

CLONING OF RAT K_{ATP-2} CHANNEL AND DECREASED EXPRESSION IN PANCREATIC ISLETS OF MALE ZUCKER DIABETIC FATTY RATS

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Summary: ATP-regulated potassium channels play a key role in regulating insulin secretion. We have isolated cDNA clones from a RINm5F insulinoma cell cDNA library that encode a protein, K_{ATP-2} , whose sequence shows 72% identity with the rat heart potassium channel K_{ATP} . RNA blotting showed that K_{ATP-2} mRNA was present at high levels in brain and undetectable in heart, spleen, lung, liver, skeletal muscle, kidney and testis. A quantitative RT-PCR assay indicated that there were $1.85 \pm 0.32 \times 10^5$ molecules of K_{ATP-2} mRNA per microgram of total RNA in pancreatic islets from nondiabetic rats. The levels of K_{ATP-2} mRNA were reduced by 34% in islets from diabetic Zucker diabetic fatty male rats, a model of non-insulin dependent diabetes mellitus, compared to their lean nondiabetic littermates ($p < 0.05$), suggesting that decreased expression of K_{ATP-2} may contribute to β -cell dysfunction in this animal model. © 1995 Academic Press, Inc.

ATP-regulated K^+ -channels (K_{ATP}) play an important role in the regulation of insulin secretion by coupling metabolism to plasma membrane potential (1). The metabolism of glucose, the principal physiological stimulus for insulin secretion, leads to an increase in intracellular ATP/ADP ratio and closure of K_{ATP} . This results in membrane depolarization, influx of extracellular Ca^{2+} through voltage-dependent Ca^{2+} -channels, increase in intracellular Ca^{2+} levels, and exocytosis of secretory granules.

We have recently cloned a new member of the inwardly-rectifying family of potassium channels from hamster insulinoma cells (2). The amino acid sequence of this protein which we termed K_{ATP-2} was 71% identical to a rat heart protein termed K_{ATP} or cardiac inward rectifier (CIR) that may comprise part of cardiac I_{KATP} (3, 4). Here we report the cloning of rat K_{ATP-2} , and show that expression of K_{ATP-2} mRNA is significantly decreased in islets of diabetic rats.

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MATERIALS AND METHODS

General Methods

Standard methods are described in Sambrook et al. (5). DNA sequencing was done by dideoxynucleotide chain termination procedure after subcloning suitable fragments into pGEM-3Z (Promega, Madison, WI).

Animals

Homozygous obese Zucker diabetic fatty rats (ZDF/GmiTM-*fa/fa*) and lean nondiabetic littermate controls (ZLC, *+/+* or *+fa*) were purchased from Genetic Models Inc., Indianapolis, IN. Animals were maintained on an *ad libitum* diet with commercial chow (Purina 5008) and had free access to water. Four groups of animals were studied: ZDF rats at 7 weeks of age before the onset of diabetes (tail vein blood glucose values, 9.7 ± 0.6 mM); ZDF rats at 9-12 weeks of age after the onset of diabetes (blood glucose values, 21 ± 1.8 mM); ZLC rats at 7 and 12 weeks of age (blood glucose values of 7.9 ± 0.3 and 8.1 ± 0.05 mM, respectively). Serum glucose concentrations were measured by the glucose oxidase method using a glucose analyzer (Beckman, Fullerton, CA).

Isolation of Rat K_{ATP}-2 cDNA Clones

A full-length 2.5 kilobase pair (kb) ³²P-labeled hamster K_{ATP}-2 cDNA clone (2) was used to screen a rat insulinoma RINm5F cell cDNA library. Five clones were identified, one of which, λ KC-1, contained an insert of 2.1 kb whose sequence was determined.

RNA Blotting

A rat multiple tissue Northern blot (Clontech, Palo Alto, CA) was hybridized with a ³²P-labeled 0.9 kb EcoRI fragment of λ KC-1 (nucleotides 1-901; there are EcoRI linkers at the ends of the insert as well as an internal EcoRI site) encoding the 5'-untranslated region of the mRNA and amino acids 1 to 230. The hybridization was carried out in a solution of 5 x SSC (0.75 M NaCl and 0.075 sodium citrate), 50% formamide, 2 x Denhardt's solution, 100 μ g/ml of sonicated and denatured salmon testes DNA, 20 mM sodium phosphate buffer, pH 6.5, 10% dextran sulphate, and 1 x 10⁶ cpm/ml of probe at 42°C for 16 hrs. The blot was washed in 0.1 x SSC and 0.1% sodium dodecyl sulfate at 67°C for 40 min before exposure to X-ray film.

