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Potassium channels from NG108-15 neuroblastoma-glioma hybrid cells

Primary structure and functional expression from cDNAs

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The complete amino acid sequences of two potassium channel proteins from NG108-15 neuroblastoma-glioma hybrid cells have been deduced by cloning and sequencing the cDNAs. One of these proteins (NGK2) is structurally more closely related to the *Drosophila Shaw* gene product than to the *Shaker* and *Shab* gene products, whereas the other (NGK1) is identical with a rat brain potassium channel protein (BK2) which is more closely related to the *Drosophila Shaker* gene product. mRNAs derived from both the cloned cDNAs, when injected into *Xenopus* oocytes, direct the formation of functional potassium channels with properties of delayed rectifiers.

Potassium channel; cDNA cloning; cDNA expression; Delayed rectifier; Phylogeny; (NG108-15 cell)

1. INTRODUCTION

Voltage-gated potassium channels in excitable membranes play important roles in the control of cellular excitability [1]. Recently, the primary structures of some potassium channel proteins from *Drosophila* [2–6] and from mammalian brain [7–11] have been elucidated by cloning and sequencing the cDNAs. mRNAs derived from *Drosophila Shaker* cDNAs [12–14] and from rat brain cDNAs [10,11,15,16] direct the formation of functional potassium channels in *Xenopus* oocytes. The present investigation deals with cloning and sequence analysis of two cDNAs encoding distinct potassium channel proteins from mouse neuroblastoma × rat glioma hybrid cells (NG108-15) [17] and with functional expression of the cloned cDNAs. The evolutionary relationships among voltage-gated potassium channel proteins are discussed.

2. MATERIALS AND METHODS

2.1. *Cloning and sequencing of cDNAs*

NG108-15 cells were cultured and differentiated in the presence of 1 mM theophylline and 10 μM prostaglandin E_1 as in [17]. Total RNA was extracted [18], and poly(A)$^+$ RNA isolated [19]. Randomly primed cDNA libraries were constructed in phage λZAPII (Stratagene) using poly(A)$^+$ RNA as template. Double-stranded cDNA, prepared with the cDNA synthesis system (Bethesda Research Laboratories), was methylated by *Eco*RI methylase, blunted by T_4 DNA polymerase and ligated with the *Eco*RI linker dGGAATTCC. After *Eco*RI digestion, the cDNA was passed through a Sepharose CL-4B column. Fractions longer than ~0.4 kilobase pairs (kb) were pooled and ligated into the vector. The initial screening of a cDNA library was effected by hybridization at 50°C with a synthetic DNA probe prepared as follows. Two overlapping 105-mers (I, II) which comprise portions of the MBK1 cDNA [7] encoding a mouse brain potassium channel protein were prepared with an automatic DNA synthesizer (Applied Biosystems); I represents nucleotides 862–966 of the message strand, and II the sequence complementary to nucleotides 937–1041. After annealing the single strands, the complementary sequences were labeled by a fill-in reaction using a Klenow fragment and [α-^{32}P]dCTP as in [20]. For the screening of a second cDNA library, cDNA clones isolated initially served as probes for hybridization at 50°C. cDNA inserts from isolated clones were subcloned into pBluescript SK(−) by in vivo excision according to the Stratagene product protocol. Nested deletions were made [21] and DNA sequencing was carried out on both strands by the dideoxy chain termination method [22]. Blot hybridization analysis of poly(A)$^+$ RNA from NG108-15 cells was performed as in [23], using the *Eco*RI(815)/*Bam*HI(1612) fragment from λNGK2·19A4 or the *Pst*I(vector)/*Bam*HI(1612) fragment from pSPNGK2 as probe; restriction endonuclease sites are identified by numbers (in parentheses) indicating the 5′-terminal nucleotide generated by cleavage.

