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Cloning of a bovine voltage-gated K^+ channel gene utilising partial amino acid sequence of a dendrotoxin-binding protein from brain cortex

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Several variants of fast-activating, voltage-dependent K^+ channels exist in the nervous system where they control cell excitability and synaptic transmission, some of which are blocked selectively by α-dendrotoxin. Cloning of a K^+ channel from bovine genomic DNA was achieved using a primer based on the N-terminal sequence of the larger subunit from the purified toxin acceptor, in conjunction with secondary primers, in the polymerase chain reaction. The resultant amino acid sequence is highly homologous to RCK 5 already cloned from rat brain, which yields a K^+ current susceptible to α-dendrotoxin, when expressed in oocytes. These findings establish conclusively that the extensively characterised α-dendrotoxin acceptor is a K^+ channel protein.

K^+ channel; Dendrotoxin; Bovine brain; Polymerase chain reaction

1. INTRODUCTION

K^+ channels represent the most diverse group of cation channels comprising A-type, delayed rectifiers, Ca^{2+}-activated, ATP-dependent and inward rectifiers (reviewed in [1]). Studies at the molecular level were hindered until the mamba snake polypeptide, α-dendrotoxin (α-Dtx), was shown to inhibit certain voltage-dependent, fast-activating K^+ channels in rodent hippocampal [2,3] and ganglionic neurons [4–6]. High affinity binding sites for α-Dtx were identified in synaptosomal membranes and localized in mammalian brain [2,3,7]. When purified from bovine and rat cortex by affinity chromatography on toxin I (a homologue of α-Dtx), the acceptors/K^+ channels were found to be large hetero-oligomeric sialoglycoproteins containing two non-covalently linked subunits with apparent M_r of 78 (65 after *N*-deglycosylation) and 39 kDa, respectively [8,9]. A family of voltage-activated K^+ channels has recently been cloned from rodent brain and their predicted molecular weights [10] approximate to that of the larger subunit of the toxin acceptor protein identified biochemically in synaptic membranes [11,12]. When these were expressed in oocytes, RCK 5 produced a K^+ current with delayed-rectifier kinetics that exhibited the highest sensitivity to blockade by α-Dtx. Moreover, a sequence of 27 amino acids from the N-terminus of the large subunit of the α-Dtx acceptor [12] was found to be virtually identical to that predicted for RCK 5 and BK 2 [13].

In this study, cloning and sequencing of the bovine equivalent of RCK 5 was carried out to establish the full extent of their similarity and because of the usefulness of the primary sequence for structure/activity studies of this α-Dtx-sensitive K^+ channel which, as noted above, has been studied extensively in the nervous systems.

Abbreviations: α-Dtx, α-dendrotoxin; SDS, sodium dodecyl sulphate; PCR, polymerase chain reaction.

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2. EXPERIMENTAL

Bovine genomic DNA was isolated from bovine cortex: the tissue (1–2 g) was suspended in 10 ml of solution A (25 mM Tris-HCl, pH 8; 10 mM EDTA; 50 mM glucose) and homogenized by passage through a syringe with a large-bore needle. DNA was released by gentle lysis following the addition of equal volumes of solution B (0.5 mM EDTA; 100 μg/ml proteinase K; 0.5% (w/v) sodium dodecyl sulphate (SDS), pH 8). Protein was removed by phenol/chloroform extraction and DNA recovered by ethanol precipitation. Following digestion of DNA (3 μg) with *Eco*RI in a volume of 20 μl, 0.5 μg was used in the polymerase chain reaction (PCR) to amplify the sequence of interest. This process requires the production of primers which hybridize to opposite strands on either side of the unknown region and promote DNA extension through the desired sequence [14]. Primers were synthesised on a Pharmacia Gene Assembler and used in combinations detailed later.

