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A delayed rectifier potassium channel cloned from bovine adrenal medulla Functional analysis after expression in *Xenopus* oocytes and in a neuroblastoma cell line

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Abstract Using a cDNA library from bovine adrenal medulla, and, subsequently, a bovine genomic library, we have isolated the gene coding for a non inactivating potassium channel. This gene encodes a 597-amino acid protein which we have called BAK5 as its sequence is very similar to members of Kv1.5 potassium channel family. Neuroblastoma cells (Neuro-2a cell line) were stably transfected with BAK5 DNA. Protein expression was under the control of a heat-shock promoter. Transfected cells showed a current highly selective for potassium, insensitive to tetraethylammonium but reversibly blocked by 4-aminopyridine. Oocytes injected with BAK5 mRNA also expressed a potassium current with the same characteristics.

Key words: cDNA cloning; Bovine adrenal medulla; Potassium channel; Stable transfection; Heterologous expression

1. Introduction

Potassium channels are membrane proteins involved in the control of membrane potential, thus they regulate the level of cell excitability [1]. There is a large, diverse group of potassium channels, allowing a wide variety of membrane potential responses in different cell types [2]. Voltage-dependent potassium channels consist of several families sharing similar structures [3]. A number of different mechanisms can contribute to the diversity of potassium channels properties, from multiplicity of genes to post-translational modifications [4].

A cDNA library from bovine adrenal medulla has already been used to identify and clone [5] a fast inactivating potassium channel (BAK4). Now, using the same library, a different potassium channel has been found. Here, we report the molecular cloning of this delayed rectifier potassium channel from bovine adrenal medulla, and its functional expression, both in a neuroblastoma cell line, where it has been permanently transfected, and in *Xenopus* oocytes after injection of mRNA.

2. Material and methods

2.1. Molecular cloning and sequencing of bovine cDNA and genomic clones

The λgt10 cDNA libraries, constructed from poly(A)$^+$ RNA from adrenal medulla were a gift from Dr. P. Seeburg (University of Heidelberg, Germany). Screening of the libraries was performed at low stringency [6] with a *Sac*I fragment from a cDNA coding for the RCK3 potassium channel [7]. This probe, kindly provided by Dr. O. Pongs (University of Hamburg, Germany), codes for the central core of the protein, which contains the putative transmembrane segments.

The EMBL-3 SP6/T7 bovine genomic library was purchased from Clontech (Cat. # BL1015j). Screening of the library was carried out at high stringency, as previously described [6]. The PCR DNA fragment obtained with primers BAK5–4 (degenerate sense, 5'-AAYGARTAYTTYTTYGAYMG) and BAK5–1 (antisense 5'- ACATGGCCTGCTGCCCGTTCT) was used as hybridization probe for the screening. This fragment encodes amino acids 146–378 of the BAK5 sequence. Positive clones were isolated and DNA prepared from plaque-pure phage. The inserts were subcloned into the Bluescript vector and the regions of interest further analyzed. These regions were identified by hybridization with oligonucleotides. Labeling of cDNA probes was performed with [α-^{32}P]dCTP using Amersham's multiprime DNA labeling system. DNA sequencing using the dideoxy chain termination method [8] was performed with T7 DNA polymerase from Pharmacia, and with templates which had been prepared in the Bluescript vector (Stratagene) after DNAse I deletions, by a modification of the method of Li and Wu [9].

2.2. Cell transfection and selection of positive clones

The heat-shock expression vector p17SPneo [10,11], a kind gift of Dr. M. Dreano (Batelle Res. Institute, Geneva), was used for transfection. This vector contains the neomycin resistance gene driven by SV40 regulation; the heat-shock promoter, hsp70; and a SV40 polyadenylation site downstream from the polylinker sequence. The coding region of the BAK5 potassium channel as well as the flanking 5' and 3' untranslated regions (150 and 393 bp, respectively) were inserted in the *Xba*I site of the p17SPneo vector. Neuro-2a cells (ATCC CCL 131) were maintained in minimum essential medium (Eagle's) supplemented with 10% fetal calf serum. Two days before transfection cells were plated at a density of 10^4 cells/ml in a 6-well plate. Between 1–4 μg of plasmid DNA/well were used for transfection mediated by Transfectam (Sepracor, France) according to the supplier's instructions. One day after transfection, the medium was supplemented with 0.4 mg/ml Geneticin (Sigma). Foci were isolated, and cell clones expanded for further characterization. Genomic DNA of selected cell lines was isolated and used for PCR detection. Total RNA was prepared either from heat-shock treated or untreated control cells. Heat-shock activation was performed by incubating cells at 42°C for 2 h: controls were maintained at the usual culture temperature (37°C). BAK5 transcripts were detected by a combined reverse transcription-PCR assay [12] from poly(A)$^+$ RNA.

