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Expression of voltage-gated K^+ channels in insulin-producing cells

Analysis by polymerase chain reaction

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We have used the polymerase chain reaction (PCR) with primers against the S5 and S6 regions of voltage-gated K^+ channels to identify 8 different specific amplification products using poly(A)$^+$ RNA isolated from islets of Langerhans from obese hyperglycemic (*ob/ob*) mice and from the two insulin-producing cell lines HIT T15 and RINm5F. Sequence analysis suggests that they derive from mRNAs coding for a family of voltage-gated K^+ channels; 5 of these have been recently identified in mammalian brain and 3 are novel. These hybridize in classes to different mRNAs which distribute differently to a number of tissues and cell lines including insulin-producing cells.

Insulin-producing cell; Potassium channel; Polymerase chain reaction; Northern blot analysis

1. INTRODUCTION

Pancreatic β-cells possess a variety of potassium channels including both ligand- and voltage-gated K^+ channels [1,2]. The best characterized of the ligand-gated K^+ channels in the β-cell is the ATP-sensitive K^+ channel (G channel) [2]. This channel is directly inhibited by intracellular ATP ($[ATP]_i$) and changes in $[ATP]_i$ are believed to couple metabolic events to regulation of channel activity. It is also inhibited by sulfonylureas that bind to the channel with high affinity [3–5]. The voltage-gated K^+ channels in β-cells include two kinds of delayed rectifier channels [6], a transient (A-current) K^+ channel [7] and a Ca^{2+}-activated K^+ channel which is gated by both voltage and intracellular Ca^{2+} [8]. The role of the delayed rectifier K^+ channels is clear and, as in other tissues, activation of these channels produces repolarization of the action potential [9]. Whereas the Ca^{2+}-activated K^+ channel is believed also to contribute to action potential repolarization [10], the physiological function of the A-current is not fully understood.

Recent molecular biological studies [10–12] have identified a family of voltage-gated K^+ channels known as RCK (rat cortex) or MBK (mouse brain) which functionally resemble the delayed rectifier K^+ channels. The protein encoded by RCK1 displays some characteristics similar to those of the larger of the two delayed rectifier K^+ channels found in the β-cell. Notably, the channels display the same conductance (10 pS) under a similar ionic concentration gradient, inactivate slowly during a maintained depolarisation and have a similar sensitivity to TEA [6,10]. We have investigated the presence of mRNA sequences similar to the RCK family in insulin-producing cells using the polymerase chain reaction [13] and oligonucleotide primers directed against conserved regions of the RCK family. A similar approach has previously been successfully used to identify sequences coding for K^+ channels in genomic DNA [14]. Using cDNA derived from isolated islets of the obese hyperglycemic (*ob/ob*) mice [15] and from the insulin secreting cell lines HIT T15 [16] and RINm5F [17], we have identified 8 different cDNA sequences which show close homology to the RCK and MBK family. Three of these sequences have not been described previously.

2. MATERIALS AND METHODS

2.1. *Polymerase chain reactions*

Total cellular RNA was prepared [18] from the hamster and rat insulinoma cell lines HIT T15 [16] and RINm5F [17], the human epidermal carcinoma cell line A431 [19] and from *ob/ob* mouse pancreatic β-cells [15], isolated as previously described [20]. Poly(A$^+$)

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RNA was selected using an oligo(dT) cellulose column (Pharmacia, Uppsala) and converted to single-stranded cDNA using oligo(dT) primers as previously described [21]. Approximately 50 ng cDNA was used as a template for enzymatic amplification with a thermostable *Thermus aquaticus* (Taq) DNA polymerase (Perkin Elmer Cetus, Norwalk, CT) and a DNA thermal cycler from the same company. Alternatively, DNA prepared from a λgt11 cDNA library generated from HIT T15 cells was used as template for the amplification reaction. The first two amplification cycles had the profile 94°C, 1 min; 37°C, 3 min; 72°C, 15 s and the following 28 cycles had the profile 94°C, 1 min; 55°C, 2 min; 72°C, 15 s with an extra 5 s at 72°C added in each cycle. The following two degenerate oligonucleotide primers were employed in the reactions: 5'ATTGGATCCAT-(C/A)TT(C/T)TTCCTCTTCAT (sense primer) and ATTGAATTCAC(A/C)CC(A/C/T)GC-(A/G)AT(G/T)GCACA (antisense primer). The amplification products were analysed on agarose Tris-acetate buffer gels and DNA fragments of the expected size were excised and purified using a Geneclean kit (Bio 101, La Jolla, CA). DNA fragments were then digested with *Eco*RI and *Bam*HI (to facilitate subcloning, the 5' parts of the two primers were designed to contain an *Eco*RI and a *Bam*HI site respectively), cloned into *Eco*RI/*Bam*HI cut M13mp18/19 or Bluescript vectors (Stratagene, USA) and sequenced using the Sequenase dideoxy nucleotide sequencing [22] system (United States Biochemical Corp., Cleveland, OH, USA).