2.2. *Synthesis of specific mRNAs*

The recombinant plasmids pSPNGK1 and pSPNGK2, used for the synthesis of mRNAs specific for the NGK1 and the NGK2 protein (see section 3), respectively, were constructed as follows. pSP64(polyA) (Promega) was digested with *Eco*RI, blunted by T_4 DNA polymerase and ligated with the *Xho*I linker dCCTCGAGG. The resulting plasmid was cleaved with *Xho*I and ligated again to yield pSP64AX. The 1.8-kb *Pvu*II(−40)/*Bam*HI(vector) fragment from λNGK1·23C2

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and the 3.0-kb *Bam*HI/*Hin*cII fragment from pSP64AX were ligated to yield pSPNGK1. The *Nar*I(−7)/*Nar*I(508) fragment from λNGK2·18A1 was blunted by T_4 DNA polymerase and cleaved by *Hin*dIII. The resulting 0.23-kb fragment, the *Hin*dIII(224)/*Eco*RI(815) fragment from λNGK2·18A1, the *Eco*RI(815)/*Bam*HI(1612) fragment from λNGK2·19A4 and the 3.0-kb *Bam*HI/*Hin*cII fragment from pSP64AX were ligated to yield pSPNGK2. NGK1-specific and NGK2-specific mRNAs were synthesized in vitro, using *Xho*I-cleaved pSPNGK1 and pSPNGK2, respectively, as templates [24]. Transcription was primed with the cap dinucleotide G(5′)ppp(5′)G (0.5 mM) [25].

2.3. *Electrophysiological measurements*

Each of the specific mRNAs was injected into *Xenopus laevis* oocytes (mRNA concentration, 25 ng/μl; average volume injected per oocyte, ~50 nl). The injected oocytes were incubated at 19°C for 2–3 days in modified Barth's medium [26] containing cefmenoxime (0.1 mg/ml). Whole-cell currents were measured using a conventional two-microelectrode voltage clamp. The chamber was continuously perfused with normal frog Ringer solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 10 mM HEPES, pH adjusted to 7.2 with NaOH). For ion substitution experiments, the concentration of KCl was raised at the expense of NaCl. The final KCl concentration ranged from 5 mM to 100 mM. Effects of pharmacological agents were assessed by measuring the current amplitudes at the end of 200-ms voltage steps to 0 mV. Whole-cell current records were low-pass filtered at 3 kHz and sampled at 2.5 kHz. For single-channel recording, oocytes were immersed in a nominally Ca^{2+}-free K^+ solution (100 mM KCl, 10 mM EGTA, 10 mM HEPES, pH adjusted to 7.2 with KOH) and inside-out patches were excised into the same solution. Patch pipettes were filled with normal frog Ringer solution. All electrophysiological measurements were carried out at 22 ± 1°C essentially following the procedures described in [15].

3. RESULTS AND DISCUSSION

A randomly primed cDNA library derived from NG108-15 cell poly(A)$^+$ RNA was screened by hybridization with a synthetic DNA probe comprising a partial cDNA sequence for a mouse brain potassium channel protein (see section 2.1). From ~5×10^5 plaques, two clones (λNGK1·5C1 and λ2NGK2·5B1) were isolated. A second randomly primed cDNA library (~3×10^6 plaques) was screened by hybridization with the cDNA inserts of the initial clones, yielding two clones (including λNGK1·23C2) that hybridized with λNGK1·5C1 and six clones (including λNGK2·18A1 and λNGK2·19A4) that hybridized with λNGK2·5B1. Nucleotide sequence analysis of clones λNGK1·23C2 and λNGK1·5C1 revealed that they encode a protein composed of 499 amino residues (NGK1) whose sequence is identical with that of the rat brain BK2 protein reported previously [9].

