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Molecular cloning and permanent expression in a neuroblastoma cell line of a fast inactivating potassium channel from bovine adrenal medulla

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Using a cDNA library from bovine adrenal medulla, we have isolated cDNAs coding for a potassium channel. These cDNAs encode a 660-amino acid protein that has a molecular weight of 73,288 kDa and no amino-terminal signal peptide. We have called it BAK4. Analysis of its sequence reveals close similarity (94% homology) with a recently described potassium channel from rat brain (RCK4) and heart (RHK1). Neuroblastoma cells (Neuro-2a cell line) were stably transfected with BAK4 DNA. Expression of the DNA was under the control of a heat-shock promoter. Several clones, that were isolated by neomycin resistance selection, had integrated the plasmid DNA in a stable form. Upon heat induction, these cells produced BAK4 RNA and a potassium outward current, not present in control non-transfected cells. The current, which was transient and decayed markedly during the duration of 200 ms-pulses, can be described as a I_{to1} potassium current. The expression of these types of channels in brain (RCK4,RHK1), heart (RHK1) and adrenal medulla (BAK4) suggest their possible implication in important functions for the cell.

Bovine adrenal medulla; cDNA cloning; Transient outward potassium current; Stable transfection; Neuroblastoma

1. INTRODUCTION

Potassium channels are molecules involved in the control of the electrical potential across the cell membrane and, therefore, can regulate the flux of information between cells [1]. This is particularly true in the nervous system, where potassium channels are responsible for an enormous diversity of ionic currents, underlying a wide repertoire of neuronal firing patterns [2].

The cloning of the *Shaker*, *Shal*, *Shab* and *Shaw* genes in *Drosophila* [3–7] and of related genes in the mammalian brain [8–14] has shown that the encoded potassium channels share similar structures, at the same time resembling one of the four repeated domains of sodium and calcium channels subunits [15]. In fact, different or identical potassium channels polypeptides may co-assemble in tetrameric structures to give rise to an almost unlimited amount of channel proteins [16–18]. This fact, together with the multiple possibilities of regulation of potassium channels [19–23], could constitute the molecular ground where the functional variety of potassium channels is based. However, the ubiquity and diversity of potassium channels, together with the cell heterogeneity found in the nervous system, makes the molecular analysis of potassium currents difficult. For this reason we chose to study potassium channels in a more accessible and homogenous system, the adrenal medulla. This tissue includes neuronal, interstitial and vascular components, but the secretory chromaffin cells are predominant (65% of total cell volume in the adult rat [24]). These cells are of neural origin and fire action potentials which are the result of voltage-dependent Na, Ca and K currents, similar to those found in neurones and muscle fibres [25–27]. In addition, they are being used to study neuronal processes such as neurosecretion [28] and neuronal plasticity and development [29]. In the present paper, we report the molecular cloning of a fast inactivating potassium channel, probably expressed in chromaffin cells, its permanent transfection in a neuroblastoma cell line, and its electrophysiological characterization.

2. EXPERIMENTAL

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

The λgt 10 cDNA libraries, constructed from poly(A)$^+$ RNA from adrenal medulla were a gift from Dr. P. Seeburg (ZMBH, University of Heidelberg, Germany). Screening of the libraries was carried out at low stringency [30] with a *Sac*I fragment from a cDNA coding for the RCK3 potassium channel [12]. This probe, kindly provided by Dr. O. Pongs (University of Hamburg, Germany), codes for the central core of the protein that contains the putative transmembrane segments.

Chromaffin cells were isolated from bovine adrenal medullary tissue [31] and their RNA purified by using a kit (Pharmacia LKB). Purified RNA (20 μg) was separated on 1% agarose gels containing 2.2 M formaldehyde, transferred to Nytran nylon membranes (Schleicher & Schüll) and crosslinked with UV light. Prehybridization of Northern blots was performed for 4–6 h at 42°C in 50% formamide, 5× SSC, 0.1% bovine serum albumin, 0.1% polyvinylpirrollidone, 0.1% Ficoll,

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0.05M sodium phosphate, pH 7.0, 0.1% SDS and 100 μg/ml sonicated salmon sperm DNA. After hybridization in the same solution, blots were washed in 2× SSC, 0.1% SDS at room temperature and 42°C, and 0.2× SSC, 0.1% SDS at 42, 50 and 55°C.

