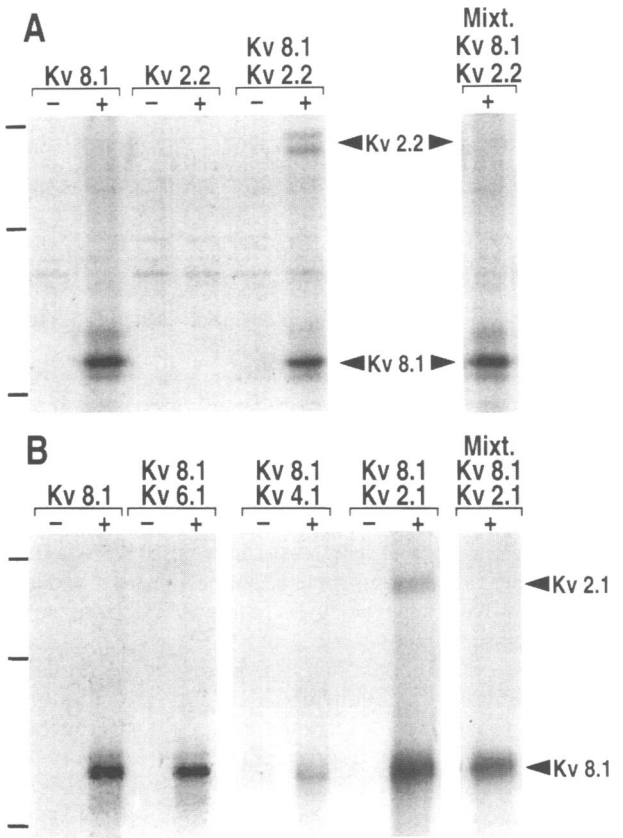


Fig. 6. Immunoprecipitation indicates the formation of heteropolymeric assembly with the Kv8.1 subunit. Autoradiograph of immunoprecipitated [^{35}S]methionine-labeled proteins extracted from (**A**) transfected COS cells and (**B**) *in vitro* translation of cRNAs. Proteins were immunoprecipitated with (+) or without (–) M_2 anti-Flag monoclonal antibody. The transfected plasmids and *in vitro* translated cRNAs are indicated above each lane. A 1:1 molecular ratio was used upon co-transfection of Kv8.1 and Kv2.2 plasmids. In *in vitro* co-translations with Kv8.1 cRNA, the molecular ratios of co-translated cRNAs were optimized in order to get about three times the amount of co-synthesized subunit (Kv2.1, Kv4.1, Kv6.1) than Kv8.1 protein. A systematic check was carried out to ensure that each subunit analyzed in this figure had been synthesized (see Materials and methods). Migration of molecular weight standards is indicated by bars on the left side [from top to bottom, 120 kDa (β-galactosidase), 84 kDa (fructose-6-phosphate kinase), 55 kDa (glutamic dehydrogenase)]. Mixt.: mixture (50:50 v/v) of the indicated proteins carried out before solubilization.

by the M_2 antibody (Figure 6A). When tagged Kv8.1- and Kv2.2-expressing vectors were co-transfected in COS-7 cells, new bands were observed following immunoprecipitation with the M_2 antibody, corresponding to the expected migration profile of the Kv2.2 subunit (Figure 6A). Since no immunoprecipitation was seen when Kv2.2 was expressed alone, the conclusion is that the Kv8.1 protein can associate with Kv2.2 in COS-7 cells. This view is confirmed by the observation that, upon solubilization of a mixture of two different extracts of COS-7 cells, one containing the tagged Kv8.1 and the other one the Kv2.2 protein, no assembly of subunit can be detected (Figure 6A). Clearly then the interaction of Kv8.1 with Kv2.2 is achieved during translation or/and maturation of the K^+ channel subunits.

The ability of Kv8.1 to associate with other types of Kv subunits was also assayed by immunoprecipitation

after co-synthesis *in vitro* in the presence of microsomal membranes. Following co-synthesis of Kv8.1 with Kv4.1 and Kv6.1 (Figure 6B), the M_2 antibody could only immunoprecipitate Kv8.1. Conversely, co-translation of Kv8.1 and Kv2.1 led to immunoprecipitation of both K^+ channel subunits, demonstrating again the specific assembly of these two proteins (Figure 6B).