RT-PCR Amplification of K_{ATP}-2 mRNA

Pancreatic islets were isolated as described previously (6) and RNA was prepared from islets obtained from individual ZDF and control rats using the acid-guanidinium thiocyanate-phenol-chloroform procedure (7). cDNA was prepared using 1 μ g of total islet RNA and 20 pmol of oligo d(T)₁₆ primer (Perkin Elmer, Norwalk, CT) in a 20 μ l solution containing 50 mM Tris-Cl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 0.125 mM each dNTP, 2 units of RNasin (Promega) and 200 units of Moloney murine leukemia virus reverse transcriptase (RT) (GIBCO/BRL, Gaithersburg, MD). After incubation for 1 hr at 37°C, the reaction mixture was heated to 65°C for 5 min, the cDNA diluted 1:100 with water, and stored at -20°C. K_{ATP}-2 mRNA levels were determined using a quantitative polymerase chain reaction (PCR) assay (6, 8). Known amounts of a synthetic competitor DNA template, prKATP-2, that generated a PCR product different in size from K_{ATP}-2 mRNA were added to each reaction. The cDNA target and competitor template were coamplified using primers rKATP2-F (5'-TAACCAACAGAGTCCCTTCTG-3') and rKATP2-R (5'-GTTAGCCAGCTCGGCTAGCTCT-3'). PCR was carried out in a GeneAmp PCR system 9600 (Perkin Elmer) in a volume of 50 μ l containing cDNA, competitor DNA, 10 μ M dNTP, 10 pmol of each oligonucleotide primer, 50 mM KCl, 10 mM Tris-Cl (pH 8.3), 1.5 mM MgCl₂, 2 μ Ci (12 nM) of [α ³²P]dCTP (3000 Ci/mmol) (Amersham, Arlington Heights, IL), and 1 U of Taq DNA polymerase. The PCR conditions were initial denaturation at 94°C for 5 min, followed by 34 cycles of denaturation at 94°C for 45 sec, annealing at 58°C for 1 min, and extension at 72°C for 1 min, with a final extension step at 72°C for 10 min. The PCR products were separated by electrophoresis in a 5% polyacrylamide gel and the radioactivity incorporated into each fragment determined directly using a PhosphorImagerTM and ImageQuantTM software (Molecular Dynamics, Sunnyvale, CA). Control studies using synthetic rat insulin mRNA indicated the efficiency of cDNA synthesis was 42% which was taken into account when determining the number of mRNA molecules/ μ g of total RNA.

Data Analysis

Differences between groups were analyzed by Student's t-test or, where appropriate, by ANOVA and differences were considered significant at $p < 0.05$.

RESULTS

Cloning and Sequence of Rat K_{ATP-2}

Rat K_{ATP-2} cDNA clones were isolated from rat insulinoma RINm5F cell cDNA library by hybridization with a Syrian hamster K_{ATP-2} cDNA probe. Five clones were identified, λ KC-1 to -5 with inserts of 0.9, 1.3 and 2.1 kb; three clones had inserts of 0.9 kb and were likely different isolates of the same cDNA clone. The sequence of the 2.1 kb insert in λ KC-1 (GenBank accession no. U21087) had 84.5% overall nucleotide sequence identity and 91.7% identity in the coding region of the mRNA with that of hamster K_{ATP-2} (2). The longest open reading frame in the rat K_{ATP-2} cDNA sequence started at nucleotide 212 and encoded a protein of 425 amino acids (M_r 48,684) (Fig. 1). There were three ATG codons in the putative 5' untranslated region, all which were closely followed by translation termination codons. The amino acid sequence of rat K_{ATP-2} showed 99%, 72%, and 57% identity with hamster K_{ATP-2} , rat heart K_{ATP} (K_{ATP-1}) and rat GIRK1, respectively (Fig. 1). Regions of high sequence homology include the membrane-spanning regions M1 and M2 (amino acid residues 95-118 and 169-190 of rat K_{ATP-2} , respectively), as well as the putative pore-forming domain H5 (amino acid residues 144-160 of rat K_{ATP-2}). The intracellular N- and C-termini are the most divergent regions and differ considerably in sequence and length. Amino acid residue 184 is Asn in both K_{ATP-1} and -2 and Asp in GIRK1 and related proteins; this residue controls gating properties of inwardly-rectifying K^+ -channels (9, 10).