Labelling of cDNA probes was carried out with [α-^{32}P]dCTP using the multiprime DNA labelling system of Amersham. Sequencing of DNA was performed by the dideoxy method [32] with Sequenase (US Biochemicals). Both DNA strands were sequenced. Templates for sequencing were prepared in the Bluescript vector after DNaseI deletions [33].

2.2. *Cell transfection and selection of positive clones*

The heat-shock expression vector pl7SPneo [34,35], a kind gift of Dr. M. Dreano (Batelle Research Institute, Geneva), was used for transfection. This vector contains the neomicin resistance gene driven by SV40 regulation, the heat-shock promoter, hsp70, and an SV40 polyadenylation site downstream from the polylinker sequence. The coding region of the BAK4 potassium channel, as well as the flanking 5′ and 3′ untranslated regions were isolated by *DraI–RsaI* digestion and inserted into the *SmaI* site of Bluescript vector. The right orientation of the cDNA was then digested with *SalI* and *BamHI* and inserted in the corresponding *SalI* and *BamHI* sites of p17SPneo. 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 10^5 cells/ml in a 6-well plate. Between 1–4 μg of plasmid DNA/well were used for transfection mediated by Transfectam (IBF) according to suppliers instructions. One day after transfection, the medium was supplemented with 0.4 mg/ml geneticin (Sigma Co.). 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 while maintaining controls at the usual culture temperature of 37°C. BAK4 transcripts were detected by a combined reverse transcription-PCR assay [36]. Further details can be found at the corresponding figure legends.

2.3. *Electrophysiological methods*

Whole-cell outward K^+ currents were recorded under voltage clamp using the nystatin-perforated patch-clamp technique [37]. About 6 × 10^4 Neuro-2a cells were plated in 35 mm diameter Petri dishes (Corning) the day before induction. After heat shock, cells were incubated at 37°C until electrophysiological recording. Then, cells were washed several times with 1 ml bath solution composed of (in mmol/l): NaCl 140; $CaCl_2$ 2.5; $MgCl_2$ 1; KCl 5; HEPES 10; Glucose 10; pH 7.4. The pipette solution contained (in mmol/l):KCl 70; potassium asparate 70; $MgCl_2$ 1; Mg-ATP 2.5; HEPES 10; nystatin 200 μg/ml; pH 7.2. Capacity transients were cancelled using the built-in circuitry of the patch-clamp amplifier (EPC-7, List, Darmstadt, Germany) and the pipette potential set at −80 mV. Shortly after gigaseal formation, small capacity transients appeared, indicating electrical continuity between the cell interior and pipette solution. The size of these transients stabilized about 7 min after gigaseal formation. At this moment, current transients were elicited every 10 s by increasing depolarizing steps (10 mV, 200 ms) to a maximal membrane potential of 100 mV. Voltage pulses were under computer control. Current transients were acquired at a sampling rate of 5 kHz. The linear component of the capacity transient was subtracted using a P/8 protocol [38]. Non-linear components were digitally subtracted [39].

3. RESULTS AND DISCUSSION

3.1. *Cloning of potassium channels from adrenal medulla*

The screening of 10^6 recombinants led to the isolation of fourteen cDNA clones. Most of them reacted in Southern blots with probes coding for RCK1, RCK3, RCK4 and RCK5 potassium channels [12]. Sequence analysis of these clones showed that all of them were apparently coding for the same protein, with only one exception. Since none of these clones encoded the full protein coding region, a second library from adrenal medulla was screened with a probe corresponding to the 5′ region of the longest clone previously isolated. Three clones were obtained, one of them extending into the 5′ untranslated region of the mRNA (clone 52). The deduced amino acid sequence contained in clone 52 encodes one long open reading frame of 660 amino acid residues with a molecular weight of 73,288 kDa nucleotides 420–2,399 of sequence submitted to the EMBL data library under accession number X57033). The 3′ non-coding region, beginning after the TGA stop codon, is 632 bp long. A canonical AATAAA polyadenylation signal [40] was found 176 bases downstream of the stop codon. Interestingly, several clones found during the first screening contained polyadenylation sites at two different positions, 19 and 30 bases downstream of the polyadenylation signal.