2.3. Electrophysiology

Whole-cell currents were recorded using the patch clamp technique [13] with an Axopatch 200A amplifier (Axon Instruments Inc. Foster City. CA). Electrodes of 2–4 MΩ were used and usually up to 80% of series resistance compensation was achieved. The composition of the solutions, in mM, was as follows: Pipette solution: KCl 150, $MgCl_2$ 2, EGTA 11, HEPES 10, pH 7.3 (KOH). Bath solution: NaCl 142, KCl 5, $CaCl_2$ 2.5, HEPES 10, pH 7.4 (NaOH).

The methods we followed for the preparation of the oocytes and injection of mRNA have been described [14]. Oocytes were voltage

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clamped with a two microelectrode voltage-clamp amplifier. Electrodes were filled with a 2 M KCl solution. Bath solution (in mM): NaCl 115, KCl 2.5, CaCl2 1.8, HEPES 10, pH 7.2 (NaOH).

In both cells and oocytes experiments, the holding potential was −80 mV. Data acquisition and pulse application were controlled with a pClamp interface (Axon Instruments Inc., Foster City, CA). Current records were filtered with an 8 pole Bessel filter (Frequency Devices Inc. Haverhill, MA) at 10 kHz (cells) or at 2 kHz (oocytes) and sampled at 0.1 ms/point (cells) or at 1 ms/point (oocytes).

3. Results

3.1. Cloning of the potassium channel BAK5 from adrenal medulla

The screening of 10^6 recombinants led to the isolation of fourteen cDNA clones. Sequence analysis of these clones revealed that, with only one exception, all were apparently coding for the same potassium channel, which was then further characterized [5]. The remaining clone was also coding for a potassium channel, which was very similar to the previously reported channels Kv1 [15] and hPCN1 [16]; these are rat and human isoforms, respectively, of the so-called Kv1.5 potassium channel [17].

For this reason we called this channel BAK5 (Bovine Adrenal Potassium Channel Kv1.5). Since the aforementioned clone only encodes the protein region between the fourth transmembrane domain S4 and the stop codon, a second screening of another library was carried out with practically identical results: although the 3′ non-coding region was complete, reaching the poly(A) tail, the 5′ end of the clone ended at the junction between the S3 and S4 transmembrane fragments. Thus a genomic library was screened, several clones were found which apparently coded for the BAK5 protein. Further characterization of one of these clones yielded two *Sac*I fragments of 3 and 4 kbases, which covered the complete protein-coding region of the BAK5 potassium channel. The derived amino acid sequence is shown in Fig. 1, aligned with the rat and human analogs. Sequence similarity between the three proteins is particularly high within the core structure, i.e. the S1-S6 segments and the regions immediately adjacent to them. However, the N-terminal region shows more differences and includes several insertions and deletions. In fact, of the first 100 amino acids only about half are identical, which is unusually low between channels of the same group. It is important to note the abundance of prolines in this segment (up to fifteen between P32 and P99).

We were not able to detect the expression of this channel in the adrenal gland by Northern blot, but it was possible to detect it by reverse transcription combined with PCR (RT-PCR, data not shown), thus suggesting that the mRNA coding for this channel is present at low levels in this tissue. Omission of reverse transcriptase during RT-PCR resulted in no DNA amplification, indicating that the amplified DNA arised from RNA and not from a potential contamination with genomic DNA.