Fig.1 shows the cDNA sequence encoding the NGK2 protein, determined with clones λNGK2·18A1, λNGK2·19A4 and λNGK2·5B1, together with the deduced amino acid sequence. The translational initiation site was assigned to the first ATG triplet that appears downstream of a nonsense codon, TAA (nucleotide residues −15 to −13). The nucleotide sequence surrounding the initiating codon agrees reasonably well with the consensus sequence [27]. A translational termination codon, TGA, occurs in the frame after codon 511 specifying isoleucine. Thus the NGK2 protein is composed of 511 amino acid residues (including the initiating methionine) and has a calculated M_r of 57 925. The size of the NGK2 mRNA was estimated to be ~7000 nucleotides by blot hybridization analysis of poly(A)$^+$ RNA from NG108-15 cells.

The NGK2 protein, like other potassium channel proteins [2–11], has a basic structure corresponding to a single internal repeat of the sodium channel and the calcium channel [28,29]. It contains five hydrophobic segments (S1, S2, S3, S5 and S6) and one positively charged segment (S4). The conserved charged residues in segment S2 and in segment S3 are also retained. The sequence similarity observed suggests that the NGK2 protein has the same transmembrane topology as proposed for the single internal repeats of the other voltage-gated ionic channels and that it forms a homo- or heteromultimer to surround a central pore as a channel. This model is consistent with both the two potential *N*-glycosylation sites (asparagine residues 220 and 229) being located on the extracellular side of the membrane.

Fig.2 shows an alignment of the amino acid sequence of the NGK2 protein with those of the *Drosophila Shaw*, *Shaker* and *Shab* proteins and of the mammalian RCK1, BK2 and drk1 proteins. The amino acid sequence of the NGK2 protein is more closely related to that of the *Shaw* protein (56% amino acid identities) than to those of the other potassium channel proteins (43%–45% amino acid identities). On the basis of the sequence alignment, a phylogenetic tree representing the evolutionary relationships among the seven potassium channel proteins was inferred by the neighbor-joining method [30], as shown in fig.3. According to the phylogenetic tree, known members of the potassium channel family are classified into three subfamilies: NGK2/*Shaw* subfamily, RCK1·BK2/*Shaker* subfamily and drk1/*Shab* subfamily. From the phylogenetic positions of the divergence of mammalian and *Drosophila* genes, it is evident that the three subfamilies diverged before the separation of vertebrates and insects. After this separation, mammalian genes belonging to the RCK1·BK2/*Shaker* subfamily further diverged during vertebrate evolution. The relationship between NGK2 and *Shaw* (56% amino acid identities) is less close than that between drk1 and *Shab* (72%), between RCK1 and *Shaker* (82%) or between BK2 and *Shaker* (80%). Thus it remains possible that NGK2 is not a mammalian counterpart of *Shaw*. Of interest would be the evolutionary relationships among *Shal*, another *Drosophila* potassium channel gene [6], and NGK2 and the other members of the potassium channel family.

mRNAs specific for the NGK1 and for the NGK2 protein were synthesized by transcription in vitro of the cloned cDNAs and were injected into *Xenopus* oocytes.