Discussion

Most of the cloned outward rectifier K^+ channel subunits with six transmembrane structures fall into one of the four classical subfamilies *Shaker* (Kv1), *Shab* (Kv2), *Shal* (Kv3) and *Shaw* (Kv4). However, new types of potential K^+ channel subunits (Drewe *et al.*, 1992; Zhao *et al.*, 1994) have been described recently which further increase the number of subfamilies, and these can be classified in the new Kv5, Kv6 and Kv7 subfamilies. Most members of these many types of structures can express K^+ channels when their cRNAs are injected into *Xenopus* oocytes. Some, however, such as Kv6.1 and Kv7.1, have never been expressed successfully.

Here we describe the cloning from an insulin-secreting cell line of a new subunit which belongs structurally to the large family of Kv channel structures. It has been called Kv8.1 because it does not fall into any of the previously described families of subunits. The sequence which is most closely related to Kv8.1 is that of Kv2.1 (a *Shab* channel). However the two proteins share only 44% identity (Figure 2B). Although Kv8.1 has been cloned from insulinoma cells, it appears to be present mainly in brain. It is not present in other insulin-secreting cells, and its presence in HIT cells is probably due to an aberrant expression profile in this tumorigenic cell.

Injection of the Kv8.1 cRNA into *Xenopus* oocytes or expression in other cell types did not lead to K^+ channel detection. However, expression of Kv8.1 could inhibit specifically the expression of other 'functional' subunits such as Kv2.1, Kv2.2 and Kv3.4, while it had no effect on the expression of members of the Kv1 or Kv4 subfamilies.

The formation of heteromultimeric assemblies of K^+ channel subunits has been studied carefully, and it has been proposed that only members of the same subfamily can co-assemble (Christie *et al.*, 1990; Covarrubias *et al.*, 1991). However, an exception to this model has been reported recently, indicating that Kv1.2 and Kv3.1 can form functional heteromultimeric assemblies (Shahidullah *et al.*, 1995). The specific inhibitory effects of Kv8.1 on Kv2.1, Kv2.2 and Kv3.4 expression are other examples of these possible heteromultimeric assemblies. The first 213 N-terminal amino acids of Kv8.1 contain the necessary information for an interaction with other subunits, since the (N_t Kv1)–Kv8 chimera with the N-terminal portion of Kv8.1 replaced by the N-terminal portion (181 amino acids) of Kv1.3 lost its capacity to inhibit currents elicited by Kv2.1 and Kv3.4 and gained the ability to inhibit the Kv1.3 current (Figure 4B). On the other hand, the (N_t Kv8)–Kv1 chimera containing the N-terminal part of Kv8.1 (213 amino acids) followed by the structure of the Kv1.3 subunit from domain S1 to the C-terminus (i) can give rise to a functional K^+ channel and (ii) this K^+ channel activity can be inhibited by Kv8.1 (Figure 4A and B). This observation indicates again the key role of this N-terminal part in K^+ channel assembly, as previously observed for subunit assembly within *Shaker* and *Shab* subfamilies (Li *et al.*, 1992). This N-terminal domain permits a functional self-assembly of (N_t Kv8)–Kv1 as well as the heterologous (N_t Kv8)–Kv1/Kv8.1 assembly which leads to inhibition of (N_t Kv8)–Kv1 function. All these results taken together strongly indicate that the cytoplasmic N-terminal part (213 amino acids) of Kv8.1 is involved in associations that lead to inhibition of Kv2.1, Kv2.2 and Kv3.4 expression.

The next questions to address is why is Kv8.1 unable to express K^+ currents? Which are the regions in this structure which are responsible for this lack of current?