3.2. Transfection of neuroblastoma cells with BAK5 DNA

Neuro-2a cells were transfected with BAK5 DNA. Several clones were selected with Geneticin and expanded. Incorporation of BAK5 DNA into the genome of the best growing line was confirmed by PCR using specific primers for the BAK5 sequence (Fig. 2A). The signal was absent in the parent neuro-2a cells (Fig. 2A). After heat shock induction, poly(A)$^+$ RNA from transfected cells was purified and the levels of BAK5 transcripts were analyzed by RT-PCR. A control with non-induced BAK5 transfected cells did not show any expression of BAK5 transcripts (data not shown). However, in heat induced cells the presence of BAK5 mRNA was observed 1 h and 3 h after heat shock (Fig. 2B).

3.3. Functional properties of BAK5 channels in transfected cells and injected oocytes

Transfected Neuro-2a cells were recorded from one day after heat- induced BAK5 expression. Currents could be recorded for up to one week. Fig. 3A shows a typical family of current records obtained by depolarizing to different voltages. Currents

Fig. 1. The deduced BAK5 amino acid sequence compared with Kv1 and hPCN1 sequences. Identical amino acid residues have been indicated by dashes, gaps (*) have been introduced for maximal alignment. Putative transmembrane segments (S1-S6) are indicated by lines.

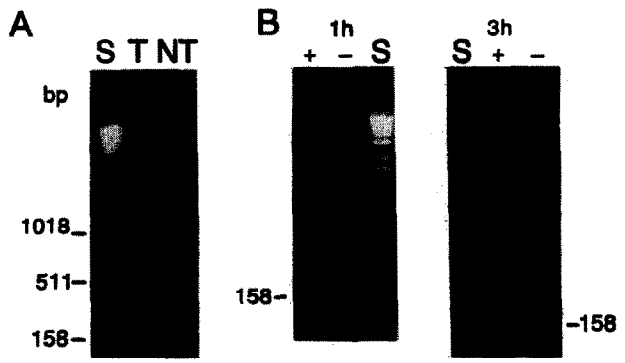

Fig. 2. DNA and RNA analysis of a cell line transfected with BAK5 DNA. (A) Genomic DNA analysis by amplification of a segment of the BAK5 channel with primers comprising amino acid residues 394–446 (sense: 5'CATCTTCAAGTTGTCCCGCCA; antisense 5'ATCTCCTGGTTGTCGGCCTCT). Template genomic DNA (0.5 μg) extracted from transfected (T) and non-transfected (NT) neuro-2a cells was processed in a standard PCR amplification for 32 cycles at the following temperature profile: 1 min at 94°C; 1 min at 62°C; 1 min at 72°C. (B) RNA analysis by a reverse transcriptase-polymerase chain reaction assay. Transfected cells were submitted to a heat shock for 2 h at 42°C, and their RNA extracted after 1 or 3 h. Samples of poly(A)$^+$ RNA (0.3 μg) were reverse transcribed (10 min, 50°C), then PCR was performed for 25 cycles with the same primers and conditions than those used in panel A (lanes labelled with +). To control specific amplification of BAK5 RNA, a control (−) without reverse transcriptase was included. Size standards are included (S).

start to activate at voltages positive to −40 mV and inactivate very little in 100 ms pulses. The *I–V* curve in part B reflects the average values and variation in 14 cells.

Xenopus oocytes injected with BAK5 mRNA were recorded two days after injection, for 5 days. Fig. 3A shows a family of current records obtained with 350 ms pulses. Currents start to activate at potentials positive to −40 mV, activate faster at more depolarized potentials and inactivate very slowly. The *I–V* curve in Fig. 3B, with data from 34 oocytes from 6 different donors, shows average level of expression and its variability.

Estimations of elementary conductance (γ) were made from noise analysis of current records obtained in cells (5.3 ± 1.7 pS, $n = 10$), and from single channel recordings in oocytes (7.9 ± 0.7 pS, $n = 2$), data not shown.

The macroscopic conductance vs. voltage curve could be fitted to a Boltzmann equation with $V_{1/2} = 2.4$ mV and slope factor k = 11.4 mV for cells and $V_{1/2} = -1.4$ mV, $k = 10.3$ mV for oocytes. The corresponding values for Kv1, where the conductance–voltage curve was estimated by the same procedure we have used, were $V_{1/2} = -3$ mV and $k = 15$ mV [15]. For hPCN1, where the conductance–voltage curve was obtained by measuring tail currents, the results were similar $V_{1/2} = -5.7$ mV, $k = 6.9$ mV [16].