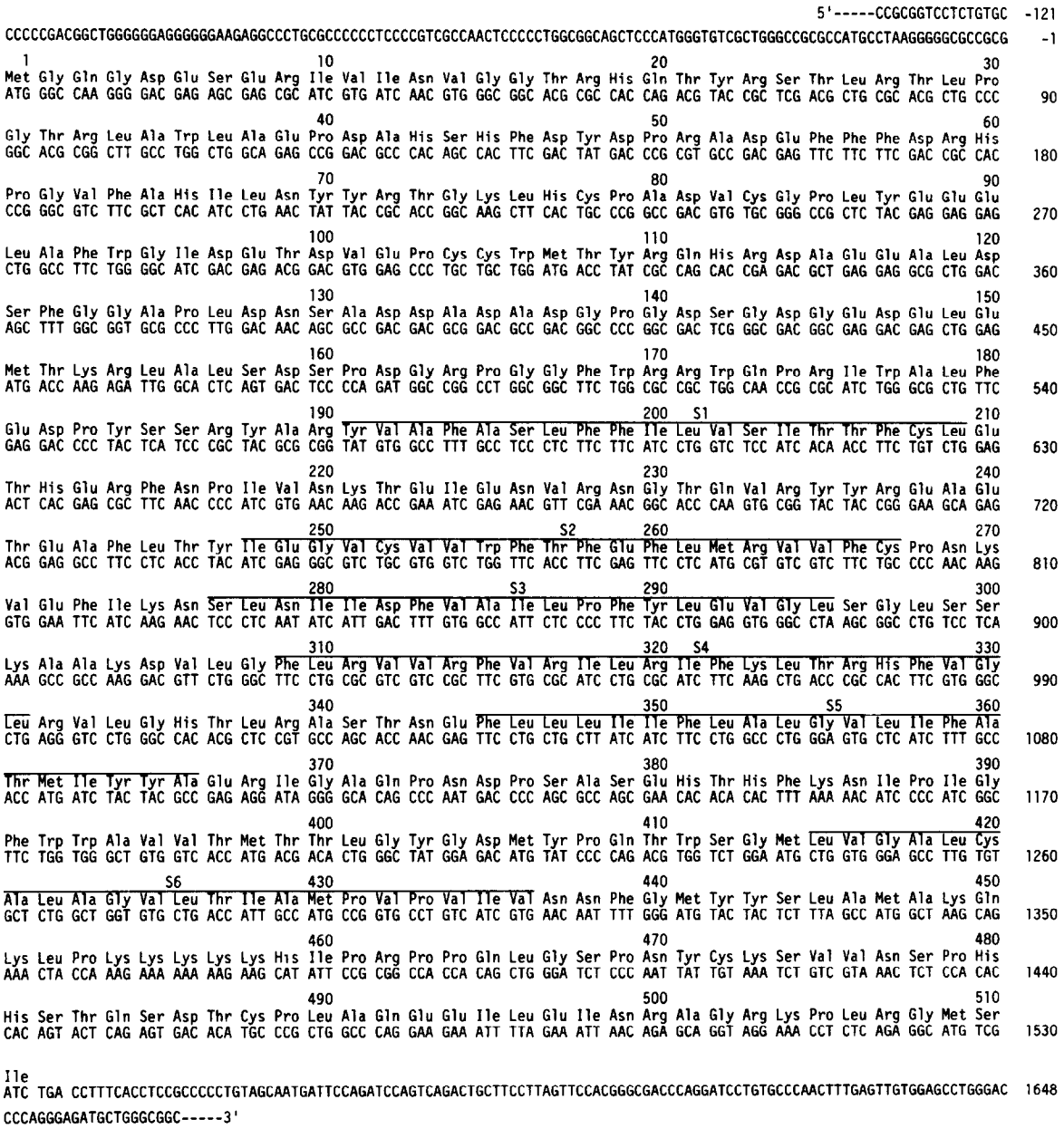

Fig.1. Nucleotide sequence of the cDNA encoding the NGK2 protein and the deduced amino acid sequence. The nucleotide sequence was determined using clones λNGK2·18A1 (carrying nucleotides −136 to 1014), λNGK2·19A4 (665–1669) and λNGK2·5B1 (925–1362). Nucleotide residues are numbered in the 5′ to 3′ direction from the first residue of the ATG initiation triplet and the preceding residues are indicated by negative numbers. Numbers of the nucleotide residues at the right-hand end of the individual lines are given. Amino acid residues are numbered from the intitiating methionine. The putative transmembrane segments S1–S6 are overlined; the termini of each segment have been tentatively assigned.