Results obtained with (N_t Kv1)–Kv8 and (N_t Kv8)–Kv1 chimeras revealed that the N-terminal region of Kv8.1 is able to direct the correct assembly of the channel and indicated that structural characteristics in the S1–C-terminal region prevent the generation of a K^+ channel activity (Figure 4A). A more accurate analysis of the properties of this domain was completed by replacing the S6–Cter region of Kv8.1 by the corresponding regions of Kv1.3 in the (N_tH5 Kv8)–Kv1 chimera and vice versa by exchanging the S6–Cter region in Kv1.3 for the corresponding region of Kv8.1 in the (N_tH5 Kv1)–Kv8 chimera. None of these chimeras gave rise to detectable currents (Figure 4A). Therefore, both S1–H5 and S6–Cter regions of Kv8.1 have specific features that prevent current generation in oocytes. Examination of the sequence in these regions points to some amino acids that may be involved in the prevention of functional expression of Kv8.1. In S1–S5 domains and the H5 pore region of Kv 8.1 a number of amino acids diverge from the very conserved residues found in functional Kv subunits. They are S235T, K269P, V383I and W387F. Four other variations from very conserved amino acids are observed in the S6 and Cter domains of Kv8.1: A413G, I421V, A429P and R434N. These particular changes might have a great influence on channel properties since the S6 domain is known to be part of the pore of the K^+ channel (Lopez *et al.*, 1994; Taglialatela *et al.*, 1994). It is remarkable to observe that variations from the consensus sequence at these four positions of S6 are also observed in Kv6.1 and Kv7.1, i.e. in two other subunits which, like Kv8.1, fail to give rise to K^+ current generation in oocytes (Drewe *et al.*, 1992). Finally, among all these replacements, K269P and A429P might be those which are particularly interesting since, in each case, one observes a replacement by a proline, i.e. a residue that probably has a peculiar role in folding and thereby a potential effect on channel structure.

What could be the function of Kv8.1 in vivo?

First, one cannot eliminate the possibility that there are other subunits in the Kv family that, on co-assembly with Kv8.1, could create channel activity. Examples of that sort exist for other channel types. For example, the NR_2 subunit of the *N*-methyl-D-aspartate (NMDA) receptor does not have channel activity by itself but channel activity appears when it associates with the NR_1 subunit (Monyer *et al.*, 1992). Another example is the amiloride-sensitive Na^+ channel which is made up of three parent subunits $\alpha\beta\gamma$ (Canessa *et al.*, 1994) or $\delta\beta\gamma$ (Waldmann *et al.*, 1995). Only one of them, α or δ, has intrinsic channel activity

but the two other subunits, β and γ, that are inactive by themselves have the capacity to increase greatly the Na^+ channel activity (Canessa *et al.*, 1994; Lingueglia *et al.*, 1994; Waldmann *et al.*, 1995). The nicotinic receptor (Bertrand and Changeux, 1995; McGehee and Role, 1995) or the G protein-activated inward rectifiers (Duprat *et al.*, 1995) are other examples of this situation. However, in none of these cases it has been observed that a particular subunit which is unable to produce channel activity in *Xenopus* oocytes by itself is capable of inhibiting channel activity normally produced by the other parent subunits.

This is why of course it is most tempting to propose from our electrophysiological results that the normal function of Kv8.1 is to inhibit the K^+ channel activity of channels made from subunits belonging to the Kv2 and Kv3 families. It turns out that the localization of the Kv8.1 transcript in brain is compatible with the interaction of Kv8.1 with Kv2.1, Kv2.2 and Kv3 subunits. Indeed, Kv2.1 and Kv2.2 have been clearly detected in regions where Kv8.1 transcripts have been found, i.e. in Purkinje and granular cells of cerebellum, in pyramidal cells of hippocampus, in granular cells of dentate gyrus (Kv2.1 only) and in the olfactory bulb (Hwang *et al.*, 1993). Moreover, the pattern of expression of Kv8.1 transcripts in the brain also overlaps the pattern of expression of the various Kv3 subunits (Weiser *et al.*, 1994).

The type of inhibition observed here for the K^+ channel has also been observed recently for the NMDA receptors. A novel subunit called χ-1 (Ciabarra *et al.*, 1995) or NMDA-L (Sucher *et al.*, 1995), specifically interacts with the assembly of NMDAR1 or the assembly of NMDAR1, NMDAR2B or 2D subunits to inhibit their functional expression. Inhibitory effects obeying the same general principle have also been observed in other fields of molecular biology. For example, proteins with parent structures displaying opposite functional effects associated with homo- or heteromultimeric formation have been described in investigations concerning regulation of gene expression (Chiu *et al.*, 1989; Benezra *et al.*, 1990; Foulkes *et al.*, 1991; Ron and Habener, 1992). Another particularly spectacular example of this situation has been found in cell apoptosis. Bcl-2, the founder member of a growing family of cytoplasmic proteins that control cell survival, prevents death of neurons deprived of particular neurotrophic factors *in vitro* and rescues developing neurons that would otherwise die *in vivo*. Bax is a Bcl-2-associated protein that has structural similarity to Bcl-2. Bax heteropolymerizes with Bcl-2, and overexpression of Bax inhibits the death-repressor action of Bcl-2. Similary, Bad, another Bcl-2 analog, can counteract the death-inhibitory effect of Bcl-x, the protein with the highest known structural homology to Bcl-2 (reviewed in Davies, 1995).