2.2. *Northern (RNA) blot analysis*

DNA fragments corresponding to each K^+ channel cDNA were subcloned in pUC vectors. Plasmids were then grown, linearized and labeled with ^{32}P using random hexanucleotide priming [23]. Total cellular RNA was prepared from various rat tissues and rat and human cell lines using the LiCl/urea method [16], poly(A^+) RNA was selected, size fractionated on 0.8% agarose/formaldehyde slab gels and transferred to nitrocellulose filters (Schleicher and Schüll, Dassel, FRG) using standard procedures [24]. Filters were dried and baked under vacuum, prehybridized and hybridized in 50% formamide at 42°C against the ^{32}P-labeled DNA probes as previously described [24]. Following washings under high stringency conditions (3 × 30 min at 55°C in 0.1 × SSC, 0.1% SDS), filters were exposed to Hyperfilm (Amersham, UK) using intensifying screens for 2–5 days at −70°C.

3. RESULTS

We designed our PCR primers against the two best conserved membrane-spanning domains S5 (sense primer) and S6 (antisense primer) of the RCK family of voltage-gated brain K^+ channels [11,12]. Sequencing of

FIGURE 1a

S5 region

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RCK-1 GGC GTC ATA CTG TTT TCT AGT GCA GTG TAC TTT GCG GAG GCG GAA GAA GCT GAG TCG CAC TTC TCC
MK-1            G               C

RCK-2   A       C   C   C   C   C   T   C       C   A       A   T   C   T   C       T       C T
MK-2    A       C   C       C   C   T   C       C   A       T   T   T   T   C       T       C T

RCK-3   G       C   T   C   C           C           T       A   C   C C   TCT     GGT     T AA
MK-3    G       C   T   C   C           C           T       A   C   C C   TCT     GGT     T AA

RCK-4   G       C   C       C   C   T       T       A       A   T     C   ACC A C   T       CAA
MK-4    G   T   C   C       C   C   T       T       A           T   C C   ACT A C   T       CAA

RCK-5   G       C   C   C           T   C   T       A   A   T   T   G CGA   T   C   G       C
MK-5    G       C   C   C           T   C   T       A   A   T   T   G AGA   T   C   G       C

MK-6        G G C   C       C   C       C           T   A T     C CGG   TG    C A C   T       A
HaK-6       G G C   C       C   C       C           T   A  T    C CGG   TG    C A C   T       A
RK-6    T   G G C   C       C   C   C   C           C   A  TT   C CGG   TG    C T C   T       A T

HaK-7 ------------------------------------      C   A           C AAC CAG  G    C

RK-8            C   C               T       T   T   A           T     C   ACT A C   T       CAA
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RCK-1 AGT ATC CCC GAT GCT TTC TGG TGG GCG GTG GTG TCC ATG ACC ACT GTG GGA TAC GGT GAC ATG TAC
MK-1

RCK-2   C       A       C   T           T       T A A           A   A   T   T   G
MK-2    C       A       C               T       T A A           G   A   T   T   G

RCK-3           G       C               C       A A         A       C   T   T       T   C
MK-3            G       C               A   A   A A         A       T   T   T       T   C

RCK-4   C   T   A       G   T           T       A A         A           C       G       A G
MK-4    C   T   A       G   T           T     CA A          A           C   T   G       A G

RCK-5   C   C   G       C               A   C   C           A       A   C   T   A       GTT
MK-5    C       G                       A   C               A       A   C   T   A       GTT

MK-6    C       G   G   C   T           A       C A     C       G   T   C   T   G       GCA
HaK-6   C       G   G T C   T           A       C A             G   T   C   T   G       GCA
RK-6    C       T   C T C               G   A   C A         T   A   T   C   T   A       GCA