Large outward currents were elicited in injected oocytes upon depolarization from a holding potential of −60 mV under voltage clamp (fig.4A,B). The size of the current at 0 mV was 18.0±10.7 μA (mean±SD, $n=29$) for oocytes injected with the NGK1-specific mRNA and 25.4±10.9 μA ($n=26$) for oocytes injected with the NGK2-specific mRNA. Under the same experimental conditions, non-injected oocytes showed small outward currents, but the size of the current was at most 0.15 μA (0.07±0.05 μA, $n=30$). When the test potential was varied, the outward current began to appear at −30 mV in oocytes injected with the NGK1-specific mRNA, whereas the outward current was elicited only at more positive potentials in oocytes injected with the NGK2-specific mRNA (fig.4A–C). The outward currents in oocytes injected with the NGK1-specific and with the NGK2-specific mRNA showed fast activation, attaining maximal amplitudes in tens of milliseconds,

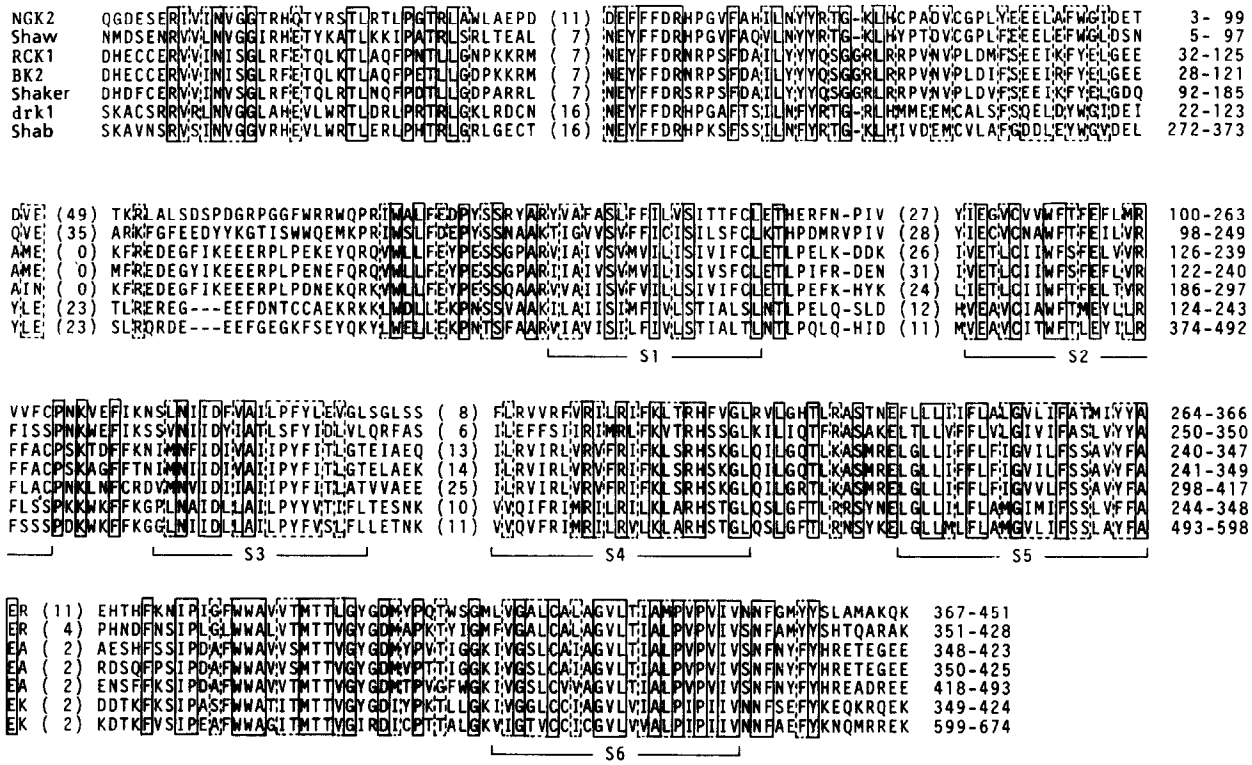

Fig.2. Alignment of the amino acid sequences of different potassium channel proteins. The one-letter amino acid notation is used. The seven sequences compared (from top to bottom) are as follows: NGK2; *Shaw2* [6]; RCK1 [8]; BK2 [9] (NGK1); ShA1 [2]; drk1 [10]; *Shab*11 [6]. Amino acid residues are numbered from the initiating methionine, and numbers of the residues on each line are given on the right-hand side. Sets of seven identical residues at one aligned position are enclosed with solid lines, and sets of seven identical or conservative residues [31] at one aligned position with broken lines. Highly divergent segments are represented by the sum of residues in each segment given in parentheses. For evaluating sequence similarity, a continuous stretch of gaps (-) was counted as one substitution regardless of its length. The positions of segments S1-S6 are indicated (see the legend to fig.1).