3.3.1. Potassium selectivity. The selectivity of the channel for potassium was assessed by measuring the shift in the reversal potential produced by increasing the external potassium concentration from 5 mM to 40 mM. The reversal potential was obtained from instantaneous *I–V* curves as shown in Fig. 4.

Under control conditions, i.e. 5 mM external K, a reversal potential of −88 ± 8 mV ($n = 3$) was obtained. In 40 mM external K the E_{rev} was −29 ± 4 mV. The individual reversal potential shifted 52.7 ± 6.4 mV for an eight-fold increase in external K concentration, the theoretical result for a pure potassium conductance is 53 mV.

3.3.2. Sensitivity to external potassium, TEA and 4AP. As can be seen in Fig. 4, the magnitude of the current during a 30 ms pulse to +70 mV is somewhat smaller in 5 mM K than in 40 mM external K despite the decrease of electromotive force in the latter ionic condition. This result suggests that the potassium conductance is dependent on the external potassium concentration, increasing when this concentration is raised. The same figure provides a direct confirmation of this hypothesis, when the results of the instantaneous *I–V* curves for the two concentrations are compared. In both cases the *I–V* relation is linear, with a conductance of 4.9 nS in 5 mM K and 17.1 nS in 40 mM K, i.e. the conductance is about 3.5 times larger in high potassium. When individual cells are considered, the increase in conductance in the same cell, upon increasing the K concentration from 5 to 40 mM was 3.58 ± 0.56 times.

On the other hand, BAK5 currents were insensitive to external TEA but sensitive to 4-AP. 40 mM TEA did not produce any change in BAK5 currents expressed in *Xenopus* oocytes, however 4-AP reversibly blocked the currents in a dose dependent manner. The average dose response data obtained for the block could be fitted by a 1:1 binding model with apparent K_d of 0.49 ± 0.12 mM, data not shown.

4. Discussion

BAK5 channel is a delayed rectifier type channel similar to the rKv1.5 (Kv1) expressed in rat brain [15] and hKv1.5 (hPCN1) expressed in human [16], all belong to the Shaker related subfamily Kv1.5. The voltage dependence of BAK5, is very similar when expressed in cells or oocytes and is also similar to the channels mentioned above. The midpoint activa-

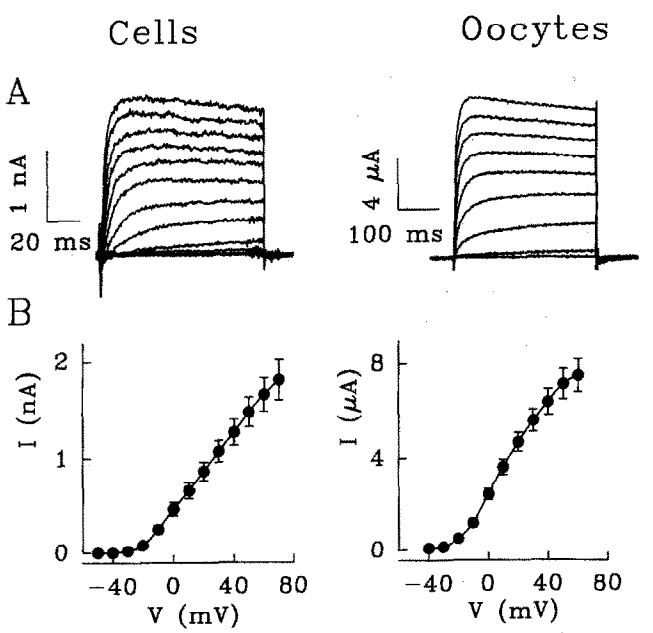

Fig. 3. BAK5 Currents. (A) Current records obtained in Neuro-2a cells (left) or *Xenopus* oocytes (right) that expressed BAK5 channels. Voltage steps start at −30 mV and are 10 mV apart. Notice different time and current scales. (B) Average *I–V* currents from 14 cells and 34 oocytes.