The deduced amino acid sequence is shown in Fig. 1. We have designated this protein BAK4 to distinguish it from previously described potassium channels. An analysis of the amino acid sequence of BAK4 for local hydropathy reveals the presence of typical membrane-spanning segments (designated S1 to S6 in Fig. 1), already found in other potassium channels and in the repeated domains of sodium and calcium channels. The sequences of the proposed transmembrane fragments S1–S6 are highly conserved between all potassium channels sequenced to date, including the BAK4 polypeptide. This protein is particularly homologous (94%) to the designated RCK4 potassium channel from rat brain [12] (see Fig. 1) and its highly homologous RHK1 found in heart [41]. Thus, the core structure of both polypeptides, i.e. the S1–S6 fragments and the regions immediately adjacent to them, are almost identical (Fig. 1). The C-terminal segment is also very similar, with only one insertion and two non-conservative substitutions, whereas the N-terminal region shows more differences. The most notable differences are frameshifts (residues 84–88) leading to RRRRP (BAK4) and RRRRQ (RHK1) vs. EEEAT (RCK4). Of relevance is also an insertion of six glycines and one serine in BAK4 (residues 167–173), not present in RCK4 and RHK1. On the other hand, the homology to other potassium channels is considerably reduced. For instance, with RCK1 [9] there is 51% homology (see Fig. 1).

Blot hybridization analysis of RNA from bovine adrenal medulla and fractionated chromaffin cells were performed and compared. Fig. 2 shows a Northern blot of the mentioned RNAs, visualized by hybridization to a ^{32}P random-labelled fragment of BAK4. This fragment codes for the region between amino acids 1 and 146, not present in other potassium channels, with the exception of RCK4 and RHK1. A predominant band