but were inactivated very slowly during 1-s voltage steps to 0 mV, the decrease in current size being $9\pm3\%$ ($n=9$) and $13\pm7\%$ ($n=9$) of the peak value, respectively.

Ion substitution experiments showed that the reversal potential of the tail currents shifted by $+55.6\pm1.8$ mV ($n=6$) and by $+53.1\pm2.0$ mV ($n=6$) per 10-fold increase of external K^+ concentration, respectively, in oocytes injected with the NGK1-specific and with the NGK2-specific mRNA. These values are close to the theoretical value of +58.6 mV, calculated from the Nernst equation, and indicate that the outward currents observed in injected oocytes are carried by potassium ions.

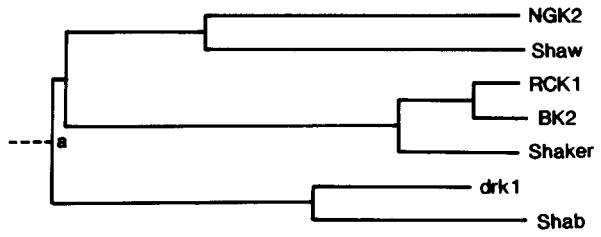

Fig.3. Phylogenetic tree of the potasium channel family. The neighbor-joining method [30] was used; the calculation procedure for the phylogenetic tree inference has been described previously [32]. The deepest root 'a' was determined by comparing the amino acid sequences of the potassium channel proteins with those of sodium channels and calcium channels (data not shown). Lengths of horizontal lines of the tree are proportional to estimated numbers of amino acid substitutions.

The outward currents were examined for sensitivity to pharmacological agents added to the bathing solution. The concentrations required for inhibiting the currents by 50% (IC_{50}) were as follows (the former and latter values refer to oocytes injected with the NGK1-specific and with the NGK2-specific mRNA, respectively): 0.2 mM and 0.6 mM 4-aminopyridine; >10 mM and 0.1 mM tetraethylammonium; and 0.8 mM and 1 mM quinine. Apamin (1 μM) and tolbutamide (100 μM) were ineffective.

Single-channel currents were recorded from inside-out membrane patches isolated from injected oocytes using a nominally Ca^{2+}-free K^+ solution on the cytoplasmic side (fig.4D,E). The channel in patches of oocytes injected with the NGK1-specific mRNA showed a slope conductance of 17.5 ± 0.4 pS (5 patches), but smaller conductance levels were frequently observed

