PANCREATIC ISLET CELLS EXPRESS A FAMILY OF INWARDLY RECTIFYING K⁺ CHANNEL SUBUNITS WHICH INTERACT TO FORM G-PROTEIN-ACTIVATED CHANNELS

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Insulin secretion is associated with changes in pancreatic β-cell K⁺ permeability. A degenerate polymerase chain reaction strategy based on the conserved features of known inwardly rectifying K⁺ (KIR) channel genes was used to identify members of this family expressed in human pancreatic islets and insulinoma. Three related human KIR transcript sequences were found: CIR (also known as cardiac KATP-3), GIRK1, and GIRK2 (KATP-2). The pancreatic islet CIR and GIRK2 full-length cDNAs were cloned, and their genes were localized to human chromosomes 11q23-ter and 21, respectively. Northern blot analysis detected CIR mRNA at similar levels in human islets and exocrine pancreas, while the abundance of GIRK2 mRNA in the two tissues was insufficient for detection by this method. Using competitive reverse-transcription polymerase chain reaction, CIR was found to be present at higher levels than GIRK2 mRNA in native purified β-cells. Xenopus oocytes injected with M2 muscarinic receptor (M2) plus either GIRK2 or CIR cRNA expressed only very small carbachol-induced currents, while co-injection of CIR plus GIRK2 along with M2 resulted in expression of carbachol-activated strong inwardly rectifying currents. Activators of KATP channels failed to elicit currents in the presence or absence of co-expressed sulfonylurea receptor. These results show that two components of islet cell KIR channels, CIR and GIRK2, may interact to form heteromeric G-protein-activated inwardly rectifying K⁺ channels that do not possess the typical properties of KATP channels.

The permeability of K⁺ ions plays a crucial role in the control of pancreatic islet β-cell excitability and insulin secretion (1, 2). Electrophysiological studies have revealed at least four classes of functionally distinct K⁺ currents in β-cells: 1) ATP-sensitive K⁺ channels that close in response to increased intracellular ATP/ADP ratios generated by increased metabolic flux, 2) voltage-gated K⁺ channels activated by depolarization, 3) large and small conductance calcium-activated K⁺ channels, and 4) ligand-gated K⁺ channels that respond to physiological agonists acting through G-protein-coupled receptors (1, 3–5). Because pancreatic islet K⁺ channel genes are only beginning to be identified, the molecular basis for most of these currents remains unknown (6–8). Furthermore, their precise contribution to insulin secretory activity is largely not understood. The characterization of K⁺ channel proteins synthesized in islet cells is of great practical interest, as it will contribute to understanding β-cell electrophysiology and potentially enhance the development of more effective and specific drugs to manipulate insulin secretory function. Furthermore, because defective insulin release is central to the pathogenesis of non-insulin-dependent diabetes mellitus (9), these molecules provide a valuable source of candidate genes to study the inherited basis of this disorder.

A novel superfamily of genes encoding inward rectifying K⁺ (KIR) channels has been recently identified (10–13). Unlike voltage-activated K⁺ channels of the Shaker gene family which are opened by membrane depolarization (14), KIR channels are open at hyperpolarized potentials. These channels share an underlying conserved structure, with two predicted membrane spanning domains, homologous to the fifth (S5) and sixth (S6) transmembrane domains of voltage-gated channels, encompassing a region homologous to the pore-forming portion of voltage-activated channels (14). KIR channels, however, lack a portion homologous to the amino-terminal region of voltage-gated channels (S1–S4).

β-cells contain K⁺ channels which have gating properties similar to members of the KIR family of channels, including ATP-sensitive channels (KATP) and G-protein-activated K⁺ channels that do not possess features of KATP channels (2, 4). These channels may be involved in the regulation of insulin secretion by glucose and/or neurotransmitters acting through G-protein-coupled receptors (1, 4, 15, 16). Because islet KIR proteins are likely to share homology to other KIR molecules, we have employed a degenerate polymerase chain reaction strategy based on the conserved features of known KIR genes to identify and clone members of this family expressed in pancreatic islets. We demonstrate here the presence of three related KIR transcript sequences, CIR, GIRK1, and GIRK2, in human pancreatic islet cells. In contrast to previous work based on studies with cultured tumor β-cell lines which disclosed the presence of GIRK2, but not CIR (15, 17), CIR was found to be more abundant than GIRK2 in native purified pancreatic β-cells. Cloned islet cell CIR and GIRK2 cDNAs are shown to express heteromultimeric G-protein-activated KIR channels that do not possess characteristic features of KATP channels in