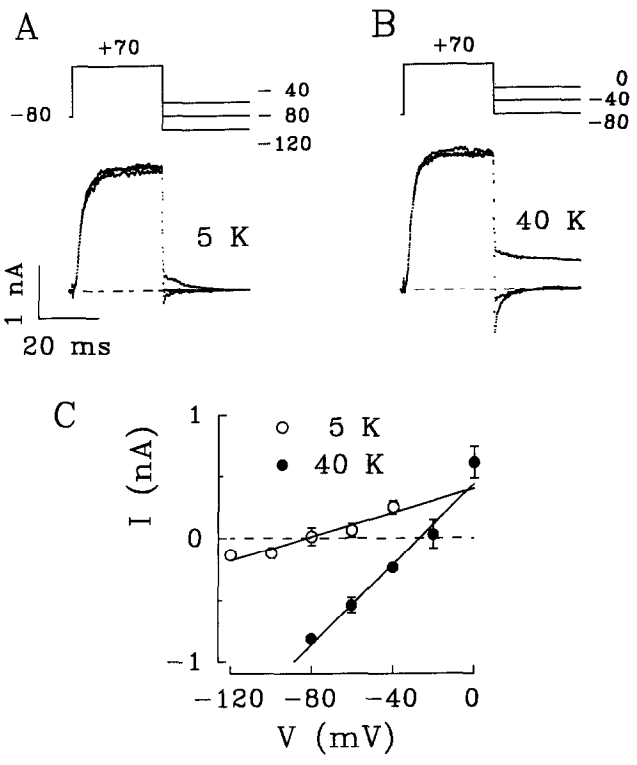

Fig. 4. Reversal potential and conductance dependence on external K. (A) Pulse protocol used to measure reversal potential, and current records obtained in Neuro-2a cells in 5 mM external K. (B) Id. in 40 mM external K. (C) Peak tail currents vs. repolarizing membrane potential for 5 mM $[K]_o$ (○) or 40 mM $[K]_o$ (●). Straight lines are weighted least squares fits of the data.

tion voltage is close to −4 mV in all three channels, but the steepness is somewhat more variable, the BAK5 being intermediate (≈10 mV) between the hPCN1 (7 mV) and Kv1 (15 mV).

It has been shown that the TEA sensitivity of voltage dependent potassium channels expressed in oocytes depends on the amino acids present in the H5 region between S5 and S6 segments [18]. Point mutations of Shaker to replace T449 with Lysine produced a decrease in sensitivity to external TEA, and changed the IC_{50} concentration from 17 mM in wild type to >200 mM. The H5 region in BAK5 only differs from Shaker at the end of the sequence, where the amino acid corresponding to T449 in Shaker is arginine R471 in BAK5. Our result that BAK5 lacks sensitivity to TEA is consistent with the presence of this positively charged amino acid. The homologous channel Kv1 is also insensitive to TEA: 40 mM only partially blocked currents [15], and 20 mM TEA had no effect on the hPCN1 channel [16].

The same amino acid seems to be related to the modulatory effect of external potassium concentration on the magnitude of the current. In Shaker channels, with the mutation T449K, external potassium alters the number of channels that open on depolarization, i.e. higher external potassium concentration increases the potassium conductance [19]. This property was first observed in RCK4 type channels [20] where, again, the amino acid implicated in $[K]_o$ modulation, in this case K533, is the same as that conferring TEA insensitivity.

BAK5 channels are blocked by 4AP with an IC_{50} of 0.5 mM, again this result is very similar to the effect of 4AP on the homologous channels, Kv1 (IC_{50} = 0.4 mM), and hPCN1 (IC_{50}≈0.1 mM). Studies using chimeric channels obtained by combining sequences from high and low 4AP sensitivity clones have identified a region in the S5 and S6 segment, that forms the cytoplasmic vestibule of the pore as the determinant for 4AP block [21]. Both regions are identical in BAK5, Kv1 and hPCN1, and in these channels show more similarity with the amino acids of the high sensitivity clone (Kv3.1) that with the low sensitivity clone (Kv2.1).

The BAK5 channel reported here adds to the repertoire of potassium channels present in chromaffin cells [22], [5], and its possible role, in the physiology of these cells remains to be determined.

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