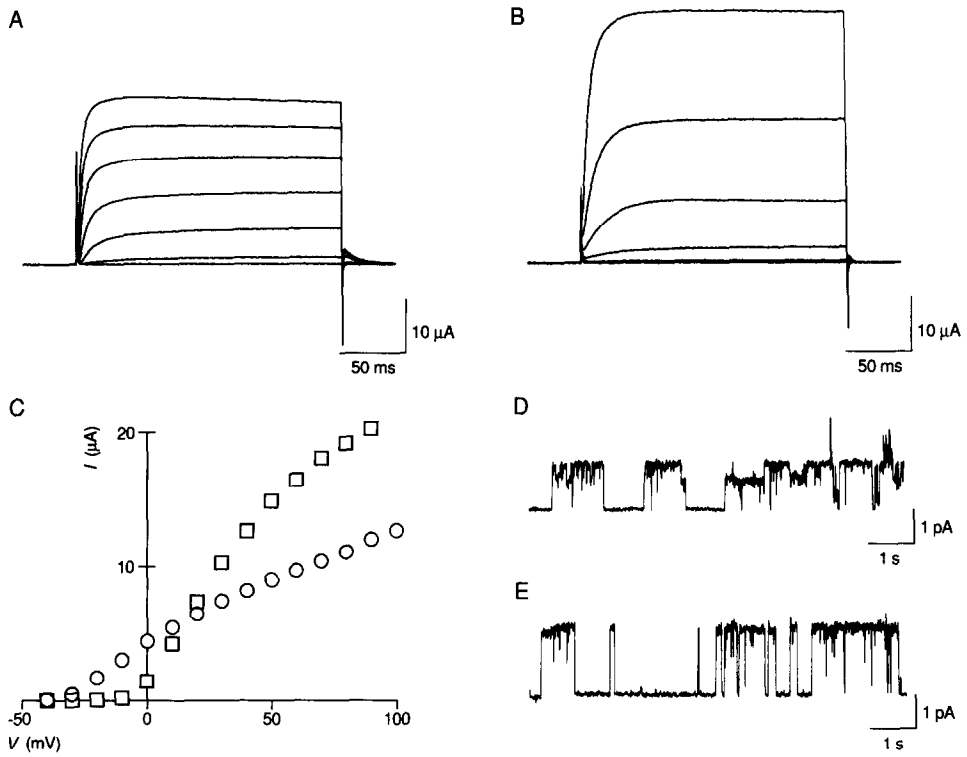

Fig.4. Properties of outward currents in *Xenopus* oocytes injected with the NGK1-specific and with the NGK2-specific mRNA. (A,B) Outward currents in response to depolarizing voltage steps in an oocyte injected with the NGK-1 specific mRNA (A) or with the NGK2-specific mRNA (B). The membrane potential was held at −60 mV and stepped to test potentials ranging from −40 mV to +20 mV in 10-mV increments. In B, overlapping records were obtained upon depolarization to −40 mV, −30 mV and −20 mV. (C) The whole-cell current-voltage relation obtained from an oocyte injected with the NGK1-specific mRNA (circles) or with the NGK2-specific mRNA (squares). The oocytes used for C were different from those used for A and B. (D,E) Single-channel currents from an oocyte injected with the NGK1-specific mRNA (D) or with the NGK2-specific mRNA (E). Inside-out patch. Outward current upwards. Membrane potential, 0 mV. Records were low-pass filtered at 0.1 kHz and sampled at 0.25 kHz.

(fig.4D). The channel in patches of oocytes injected with the NGK2-specific mRNA showed a larger slope conductance (26.4±1.7 pS; 6 patches), and subconductance levels rarely occurred (fig.4E).

The cDNA expression studies indicate that both the NGK2 protein, which is closely related to the *Drosophila Shaw* gene product, and the NGK1 protein, which is a member of the RCK1 · BK2/*Shaker* subfamily, form potassium channels with properties of delayed rectifiers, although the NGK2 and NGK1 potassium channels exhibit some kinetic and pharmacological differences. It has been reported that *Drosophila Shaker* cDNAs are expressed to produce A-type potassium channels [12–14], while expression of mammalian RCK1 and related cDNAs of the same subfamily yields potassium channels with properties of delayed rectifiers and possibly A-type channels [11,15,16]. The drk1 protein, which is a mammalian counterpart of the *Drosophila Shab* gene product, is expressed as a delayed rectifier class channel [10]. The molecular basis for the electrophysiological differences between these voltage-gated potassium channels remains to be elucidated.

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