**References**

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conditions described above except for modifications of annealing temperature and cycle number. A limited number of cycles, 25–28, were used to avoid the plateau phase of amplification. Reactions with H2O instead of cDNA and RNA lacking reverse transcriptase were used as controls. For human islet CIR, primers were 5'-GAATAAGAGG-GAGACC-3' and 5'-GGGCTATCTTCTCTTCG-3' (annealing temperature, 62°C). Similarly, for human islet Girk2, were 5'-CCTGCTATCTCCTCCACA-3' and 5'-CTATGCTTATTTATTAT-3' (annealing temperature, 58°C). Primers to co-amplify rat CIR and Girk2 were 5'-(AT)AGACAGAAAGAC(C/A)ACCATT-3' and 5'-T/C)TGCC(T/C)CATC+GCTGGA-3' (annealing temperature, 58°C). PCR products were resolved on an ethidium bromide-stained 2% agarose gel and visualized under ultraviolet light.

The third amplification of human islet KATP-1/CIR cDNA was performed as follows: reverse transcription of RNA was primed with T7(A/G/C/IN, where) N is A, G, C, and T. PCR amplification was performed for 35 cycles at 94, 48, and 72°C for 1 min each step with primer 5'-GGGCTATCTTCTCTTCG-3', derived from the insulinoma CIR degenerate PCR fragment sequence, and T7(A/G/C/IN. A unique 2.4-kb band was purified and partially sequenced directly.

Northern blots were prepared with selected public poly(A)-RNA-enriched samples, hybridized with 32P-labeled probes, and washed at a final stringency of 0.1× SSC, 0.1% SDS, 65°C before exposure to x-ray film for 48 h.

Chromosomal Localization of islet KIR Genes—The chromosomal localization of islet KIR genes was determined by PCR amplification of DNA from a panel of rodent/human somatic cell hybrids, each of which contained one of the 24 different human chromosomes (22). Primers and PCR conditions were identical to those used in tissue distribution studies. Sublocalization within chromosome 11 was achieved by typing a panel of well-characterized human chromosome 11-Chinese hamster ovary cell hybrids containing only defined portions of human chromosome 11. A description of the chromosomal breakpoints is described in detail in Refs. 23–25.

Oocyte Expression of KIR Channels—Capped RNAs were transcribed in vitro from linearized cDNAs using T3 RNA polymerase (Promega, Madison, WI). Stage V–VI Xenopus oocytes were isolated by partial dorsal aortotomy under tricaine anesthesia and then defolliculated by treatment with 1 mg/ml collagenase (Sigma Type 1A, Sigma) in zero Ca2+ ND96 for 1 h. Two to 4 h after defolliculation, oocytes were pressure-injected with 5–100 nl of 1–100 ng/μl cRNA. Oocytes were kept in ND96 + 18 mM Ca2+ (below), supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml) for 1–7 days prior to experimentation.

Electrophysiology—Oocytes were voltage-clamped using a commercial voltage-clamp amplifier (Warner Instruments, Inc.) in a small chamber (volume 200 μl) mounted on the stage of a SMZ-1 microscope (Nikon Instruments). The standard extracellular solution (KD98) contained (in mM): KCl, 98; MgCl2, 1; HEPES, 5; pH 7.5. Additional solutions are described in the text. Electrodes were filled with 3 × KCl and had tip resistances of 1–5 MΩ. Experiments were performed at room temperature. PClamp software and an Axon Instruments model TL125 D/A converter were used to generate voltage pulses. Data were normally filtered at 1 kHz, signals were digitized at 22 kHz (Neurodyne, Neurodata, New York) and stored on video tape. Experiments could then be replayed onto a chart recorder or digitized into a microcomputer using AxoPatch software (Axon Instruments). Alternatively, signals were digitized onto-line using Pclamp and stored on disk for off-line analysis.