HaK-7           A       C               A   A   C A             C   A   C   T   G  G    AGG

RK-8    C       A       A   T           T         A         A           C   T   G       A G
```

Fig. 1. Nucleotide (a) and predicted amino acid (b) sequences of amplified K^+ channel DNA fragments. Sequences are compared with those of RCK-1 [11], RCK-2 (O. Pongs, unpublished sequence) and RCK-3,4,5 [12]. Diverging positions are indicated. Identical positions are left out. (---) Sequence not revealed.

fragments amplified from *ob/ob* mouse islets revealed 6 different, but closely homologous, sequences related to this family. Five of these appear to be the mouse islet counterparts (MK1-5) of previously identified members of the rat brain RCK family (RCK1-5) (Fig. 1). MK1 is almost identical to MBK1 which is derived from mouse brain [10]. A sixth mouse islet sequence (MK6) is, however, novel and may derive from a hitherto unidentified K^+ channel gene (Fig. 1). We repeated the same procedure on two insulin-producing tumour cell lines,

Fig. 1 continued.

FIGURE 1a (continued)

S6 region

```
RCK-1 CCT GTG ACA ATT GGA GGC AAG ATC GTG GGC TCC TTG TGT
MK-1

RCK-2   C A     G G G              T           A C
MK-2    C A     G G A   G          A           A C

RCK-3   A       C   A            T  T           T C T
MK-3    A       C   A               T           T C T

RCK-4   C ATC     G G       A       T       G     C
MK-4    C ATC     G C   G   A       T       G     C

RCK-5   A ACT   C       G   A       A       T   T C
MK-5    A ACT   C       G   G       A       T   T C

MK-6    C   C   C G G   T                       T C
HaK-6   C   C   C G G   T                       T C
RK-6    C   C   T G G   T                       ---------

HaK-7   C A C   T G G               T           G C

RK-8    C A C   T G A   G           T   C G     C
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FIGURE 1b

S5 region

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RCK-1  G V I L F S S A V Y F A E A E E A E S H F S S I P D A F W W A V V S M T T V G Y G D M Y
MK-1       M

RCK-2                          D D V D   L   P                     T
MK-2                           D D V D   L   P                     T

RCK-3                          D D P S   G   M                     T                   H
MK-3                           D D P S   G   M                     T                   H

RCK-4                          D   P T T     Q                     T                   K
MK-4                           D D P T T     Q                     AT                  K

RCK-5                          D   R D   Q   P                                         V
MK-5                           D   R D   Q   P                                         V

MK-6       V                 S D R V D T     T       E             T I                 A
HaK-6      V                 V D R V D T     T       E S           T                   A
RK-6       V                 V D R V D S     T         S           T                   A