The existence of inhibitory subunits for voltage-sensitive K^+ channels could provide a new mode of regulation of electrical activity in neuronal cells. It certainly could be used in long-term changes in the electrogenic nature of neuronal cells that are associated with memory processes.

Materials and methods

Isolation of the Kv8.1 cDNA

Two degenerate primers 5′-WTKWCNWCNNYNGGNTA-3′ and 5′-CCCTCGAGTTTAAAGCTTNGTNSWYTTNCC-3′ corresponding respectively to the H5 pore region and the putative Walker A ATP binding site (Walker *et al.*, 1982) were used in a PCR amplification on HIT cDNA. The amplification solution (25 μl) contained 30 ng of HIT cDNA, 1 μg of each oligonucleotide, 500 μM dNTPs, 20 mM Tris pH 8.4, 50 mM KCl, 2 mM $MgCl_2$ and 2.5 U of *Taq* polymerase (Gibco BRL). Reverse transcription of HIT mRNA was accomplished with a Pharmacia kit and random primers. After 30 cycles, consisting of 94°C, 30 s, 50°C, 3 min, 72°C 1 min, 1 μl of the PCR reaction mixture was submitted to a second round of amplification under the same conditions but with two nested degenerate primers, 5′-WCNWCNNYNGGNTAY-GGNGA-3′, which corresponds to the H5 domain, and 5′-CCCTCG-AGTTTAAAGCTT-3′, which represents the 5′ portion of the Walker A oligonucleotide described above. Amplification products were fractionated on a 10% polyacrylamide gel and each band was electroeluted and cloned in PCR-Script vector (Stratagene). The sequence of cloned PCR products allowed the isolation of the Kv8.1 probe that was used to screen 10^5 clones of an HIT cDNA library. The library was constructed with λ ZAP I vector (Stratagene) and sized oligo(dT)-primed cDNA derived from HIT mRNA. Recombinant phages were screened as previously described (Attali *et al.*, 1993b) by hybridization with random primed [α-^{32}P]dATP-labeled Kv8.1 fragment. Four clones containing inserts of 2.9 kb were then obtained. Sequencing of their extremities indicated that they represented four independent insertions. Deletion clones for sequencing were prepared with the Erase-A-Base system (Promega) and the sequence was determinated on both strands with a dye terminator kit (Applied Biosystem) and automatic sequencer.

Northern blot analysis and brain in situ hybridization

Total RNA was extracted from hamster tissues and various cell lines as previously described (Chomczynski and Sacchi, 1987). Northern blot analysis was performed with 5 μg of poly(A^+) RNA (Attali *et al.*, 1993b). The probe used corresponded to the 2.9 kb insert labeled with a random primed kit (Pharmacia) and [α-^{32}P]dATP (RAS = 3000 Ci/mmol). The final wash was in 0.1× SSC and 0.1% SDS at 65°C.