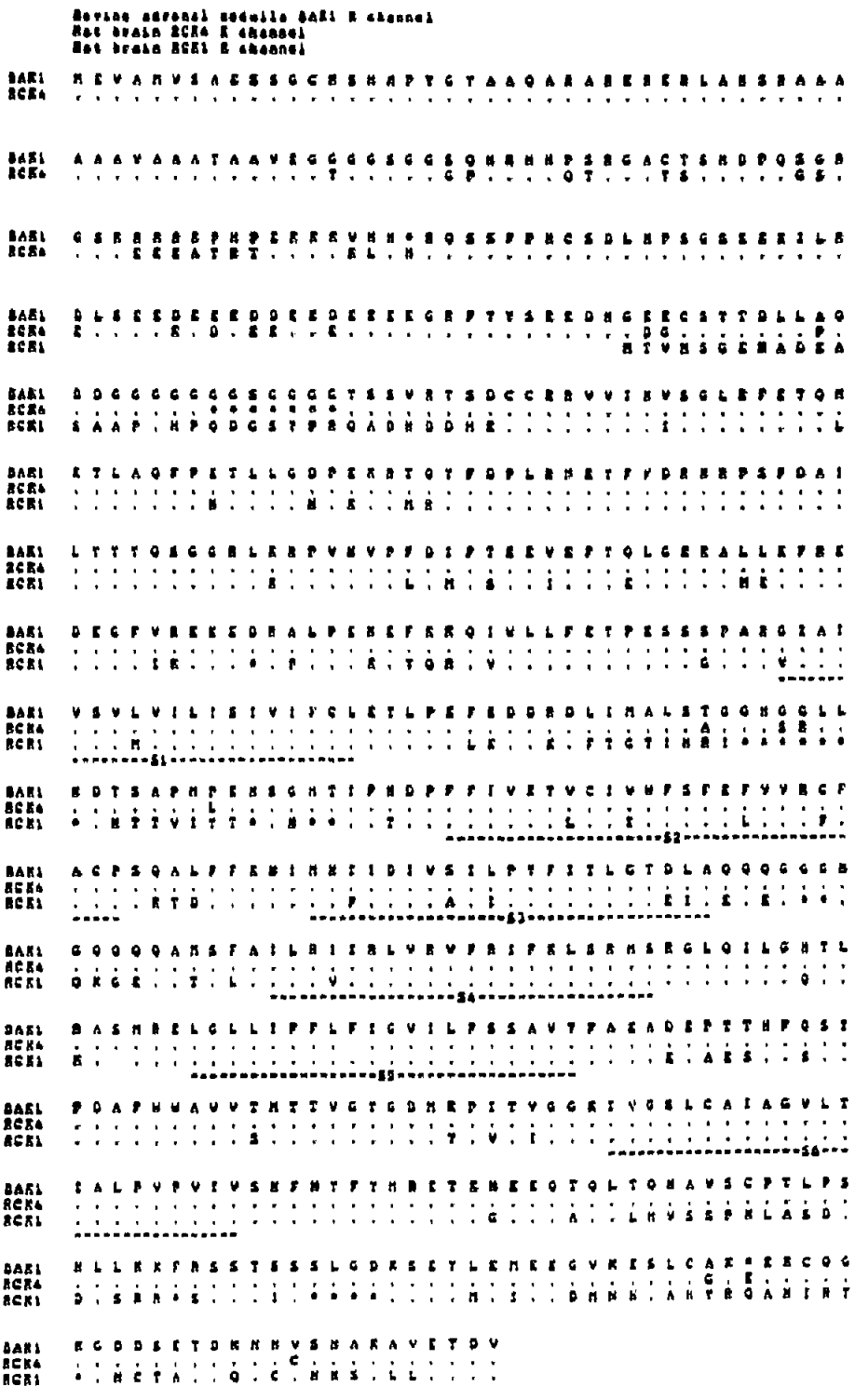

Fig. 1. The deduced BAK4 amino acid sequence compared with RCK4 and RCK1 sequences. Identical amino acid residues have been indicated by points, whereas gaps (*) have been introduced for maximal alignment. Putative transmembrane segments (S1–S6) are indicated by dashed lines. Notice the length of the N-terminal region of the RCK1 protein, which is much shorter than the ones of BAK4 and RCK4, but similar to the rest of potassium channels sequenced to date. The BAK4 nucleotide sequence is available from the EMBL database (accession number X57033).

of approximately 3,100 bases is observed. In rat brain, a single mRNA species for the homologous RCK4 has been identified which has a size of 4,600 nucleotides [42].

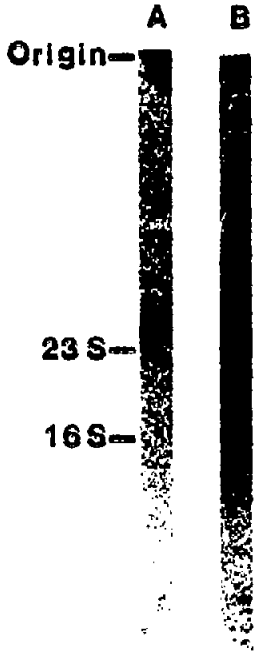

Fig. 2. Northern blot analysis of RNA from bovine adrenal medulla and chromaffin cells. Total RNA was isolated from bovine adrenal medulla or fractionated chromaffin cells, separated in formaldehyde-agarose gels and blotted onto Nytran membranes. Origin of the gel and ribosomal RNA bands (1.6 and 2.9 kb) are indicated. (A) Hybridization of 20 μg of adrenal medulla RNA. (B) Hybridization of 20 μg of chromaffin cells RNA. In both cases hybridization was carried out with a *Sty*I fragment of clone 52 (coding for amino acids residues 1–146). The blot was autoradiographed on Kodak XAR5 X-ray film with intensifying screen for 44 h.