Tissue Distribution of K_{ATP-2} mRNA

RNA blotting indicated that K_{ATP-2} mRNA was highly expressed in brain (Fig. 2). There was no hybridization signal in heart, spleen, lung, liver, skeletal muscle, kidney or testis. The probe did not hybridize to a single transcript of discrete size but rather hybridized to polydisperse series of mRNAs varying in size from 1.35-3.5 kilobases. This diffuse pattern was not observed when the membrane was hybridized with a probe for glyceraldehyde 3-phosphate dehydrogenase mRNA. The molecular basis for the diffuse hybridization signal is unknown.

Regulation of K_{ATP-2} mRNA Levels in Islets of ZDF Male Rats

The levels of K_{ATP-2} mRNA were measured in pancreatic islets isolated from four groups of male rats: prediabetic and diabetic ZDF male rats at 7 and 9-12 weeks of age, respectively; and lean nondiabetic littermates (ZLC) at 7 and 12 weeks. The ZDF animals were not markedly obese when studied and their weights at 7 weeks of age (250 ± 7 g, $n=14$) and 9-12 weeks (370 ± 9 g, $n=14$) were 14% greater than those of 7 and 9-12 week lean littermate controls (219 ± 9 g, $n=14$, and 325 ± 19 g, $n=9$, respectively) ($p < 0.05$ (ANOVA), ZDF rats vs ZLC animals of the same age). The prediabetic 7 week-old and diabetic 9-12 week ZDF rats were insulin resistant with insulin levels of 753 ± 83 ($n=14$) and 624 ± 89 pmol/L ($n=14$), respectively, values which are 8-12-fold higher than those for 7 and 12 week-old ZLC rats (63 ± 5 ($n=14$) and 81 ± 6 pmol/L ($n=9$), respectively) ($p < 0.001$, (ANOVA) ZDF vs age-matched controls) (Y. Tokuyama et al., in preparation).

The abundance of K_{ATP-2} mRNA in islets of 7 week ZLC rats was determined by competitive RT-PCR. There were $1.85 \pm 0.32 \times 10^5$ molecules of K_{ATP-2} mRNA per microgram of

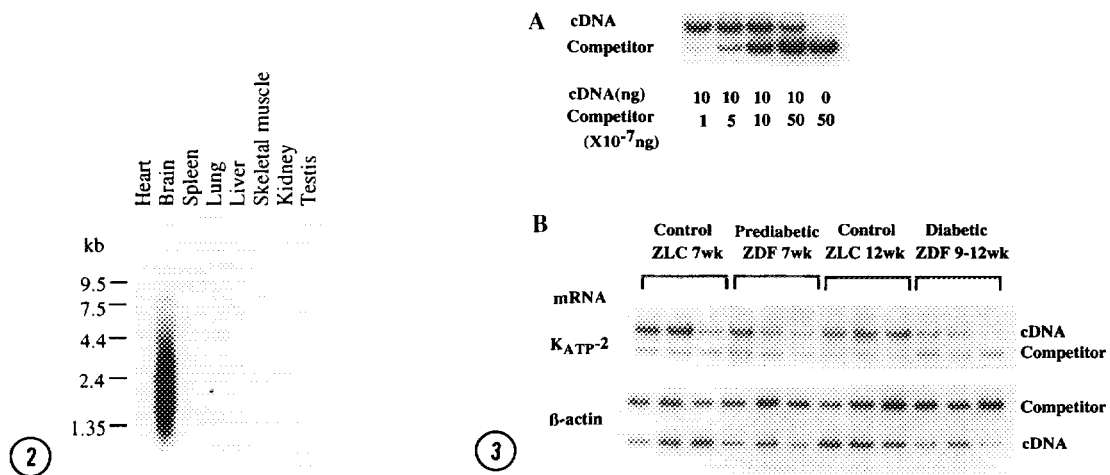


Fig. 2. Tissue distribution of K_{ATP}-2 mRNA. An RNA blot containing 2 µg of poly (A)⁺ RNA from various rat tissues was hybridized with a ³²P-labeled rat K_{ATP}-2 cDNA probe. The positions of RNA size markers are shown on the left.