Primer 1: (5-ATGACAGT$^{T}/_{G}$GC$^{T}/_{G}$ACCGGAGA$^{T}/_{C}$CC-3) corresponding to amino acids 1–8.
Primer 2: antisense (5-CC$^{A}/_{G}$TGCAT$^{A}/_{G}$TC$^{T}/_{C}$TC$^{A}/_{G}$TT$^{T}/_{C}$TC-3) corresponding to amino acids 191–197.
Primer 3: sense (5-GA$^{A}/_{G}$AA$^{T}/_{C}$GA$^{A}/_{G}$GA$^{T}/_{C}$ATGCA$^{T}/_{C}$GG-3) as primer 2.
Primer 4: antisense (5-TT$^{T}/_{C}$TC$^{T}/_{C}$TC$^{T}/_{G}$CG$^{A}/_{G}$AA$^{A}/_{G}$TC/$_{C}$TC$^{A}/_{G}$TT-3) corresponding to amino acids 468–475.

PCR conditions were 94°C, 1 min; 55°C, 1–5 min; 72°C, 2 min; 40

cycles with 10 min extension. Following electrophoresis in 1% agarose gels of the reaction products, the fragment corresponding to the correct predicted length was excised from the gels and, where necessary, subjected to reamplification. The conditions employed for the second round of PCR were 94°C, 1 min; 55°C, 1 min; 72°C, 1.5 min; 35 cycles with no extension. Approx. 10 ng of target DNA were used per reaction. After excision of PCR fragments from agarose gels, ragged ends were filled-in using T4 DNA polymerase and phosphorylated using polynucleotide kinase. Fragments were then cloned into *Eco*RV restricted Bluescript vector (Stratagene) and sequenced using the method described in [15].

3. RESULTS

For the isolation of the bovine equivalent to RCK 5 (BGK 5), PCR was chosen for its speed and simplicity. From the literature it is known that voltage-activated K^+ channel genes do not normally contain introns which, therefore, permitted the use of genomic DNA [16,17]. Primer for use in PCR were designed from regions specific to RCK 5 using codon usage frequencies described in [18] and the authors' own unpublished observations. The amplification of DNA fragments utilised *Eco*RI-digested DNA to ensure complete separation of double-standard DNA during the initial denaturing step, thereby increasing the yield. The amplification steps produced three fragments approximating in length to those expected from RCK 5 (Fig. 1). The 600 bp clone obtained using primers 1 and 2 in the PCR (Fig. 1, lane 3), was found to be highly homologous to the N-terminal of RCK 5 and BK 2. Overall homology was 99% at both the nucleotide and the amino acid levels; at position 8 (of the protein) there is a conservative substitution of alanine for valine. Use of primers 3 and 4 in PCR (Fig. 1, lane 1), yielded a 800 bp clone that was highly homologous to C-terminus of RCK 5 – 96% and 95% at the nucleotide and amino acid levels, respectively. This clone differs from the RCK 5 channel by 12 of 284 amino acids and is nearly identical to BK 2, except for substitutions at position 198 where glycine is replaced by serine, and threonine for isoleucine at position 268. The third clone (1400 bp) obtained using primers 1 and 4 in the PCR (Fig. 1, lane 2), gave a nucleotide sequence (Fig. 2) that encompassed almost the entire reading frame seen in RCK 5. The amino acid sequence corresponding to the latter (Fig. 3) shows that it lacks the last 21 amino acids from the C-terminus; this was a consequence of utilising primer 4 which was designed specifically to amplify the bovine equivalent of RCK 5.