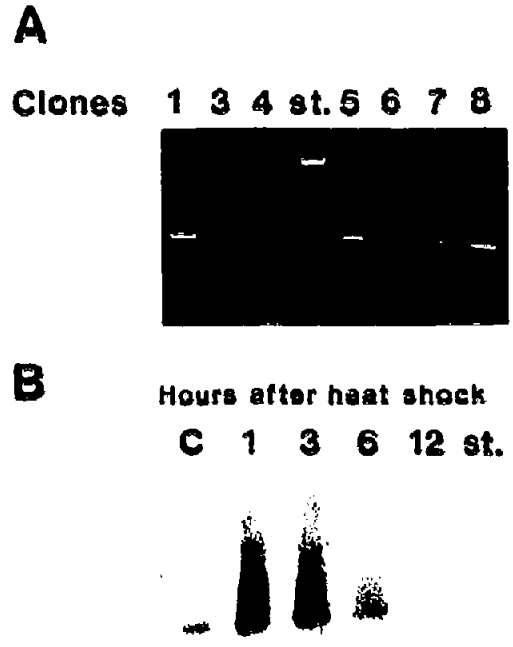

Fig. 3. DNA and RNA analysis of cell lines transfected with BAK4 DNA. (A) Genomic DNA analysis by amplification of an amino-terminal segment of the BAK4 channel with primers comprising amino acid residues 1–180. Template genomic DNA (0.85 μg) extracted from each selected clone (designated by numbers) and from non-transfected Neuro-2a cells (not shown) was processed in a standard PCR amplification for 33 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. Clone 1 cells were submitted to a heat shock for 2 h at 42°C (except for lane C, control maintained at 37°C all the time) and their RNA extracted after the indicated times. Samples of total RNA (0.8 μg) were reverse transcribed (10 min, 50°C), then PCR was performed for 25 cycles (1 min 94°C; 2 min 55°C; 2.5 min 72°C) according to [36]. The products from the PCR were separated by electrophoresis, transferred to a nylon membrane and hybridized with a digoxigenin-labeled BAK4 probe. The antisense primer used in reverse transcription and PCR was oligo $d(T)_{16}$ with a tail containing several restriction enzyme sites, and the sense primer was 5'CCATACCTCCCTTCTAAT (amino acid residues 595–600).

3.2. *Transfection of neuroblastoma cells with BAK4 DNA*

Neuro-2a cells were transfected with BAK4 DNA. Several clones were selected with geneticin and expanded. Incorporation of BAK4 DNA into the genome of these lines was tested by PCR using specific primers for the BAK4 sequence. All tested cell lines showed the presence of the BAK4 sequence, whereas in the parent Neuro-2a cells the signal was absent (Fig. 3A). As expected the amount of amplified DNA varied from clone to clone, probably indicating differences in copy number of plasmic integrated into the genome. For further studies we chose clone 1, which seemed to have integrated more DNA copies and yet was growing very well.

Total RNA from clone 1 cells was extracted at various times after heat shock and BAK4 mRNA levels analyzed (Fig. 3B). The signal is highest 1 h after heat shock and declines rapidly to control levels within 12 h following heat shock. This fact decay has also been observed with other transfected DNA controlled by the heat-shock promoter [43]. It is interesting to note that, in the absence of heat shock, clone 1 cells express low levels of BAK4 mRNA (Fig. 3B, lane c). The level of protein expression was only tested by electrophysiological methods (see below). Potassium channels were detected 2 h after heat shock (the earliest time tested) and 24 h later the measured current was still high.

3.3. *Functional properties of BAK4 channels in transfected cells*

Depolarization of control Neuro-2a cells from a holding potential of −80 mV evokes the appearance of inward currents that reached a maximum value around −500 pA at −10 mV membrane potential. The inward current was followed by a very small outward current (Fig. 4A) that disappeared when tetraethylammonium (TEA, 40 mM) was present in the incubation bath (data not shown). The ion responsible for the inward current was Na^+ because substitution of Na^+ by choline in the