HaK-7  - - - - - - - -         D M Q G                             T             G     R
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S6 region

```
RCK-1  P V T I G G K I V G S L C
MK-1

RCK-2     M   V
MK-2      M   V

RCK-3
MK-3

RCK-4     I   V
MK-4      I   V

RCK-5     T
MK-5      T

MK-6          V
HaK-6         V
RK-6          V

HaK-7     I   V
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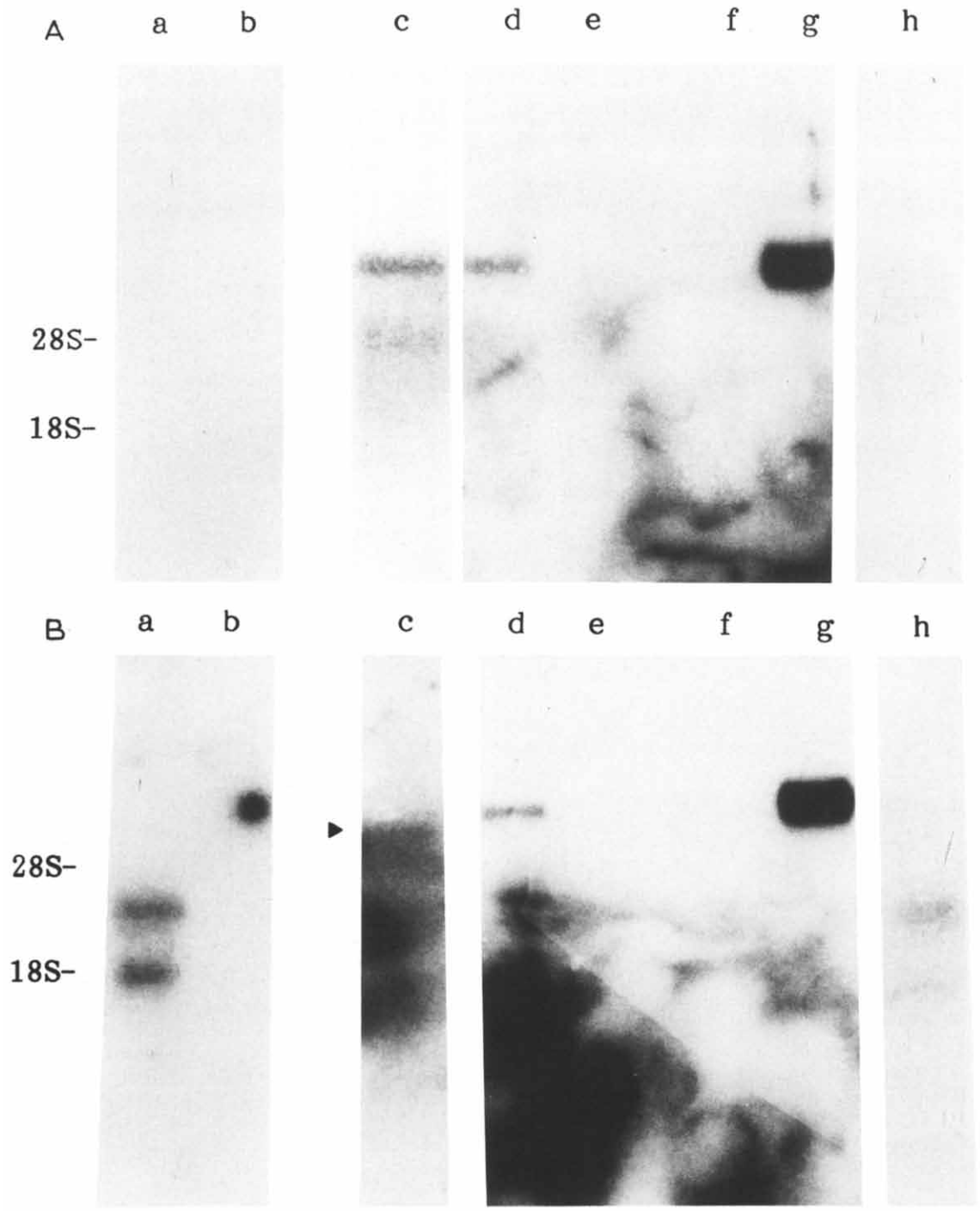

Fig. 2. Northern blot analysis using MK-1 in (A) and MK-2 in (B) as probes. Samples of 10 μg poly(A)$^+$ RNA each from RINm5F cells (lane a), HIT T15 cells (lane b), rat brain (lane c), rat liver (lane d), rat spleen (lane e), rat heart (lane f), rat skeletal muscle (lane g) and A431 cells (lane h) were electrophoresed, transferred to nitrocellulose filters and hybridized with the probes. Note that lanes a, b, c and h have been overexposed relative to the other lanes to visualize the mRNAs that hybridized. The arrow indicates a faint 6–7 kb band in brain RNA that hybridized with MK-2.

HIT T15 and RINm5F. Four different PCR amplification products were obtained from size-fractionated (>4 kb) first-strand cDNA prepared from mRNA from RINm5F cells. Two of these (RK1 and RK4) were identical to RCK-1 and RCK-4 respectively over the amplified region whereas the third (RK6) probably represents the rat counterpart to MK6 (Fig. 1). The fourth amplification product (RK8) was novel. The amino acid sequence encoded by the RK8 fragment was identical to that of RK4 despite several differences in the nucleotide sequence (Fig. 1).

Two RCK counterparts were identified in cDNA

prepared from HIT T15 mRNA. One of these, HaK1, was closely similar to RCK-1 (not shown), but the other, HaK7, was novel (Fig. 1). When DNA prepared from a HIT T15 λgt11 cDNA library was used as a source of DNA for PCR reaction, two sequences were found. One was identical to RCK-2 whereas the other (HaK6) was closely homologous to MK6 and RK6 (Fig. 1).

When the PCR amplification products were studied by Northern blot analysis, all probes tested (MK1-5) hybridized strongly to a large (approximately 8 kb) skeletal muscle mRNA (Fig. 2A, B). A fainter band of similar size was seen in rat liver mRNA, whereas no detectable hybridization occurred to spleen or HIT T15 mRNA (Fig. 2A, B). MK1 hybridized weakly to an 8 kb rat brain mRNA (Fig. 2A). MK2 hybridized weakly to a smaller mRNA (6–7 kb) in rat brain (Fig. 2B). No detectable hybridization occurred to rat brain mRNA using MK3-6, HaK7 or RK8 as probes (results not shown). Two faint bands of sizes 7–10 kb were detected in heart mRNA using MK1, MK3 and MK5 as a probe (Fig. 2B), whereas none of the other PCR amplification products (MK2, MK4, MK6, HaK7 and RK8) generated a detectable signal in heart mRNA (results not shown). Two bands of about 2 and 4 kb were seen in RINm5F and A431 mRNA when hybridizing with MK2 (Fig. 2B), MK4, MK6, HaK7 and an only 4 kb band was detected in these RNAs using RK8 as a probe (results not shown). HaK7 hybridized weakly to 2 and 4 kb mRNAs in HIT T15 mRNA (result not shown).

4. DISCUSSION

Voltage-gated K^+ channels are known to be present in pancreatic β-cells where they play an important physiological role in repolarization of the action potential. We have now identified 8 distinct but closely homologous putative K^+ channel sequences in cDNA prepared from isolated pancreatic islets or insulin-producing cell lines. These PCR amplification products show close similarity to the S5–S6 region of the RCK family of voltage-gated K^+ channels. This suggests that members of the RCK family are also expressed in non-neuronal tissue and that the novel sequences (MK6, HaK7, RK8) may constitute further members of this family.