The *in situ* hybridization procedure was carried out using animals killed by transcardial perfusion with 0.9% NaCl followed by ice-cold 1% (w/v) paraformaldehyde in 0.1 M sodium phosphate-buffered saline solution (PBS, pH 7.4). Dissected brains were post-fixed in the same solution for 2 h and then immersed overnight at 4°C in a 20% sucrose–PBS solution. Sagittal and coronal frozen sections (10 μm) were cut on a cryostat (Microm) at –25°C, collected on 3-aminopropylethoxysilane-coated slides and stored at –70°C until use. Brain sections were hybridized with an oligonucleotide probe complementary to Kv8.1 mRNA (5′ GAGAGAGCGCACAGCTGCTCCATGACGTGCAGGCG-ACCCG 3′). The sense oligonucleotide was used as a control probe. Both probes were 3′ end-labeled using terminal deoxynucleotidyl transferase (Boehringer) and [α-^{35}S]dATP (>1000 Ci/mmol, Amersham) to a specific activity of 1.5×10^9 c.p.m./μg. Sections were pre-hybridized for 1 h at room temperature in a solution containing 4× SSC and 1× Denhardt's solution. The slides were then rinsed for 10 min in 4× SSC, acetylated for 10 min in acetic anhydride 0.5 ml/200 ml of 0.1 M triethanolamine and dehydrated. Hybridization was carried out overnight at 42°C in 50% deionized formamide, 10% dextran sulfate, 500 μg/ml yeast tRNA, 20 mM dithiothreitol (DTT), 20 mM $NaPO_4$ in 2× SSC. For each slide, a 35 μl hybridization mix containing 3×10^5 c.p.m. of the denatured labeled oligonucleotide was used. Slides were washed in 1× SSC, 20 mM DTT at 55°C twice for 30 min before dehydration and apposition to Hyperfilm-βmax (Amersham) for 5 days at 4°C. Selected slides were dipped in Amersham LM1 photographic emulsion and exposed for 2 weeks at 4°C and then developed in Kodak D-19 for 4 min. All slides were counterstained with Cresyl violet. For control experiments, adjacent sections were hybridized with sense probes or digested with RNase before hybridization.

Human chromosomal mapping

In situ hybridization was carried out on chromosome preparations obtained from phytohemagglutinin-stimulated human lymphocytes cultured for 72 h. 5-Bromodeoxyuridine was added for the final 7 h of culture (60 μg/ml of medium), to ensure a post-hybridization chromosomal banding of good quality. Kv8.1 cDNA was tritium labeled by nick-translation to a specific activity of 2.5×10^8 d.p.m./μg. The radiolabeled probe was hybridized to metaphase spreads at a final concentration of 25 ng/ml of hybridization solution as previously described (Mattéi *et al.*, 1985). After coating with nuclear track emulsion (Kodak NTB2), the slides were exposed for 25 days at +4°C, then developed. To avoid any slipping of silver grains during the banding procedure, chromosome spreads were first stained with buffered Giemsa

solution and metaphases photographed. R-banding was then performed by the fluorochrome–photolysis–Giemsa (F.P.G) method and metaphases re-photographed before analysis.

Construction of epitope-tagged Kv8.1 protein and Kv1.3–Kv8.1 chimeric subunits

In order to link the Flag epitope (DYKDDDDKV) to the N-terminus of the Kv8.1 subunit, the double-stranded oligonucleotide 5′-CTAGC-ATCGATACCATGGACTACAAAGACGATGACGATAAAGTTAAC-TAA-3′ was ligated to non-regenerative *Bst*XI adaptors (Invitrogen) and inserted into a *Bst*XI-digested pRc/CMV-expressing vector (Invitrogen). This construction designated Flag-pRc/CMV vector contains *Hin*dIII, *Cla*I, *Hpa*I, *Not*I, *Xba*I and *Apa*I cloning sites used to generate N-terminal- or C-terminal-tagged proteins. An *Xba*I restriction site was added to the 3′ end of Kv8.1 cDNA by PCR amplification (PWO DNA polymerase Boehringer). This fragment was then digested by *Xba*I and inserted in *Hpa*I–*Xba*I-linearized Flag-pRc/CMV vector in order to create a tagged-Kv8.1-expressing vector.

To generate the first chimera (N_t Kv8)–Kv1, a plasmid (pKv1.3) containing the Kv1.3 coding sequence (Attali *et al.*, 1992) was digested with *Xho*I in order to linearize the plasmid in the polylinker located upstream of the open reading frame (ORF) and with *Xma*I which cleaves in the ORF. This procedure generated a plasmid deleted for the first 181 amino acids of Kv1.3 which subsequently was ligated with a DNA fragment flanked by *Xho*I and *Xma*I sites and coding for the first 213 amino acids of Kv8.1. This insert was produced by PCR amplification (PWO DNA polymerase, Boehringer) on pBTG Kv8.1 vector (see below) with T3 primer and 5′-AAGACCCGGGCTGCTGTGGAA-3′ oligonucleotide, and then digested by the two cited restriction enzymes.