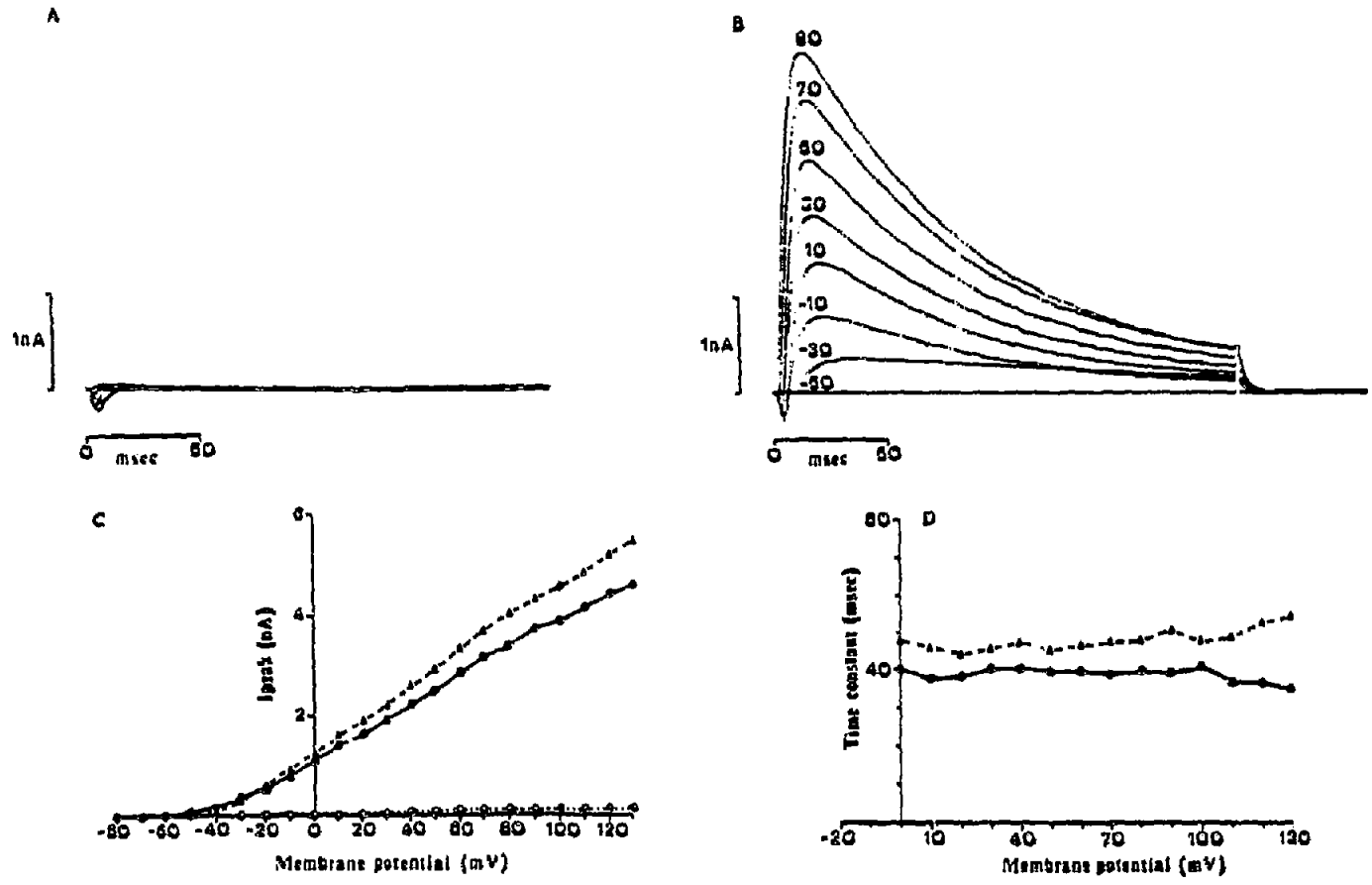

Fig. 4. Currents evoked in Neuro-2a cells transfected or not with the BAK4 DNA. Current transients in control, non-transfected (A) or transfected (B) Neuro-2a cells were obtained by depolarization from a holding potential of −80 mV as indicated in section 2. For the sake of clarity only currents evoked at certain potentials are represented. After establishing a high resistance seal between the cell and the pipette, and once nystatin treatment provided electrical continuity between the cell interior and the pipette, current transients were elicited every 10 s by depolarizing steps (10 mV, 200 ms) from a holding of −80 mV. (C) Potassium current–voltage relationship for control (○) or transfected Neuro-2a cells in the absence (●) or the presence (△) of 40 mM TEA. (D) Inactivation time constant as a function of membrane potential for transfected Neuro-2a cells in the absence (●) or presence (△) of 40 mM TEA. Time constants were calculated by fitting the decaying current, from the peak to the end of the pulse, to the following equation $I_{(t)}=I_0 \cdot \exp(t/\tau)$.

bath solution completely suppressed the inward current (data not shown).