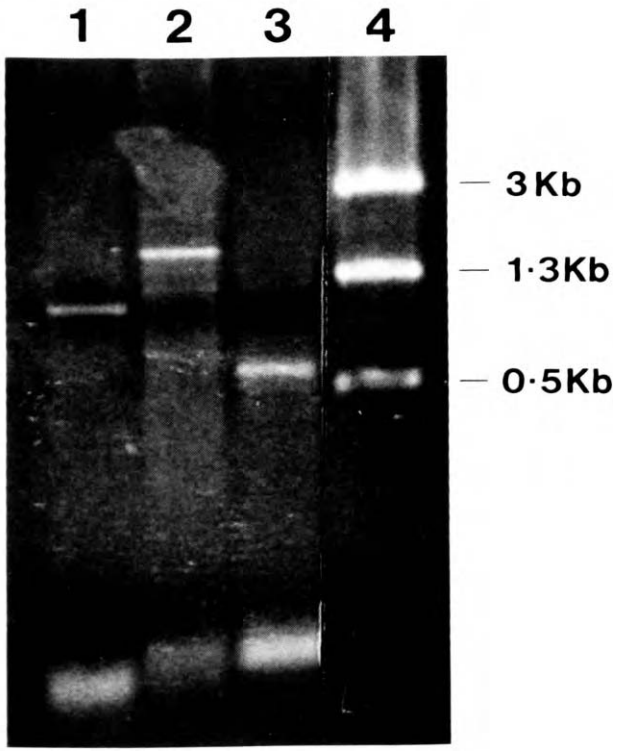

Fig. 1. Electrophoresis in agarose gel of PCR fragments from bovine genomic DNA. 10 μl aliquots of each PCR reaction were loaded per well and bands visualised by UV irradiation. PCR reaction utilising primers 3 and 4 (lane 1); 1 and 4 (lane 2); 1 and 2 (lane 3). Lane 4 represents standards with sizes indicated. Broad band at base of gel represents unused primers. Conditions employed for PCR reaction are detailed in section 2.

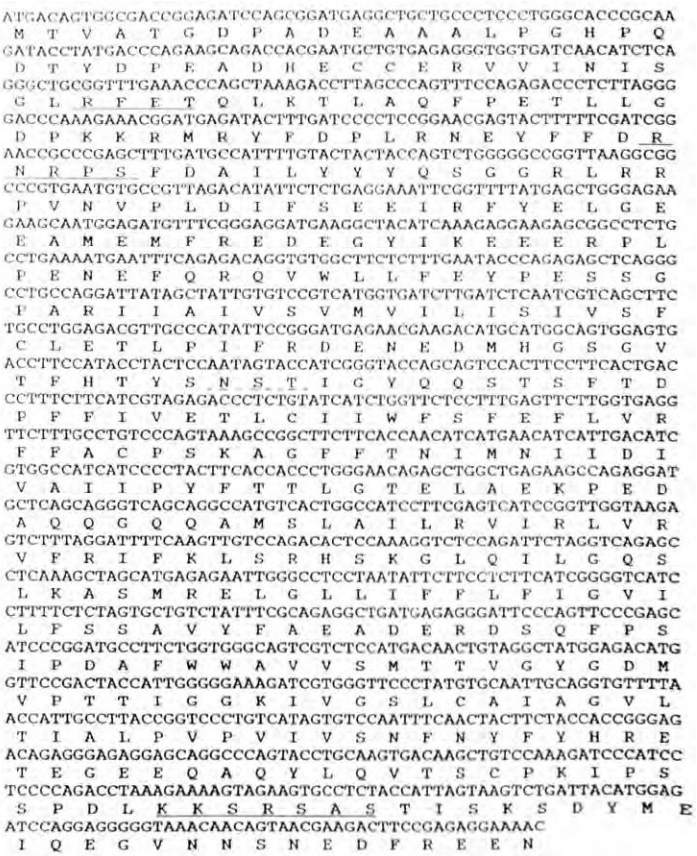

Fig. 2. DNA sequence and predicted amino acid sequence of the bovine genomic K^+ channel (BGK 5). Amino acids are listed beginning with the methionine initiation site. Putative *N*-glycosylation and phosphorylation sites are indicated by the broken and continuous bars, respectively.