Without knowledge of the gene structures, the possibility that the S5–S6 region of the RCK family of proteins is contained within a single exon, and thus the risk that one or several of the amplified sequences originate from chromosomal DNA fragments contaminating the poly(A)$^+$ RNA has to be considered. However, the differences in the spectrum of putative channel sequences obtained from the various mRNA sources (Table I) favour amplification from cDNA and not from genomic DNA, especially since no one single source amplified all 8 channels. The largest diversity of channel sequences was seen in isolated pancreatic islets. When comparing the amplification products from islets and β-cell lines, two were always detected (RK/MK/HaK1 and RK/MK/HaK6), suggesting that these are expressed in the β-cell. HaK-6 was also obtained from a λgt11 cDNA library, minimizing the risk that this product arose from amplification of genomic sequences. Finally, RK4 and RK8 were abundant amplification products in size-selected first strand cDNA, which also argues against genomic amplification, since then no bias in favour of any specific channel should exist. The absence of sequences corresponding to HaK7 and RK8 in the islet amplification reaction suggests that these are spuriously expressed in insulin-producing tumour cells. Both HIT T15 and RINm5F cells display altered characteristics of insulin secretion, however, electrophysiological studies have not indicated any differences in voltage-gated K^+ channels between normal β-cells and insulin producing tumour cell lines. The Northern blot analyses do not entirely resolve the matter of expression of specific channels in RINm5F and HIT T15 cells because of significant cross-reactivity between the different probes. Two mRNAs of 2 and 4 kb present in the insulin-producing cell lines hybridized with several of the amplification products. Since the PCR reactions using HIT T15 and RINm5F cDNAs yielded more than two products, these two bands are either heterogeneous or other, less abundant, mRNAs coding for voltage-gated K^+ channels are expressed in these cells.

Functional characterization of the channels in *Xenopus* oocytes following microinjection of in vitro transcribed RCK-specific RNAs has shown them to be a family of voltage-gated K^+ channels [12]. Despite the extensive homology at the level of derived amino acid sequence, the channels show considerable diversity in their voltage-dependent gating and toxin-binding properties. The RCK family are also homologous to the

Table I

Frequency with which each K^+ channel sequence occurred following PCR

cDNA source	Number of clones identified corresponding to different RCKs							
	1	2	3	4	5	6	7	8
ob/ob islets	5	2	10	4	7	1	0	0
HIT T15	7	0	0	0	0	0	2	0
RINm5F	1	0	0	3	0	1	0	2
HIT T15 library	0	1	0	0	0	1	0	0

Amplified DNA fragments were processed as described in section 2 and subcloned M13 or Bluescript vectors. Numerous DNA sequences without homology to the RCK-family were revealed. The table gives the number of M13/Bluescript clones containing inserts identical to or homologous with previously known RCK sequences

Shaker family of *Drosophila* voltage-gated K^+ channels. An additional mammalian K^+ channel, drk-1, less homologous to the RCK family in the S5–S6 region than the novel sequences obtained in the present study, is also voltage-gated [25]. We therefore consider it likely that the new K^+ channel-like sequences (MK6, HaK7 and RK8) identified here represent additional voltage-gated K^+ channels. Since the primers that we used did not encompass the putative voltage-sensor region (S4) of the K^+ channel, but rather an extracellular intermembranous domain, there remains the possibility that they may include other types of K^+ channels, e.g. the ATP-sensitive K^+ channel which is of relatively high abundance in the β-cell. Furthermore, diversity of K^+ channels could be generated by heteropolymer subunit arrangement in which the ATP-sensitive K^+ channel could have a subunit which does not share the overall protein structure characteristic for the RCK/MBK family. However, the nature of these channels will be resolved first by the isolation and functional expression of full length cDNA clones.

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