After heat shock of Neuro-2a cells transfected with BAK4 DNA (clone 1), the inward current was still present, but its duration was markedly decreased by the appearance of an outward current that amounted about 5 nA (Fig. 4B) at 100 mV membrane potential. This current was transient and decayed markedly during the duration of the pulse (200 ms), so it can be considered as an $I_{k(A)}$ potassium-type current [44]. The current–voltage relationship indicates that the peak current increases monotonically as a function of membrane potential (Fig. 4C). It is important to note that this current is not sensitive to TEA (40 mM) (Fig. 4C). This lack of TEA sensitivity contrasts with what has been observed in chromaffin cells in culture: 1 mM TEA blocks about 80% of total K^+ currents [27] and 30 mM blocks almost 100% of total K^+ currents (González-García, Keiser, Rojas and Ceña, unpublished results). The only inactivating K^+ current described so far, in bovine chromaffin cells, is the large conductance Ca^{2+}-dependent K^+ current ($K_{(Ca)}$) [27] and this current should be completely blocked by the high TEA concentrations (40 mM) used in these experiments. A possible explanation for this discrepancy is that the BAK4 channel is not expressed in chromaffin cells, but in fibroblasts or endothelial cells, also present in the adrenal medulla although at a lower concentration. A Northern blot of RNA from highly purified chromaffin cells shows a band hybridizing with a BAK4 probe (see Fig. 2B), suggesting that this channel is expressed in chromaffin cells. However, even in this preparation, the presence of low levels of contaminating cells cannot be excluded. An alternative explanation is that in chromaffin cells the BAK4 polypeptide would form heteromultimeric structures upon assembling with other polypeptides. The resulting channel molecule would have different pharmacological and electrophysiological profiles from the ones observed for

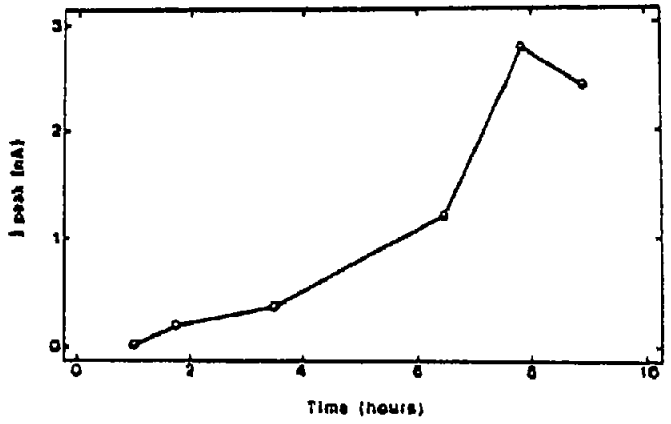

Fig. 5. Time-course of expression of the K^+ current. Neuro-2a-transfected cells were subjected to 2 h heat shock for BAK4 channel expression. At different times after heat shock, outward K^+ currents were measured and the peak current at 40 mV membrane potential was plotted as a function of time.

the BAK4 channel expressed in Neuro-2a cells. Both possibilities are being tested at the present time. On the other hand, it is interesting to note that in PC12 pheochromocytoma cells it has not been possible to find a simple correlation between homomultimeric channels expressed in oocytes and the native potassium channels recorded in PC12 cells [45].

Current inactivation time constants were independent of voltage, as can be observed in Fig. 4D. Presence of TEA does not affect the rate of inactivation of the current. The observed inactivation time constant, around 40 ms, and its voltage independence is similar to that described for a homologous channel in oocytes [12]. Single channel recordings (data not shown) indicate that the unitary conductance at 0 mV pipette potential in outside-out patches was of 1.6 pA. If an estimated reversal potential of −90 mV is assumed, then the chord conductance is about 5 pS. This chord conductance, together with the inactivation kinetics and TEA insensitivity, are similar to the ones found for the RCK4 channel expressed in oocytes [12].

Finally, the time-course of appearance of K^+ following heat-shock transcription activation was studied. To normalize the data, the peak current obtained from a pulse from a holding potential of −80 to 40 mV was plotted as a function of time after heat shock (Fig. 5). As can be observed, there is a substantial increase in the size of the current with time, reaching a plateau at about 8 h. At 24 h (data not shown) after heat shock, a robust K^+ current is still observed, in contrast with the instability observed for the BAK4 mRNA (Fig. 3B).

In summary, a potassium channel, called BAK4 by analogy to a similar channel from rat brain (RCK4), has been cloned from bovine adrenal medulla. An expression system was developed, based in: (i) a neuroblastoma cel line (Neuro-2a) with very low potassium channel activity, and (ii) regulation of exogenous K^+ channel expression by a heat-shock promoter. The permanent expression of BAK4 in this system generated a transient outward potassium current insensitive to TEA. The fact that similar channels are found in brain, heart and adrenal medulla, and that their function may be related to the metabolic state of the cell [46], suggest a crucial role for these molecules in excitable cells.

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