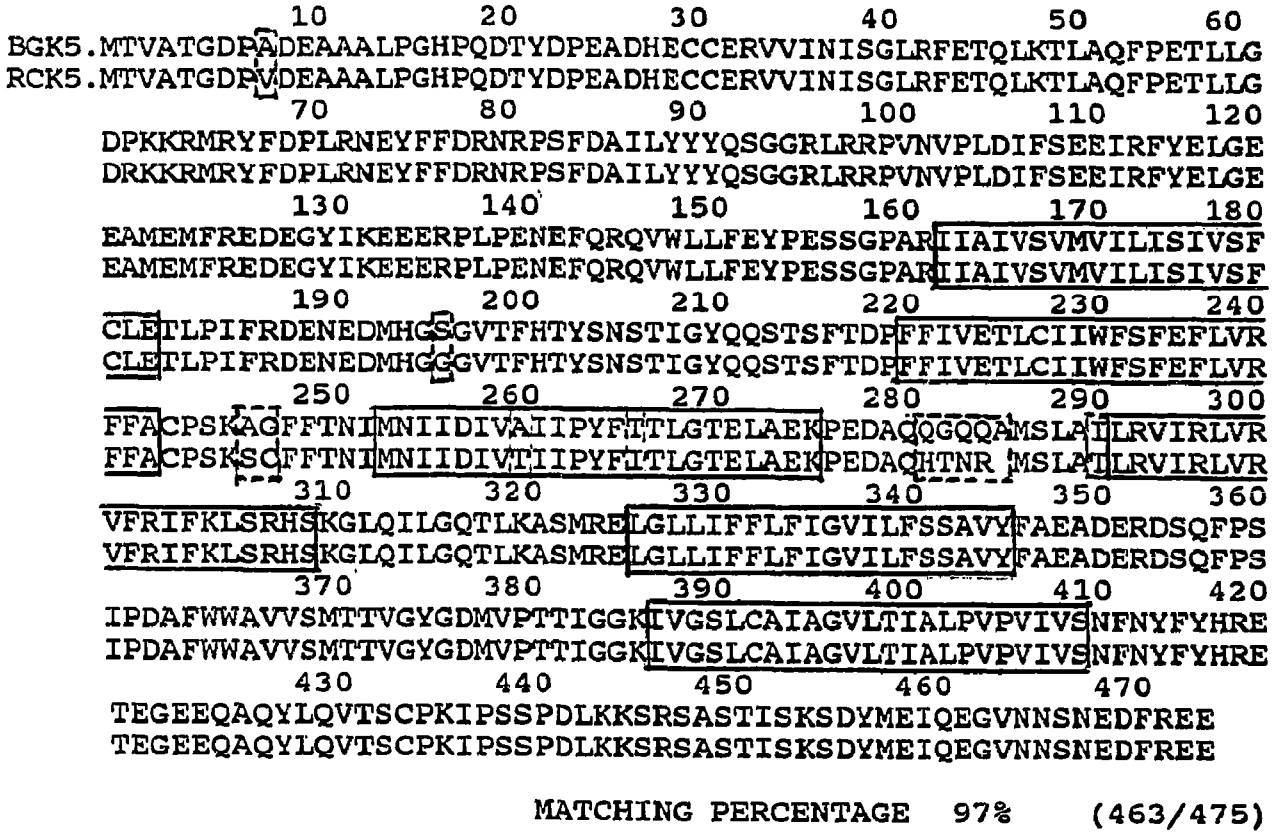

Fig. 3. Primary sequence homology between RCK 5 and BGK 5. Gaps are introduced to facilitate alignment. Amino acid substitutions are enclosed by broken lines. Putative membrane spanning regions are enclosed by boxes.

Hence, a primer corresponding to the final amino acids could not be employed due to similarities between this sequence and the other members of the RCK family and inter- species variation found at the 3 non-translating regions.