To obtain the second chimera (N_t Kv1)–Kv8, pKv1.3 was first digested with *Not*I in order to linearize the vector in the polylinker situated downstream of the ORF. This DNA was then partially digested by *Xma*I and the resulting molecule containing the Kv1.3 ORF deleted for the last 342 amino acids was gel purified and ligated with a fragment coding for the last 291 amino acids of Kv8.1. This DNA was obtained by amplification on pBTG Kv8.1 with T7 and 5′-GCAGTCCGGAT-CTTTGGAGTCA-3′ primers and then doubly digested by *Not*I and *Bsp*E1, which generated ends compatible with *Xma*I.

The third chimera (N_tH5 Kv8)–Kv1 was obtained by digestion of pKv1.3 with *Dra*III (partial cleavage) and *Xho*I. The plasmid containing the Kv1.3 ORF deleted for the first 398 amino acids was then gel purified and ligated to a DNA fragment coding for the first 402 amino acids of Kv8.1. This molecule was generated by amplification on pBTG Kv8.1 vector with T3 and 5′-GTGGTCACTGGGTGAATGTCCCCATA-GCCT-3′ primers, digested by *Xho*I and *Dra*III (partial cleavage) and gel purified.

In order to create the (N_tH5 Kv1)–Kv8 chimera, we digested the pKv1.3 plasmid with *Not*I and *Dra*III (partial cleavage) and gel purified the vector deleted for the last 122 amino acids. This molecule was then ligated to a DNA fragment coding for the last 99 amino acids of Kv8.1 which was obtained by amplification on pBTG Kv8.1 with T7 and 5′-ACATTCACCCAGTGACCACCACAGGCAAA-3′ primers and digestion by *Not*I and *Dra*III.

cRNA synthesis, injection and electrophysiological measurement in Xenopus oocytes

In order to improve the efficiency of expression in oocytes, cDNAs were inserted in a pBTG vector (an expression vector containing β-globin 5′ and 3′ non-translated sequences) (Guillemare *et al.*, 1992) containing the 5′- and 3′-non-coding regions of *Xenopus laevis* β-globin sequences. Capped cRNAs were synthesized with T3 or T7 RNA polymerase (Promega). Preparation of oocytes, cRNA injection (50 nl) and electrophysiological measurements have been described previously (Guillemare *et al.*, 1992). The variability of the results was expressed as the standard error of the mean with n indicating the number of oocytes contributing to the mean.

Immunoprecipitation of ex vivo [^{35}S]methionine-labeled proteins

Sub-confluent COS-7 cells plates (90 mm) were transfected using the DEAE-dextran method (Lopata *et al.*, 1984) with 10 μg of plasmid. Two days later, cells were incubated for 4 h with 70 μCi [^{35}S]methionine (1000 Ci/mmol) in 1.5 ml of methionine-free MEM media complemented with 10% of dialyzed fetal calf serum. After one wash with PBS, cells were harvested and disrupted by sonication with 1 ml of ice-cold buffer containing Tris pH 8.0 25 mM, NaCl 150 mM, EDTA 1 mM, phenylmethylsulfonyl fluoride 1 mM, 10 μg/ml leupeptin and aprotinin. Protein solubilization was achieved by a 2 h incubation under agitation with 1% Triton X-100, and cellular debris was eliminated by centrifugation at 100 000 *g* for 30 min. To decrease non-specific precipitation, a pre-clearing incubation of 1 h was done with 15 μl of equilibrated protein A–Sepharose beads (CL-4B Sigma). For immunoprecipitation, 500 μl of surpernatant were incubated overnight at 4°C with 5 μg of M_2 anti-Flag antibody (Kodak). Immune complexes were pelleted after an incubation for 2 h with 40 μl of protein A–Sepharose beads, washed five times for 5 min with the ice-cold solubilization buffer and then dissociated by boiling in 30 μl of the SDS sample buffer. Proteins were then fractionated on 10% SDS–PAGE.

Immunoprecipitation of in vitro labeled proteins

Translation of cRNA (50 μl) was performed with reticulocyte lysate supplemented with canine pancreatic microsome (Promega) and 20 μCi of [^{35}S]methionine. The effective co-translation of the Kv8.1 protein with other K^+ subunits (Kv2.1, Kv4.1, Kv6.1) was checked systematically by gel fractionation and autoradiography of an aliquot of each reaction mixture. After translation, the volume was brought to 500 μl with the solubilization buffer supplemented with 1% bovine serum albumin and immunoprecipitation was performed as described above.

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