Fig. 3. K_{ATP}-2 mRNA levels in islets of prediabetic and diabetic ZDF rats and aged-matched controls. A. Quantitative RT-PCR of K_{ATP}-2 mRNA in islets of 7-week-old ZLC rats. The upper band in the autoradiogram shown in this figure represents the PCR product generated from 10 ng of target cDNA (i.e. cDNA obtained from 10 ng of RNA) and the lower band represents the PCR product generated from the internal standard DNA added to each reaction, the amount of which is indicated. B. Semiquantitative competitive RT-PCR of K_{ATP}-2 mRNA. The levels in RNA preparations from islets isolated from three individual animals are shown. In the reactions to measure K_{ATP}-2 mRNA levels, each PCR contained 5 ng of cDNA target and 5 x 10⁻⁷ ng of competitor plasmid DNA. The reactions to measure β-actin mRNA levels contained 0.5 ng of cDNA and 5 x 10⁻⁴ ng of plasmid competitor DNA.

K_{ATP}-2 mRNA in islets from 7 week-ZDF and control rats. Nor were there differences in the levels of β-actin mRNA between prediabetic and diabetic ZDF rats and controls.

DISCUSSION

We have cloned and partially characterized an inwardly-rectifying potassium channel, K_{ATP}-2, which may comprise part of the pancreatic β-cell ATP-regulated potassium channel. This channel is responsible for maintenance of the membrane resting potential and its closure initiates

Table 1. Relative K_{ATP}-2 mRNA levels in islets of male prediabetic (7 wk) and diabetic (9-12 wk) ZDF and age-matched ZLC rats

ZLC - 7 wk (n = 6)	ZDF - 7 wk (n = 6)	ZLC - 12 wk (n = 5)	ZDF - 9 - 12 wk (n = 5)
100±17.6	83.1±4.9	109.7±8.5	66.3±9.2*

* p < 0.05 (Student's t-test); ZDF (9-12 wk) vs ZLC (12 wk).
 "n" denotes the number of animals studied in each group.

membrane depolarization and secretion.. In addition to pancreatic islets, K_{ATP-2} mRNA is present at high levels in brain but not in other tissues including heart, skeletal muscle and kidney.

K_{ATP-2} mRNA levels were reduced in islets of diabetic ZDF rats. This animal model has many similarities with human NIDDM (11), and affected animals demonstrate severe insulin resistance and β -cell insensitivity to glucose. K_{ATP-2} mRNA levels were reduced by 34% in islets from overtly diabetic animals compared to lean non-diabetic littermates suggesting that decreased expression of K_{ATP-2} may contribute to the β -cell dysfunction observed in these animals.

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REFERENCES

1. Ashcroft, F.M., Proks, P., Smith, P.A., Ämmälä, C., Bokvist, K., and Rorsman, P. (1994) *J. Cell. Biochem.* 55S, 54-65.
2. Tsaar, M-L., Menzel, S., Lai, F-P., Espinosa, R., Concannon, P., Spielman, R.S., Hanis, C.L., Cox, N.J., Le Beau, M.M., German, M.S., Jan, L.Y., Bell, G.I., and Stoffel, M. (1995) *Diabetes* 44, 592-596.
3. Ashford, M.L.J., Bond, C.T., Blair, T.A., Adelman, J.P. (1994) *Nature* 370, 456-459.
4. Krapivinsky, G., Gordon, E.A., Wickman, K., Velimirovic, B., Krapivinsky L., and Clapham, D.E. (1995) *Nature* 374, 135-141.
5. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning, a Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
6. Iwashima, Y., Pugh, W., DePaoli, A.M., Takeda, J., Seino, S., Bell, G.I., and Polonsky, K.S. (1993) *Diabetes* 42, 948-955.
7. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* 162, 156-159.
8. Siebert, P.D., and Larrick, J.W. (1992) *Nature* 359, 557-558.
9. Lu, Z., and MacKinnon, R. (1994) *Nature* 371, 243-246.
10. Wible, B.A., Tagliatela, M., Ficker, E., and Brown, A.M. (1994) *Nature* 371: 246-249.
11. Peterson, R.G., Shaw, W.N., Neel, M.A., Little, L.A., and Eichberg, J. (1990) *Inst. Lab. Animal Res. News* 32, 16-19.
12. Kubo, Y., Reuveny, E., Slesinger, P.A., Jan, Y.N., and Jan, L.Y. (1993) *Nature* 364, 802-806.