Hydropathy analysis [19] of the predicted amino acid sequence showed that the six hydrophobic membrane-spanning regions are present, as noted for RCK 5 (Fig. 3). This accords with models proposed for the arrangement of the protein in the membrane that place both the N- and C-termini on the cytoplasmic side, though there is recent evidence for an additional hairpin loop between segments 5 and 6 [20]. There is conservation of the single asparagine-linked glycosylation site, Asn-207, which is situated in a poorly conserved region between the S_1 and S_2 hydrophobic domains; this would be located on the extracellular side of the membrane according to suggested topographical models. Also conserved is the Lys-Lys-Ser-Arg-Ser-Ala-Ser motif and, although not in accordance with known consensus sequences for phosphorylation by cAMP-dependent protein kinase [21], the proximity of the basic residues may permit this region of the protein to serve as one acceptor site for phosphorylation. Similarly, the motif Arg-Asp-Arg-Pro-Ser at the N-terminus, conserved between RCK family members, could function as a second phosphorylation site. There are also several putative Ca^{2+}/calmodulin and casein kinase motifs located throughout the predicted amino acid sequence [22,23].

4. DISCUSSION

The predicted amino acid sequence of BGK 5 contains the protein sequence obtained [12] from the N-terminal of the larger subunit of the bovine α-Dtx acceptor. Although the N- and C-termini could contain base variations from the primer sequences used, the N-terminus from three individual PCR clones each gave an identical sequence, indicating that this had the greatest stability and, thus, is most likely to correspond to the natural gene. A similar observation was made with two clones containing the C-terminus. Notably, the protein sequence showed a single difference (at position 8) from the cloned sequence of RCK 5 (Fig. 3); the presence of an alanine residue in the BGK 5 sequence reported herein establishes that this change from a valine is due to inter-species variation between rat and cow. Genes corresponding to BGK 5 have been isolated from mouse (MK 2), rat (BK 2, RK 2, RBK 2), and neuroblastoma cells (NGK 1), which all show a strikingly high degree of homology (reviewed in [24]). Such a widespread distribution reaffirms the functional impor-

tance of these proteins that had already been revealed through the use of α-Dtx (reviewed in [25]). It is noteworthy that the amino acids found by site-directed mutagenesis to be responsible for α-Dtx binding in the RBK 1 K^+ channel expressed in oocytes [26] are conserved in BGK 5 and its counterpart in other species. Although the protein encoded by BGK 5 may represent one of the major subunits present in the oligomeric arrays of α-Dtx-sensitive, voltage-gated K^+ channels in mammalian brain, others (including those found to be sensitive and insensitive to α-Dtx following expression e.g. equivalent of RCK 1,2 or RCK 3,4) undoubtedly also contribute to the heterogeneity of this K^+ channel family unveiled by dendrotoxins, β-bungarotoxin, charybdotoxin and mast cell degranulating peptide (reviewed in [25]).

The calculated M_r for BGK 5 is 8 kDa less than that of the acceptor subunit, even though the size of the latter was reduced substantially following *N*-deglycosylation [12]. No inserts were detected that could explain this discrepancy; the presence of additional residues prior to the N-terminus is excluded because protein micro-sequencing has confirmed the identity of the N-terminal residues. Likewise, it is not suspected that an insert may be present at the C-terminal, which was not amplified in the PCR (see position of primers used), as no such inserts have been detected within any of the numerous homologous channels isolated from several other species. As dephosphorylation of the RCK 1 K^+ channel expressed in oocytes alters its electrophoretic mobility in SDS (M. Stocker and O. Pongs, unpublished observation), it is notable that the larger subunit of the α-Dtx acceptor can be phosporylated with cAMP-dependent kinase or an endogenous enzyme that co-purifies with the K^+ channel protein [27]. Such covalent modification might cause an anomalous electrophoretic migration that would underlie the apparent difference in size between the isolated protein and the cloned sequences. Another possible explanation could be provided, at least in part, by the extent of *O*-glycosylation. The observed substitution of serine for glycine at position 198 located in the S_1–S_2 loop gives a total of 9 potential sites (S/T) for *O*-linked glycosylation; its contribution to aberrant electrophoretic migration of the protein is being evaluated presently. As *O*-linked glycosylation usually occurs clustered in one region, the S_1–S_2 extracellular loop may be a prime target for this form of post-translational modification [28].

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