RESULTS AND DISCUSSION

Cloning of Human Islet KIR mRNA Using Degenerate PCR—RT-PCR amplification of human islet and insulinoma RNA was carried out with degenerate primers corresponding to two conserved regions of KIR genes. PCR products corresponding to the expected 186-bp size were subcloned, and a total of 25 subclones were sequenced. The sequences were compared with nucleic acid and protein data bases, and 13 were found to be related to previously described KIR sequences. These could be grouped into three different islet KIR-related sequences (Fig. 1). The first sequence, labeled hi-CIR.pcr in Fig. 1, was derived from human β-cell tumor RNA. It was identical to a KIR described by different groups as the cardiac inward rectifying channel (CIR) (26) or cardiac ATP-sensitive potassium channel (rat cardiac KATP-1) (15). Another islet KIR cDNA (hi-GIRK1.pcr) was represented by two clones derived from human islet RNA that had 97% nucleic acid identity with the rat
G-protein-activated inward rectifying potassium channel cDNA (GIRK1) (12). The translated open reading frame was identical to GIRK1 (12, 26). While initial reports showed that GIRK1 is expressed in rat heart, muscle, and brain (12), we now provide evidence for the expression of this mRNA in human islets. This is consistent with a recent publication that described cloning of a GIRK1 cDNA from a rat insulinoma cell line and subsequent mapping of the gene to chromosome 2q24 (13). Finally, a third islet KIR cDNA fragment, hi-GIRK2.pcr, was also generated from non-tumoral human islets and had high identity (87%) with a mouse G-protein-activated brain inward rectifier mRNA which has been designated GIRK2 (27). A total of three distinct, though closely related, KIR sequences were thus identified in human pancreatic islet and insulinoma RNA.

Isolation and Characterization of hi-GIRK2—The partial hi-GIRK2 sequence derived from degenerate PCR was radiolabeled and used as a hybridization probe to screen for full-length cDNAs in pancreatic islet-cell libraries. Three clones were isolated from a human β-cell tumor library, which contained a novel 2.1-kb cDNA insert (hi-GIRK2). hi-GIRK2 was predicted to encode a 423-amino acid polypeptide, with a calculated Mr of 48,455. Two of the GIRK2 clones, however, contained a single adenosine nucleotide insertion at amino acid position 401 (nu-
edot) which caused an open reading frame shift in the COOH terminus. This is presumed to have resulted from a cloning artifact given that human genomic and reverse-transcribed islet cDNA lacks this single nucleotide insertion (not shown). In addition, hi-GIRK2 cDNA contained 212 bp of S'-untranslated region, and a 481-bp 3'-untranslated portion. A canonical polyadenylation site was present 23 bp upstream of the terminal poly(A) stretch. Overall, the nucleic acid sequence was 92% identical to that of mouse brain GIRK2. The translated product of hi-GIRK2 displayed 95% amino acid identity with the mouse brain GIRK2 (27), with most divergent residues present in the COOH and NH2 termini (Fig. 2). mb-GIRK2 was recently cloned from mouse brain, co-expressed in Xenopus oocytes along with opioid receptors and shown to express a G-protein-activated K+ channel (27). Prior to submission of this manuscript, a hamster insulinoma dne homologous to mouse GIRK2 was reported, and designated KATP-2 based on its similarity to CIR/KATP-1 (17), and a partial identical human cDNA sequence referred to as BIR1 was deduced from genomic DNA (28). Like other KIR sequences, hi-GIRK2 had a primary structure compatible with a model that includes two hydrophobic membrane spanning domains, M1 and M2, which encompass a putative pore region, and a long COOH-terminal tail believed to be predominantly cytoplasmic (Fig. 2) (10, 26).

There are six potential protein kinase C phosphorylation sites (positions 49, 73, 212, 236, 236, 366, and 386), all of which are conserved between human and mouse. There are no putative N-glycosylation sites in regions commonly regarded as extracellular in KIR proteins. Interestingly, at residue 256 there is an N-glycosylation motif near several short stretches of hydrophobic segments of unknown topology, which is conserved among both CIR and GIRK2 in humans and rodents (Fig. 2).

No consensus ATP-binding site was encountered. Other than mouse brain GIRK2, hi-GIRK2 resembled CIR more than any other KIR channel gene in nonredundant nucleic acid and protein data bases, with approximately 69% overall amino acid identity (Fig. 2). The homology between hi-GIRK2 and CIR was most pronounced in the central 360-amino acid segment, while a lower degree of conservation was apparent in the COOH- and NH2-terminal portions. Amino acid identity of hi-GIRK2 with GIRK1 was 57%.

The expression of hi-GIRK2 in human tissues was evaluated by Northern blot and RT-PCR analysis. A distinct band of approximately 5.7 kb, and a more diffuse signal of approximately 2.4–2.8 kb, were observed by Northern analysis in poly(A)+-enriched RNA from human insulinoma (Fig. 3), while the abundance in purified human islets was insufficient to detect a similar signal. To further define the tissue distribution of hi-GIRK2 mRNA, RT-PCR analysis of a serial dilution of cDNAs was performed under reduced cycling conditions that allowed relative semiquantitation among tissues (Fig. 3). Using primers specific for hi-GIRK2, a unique PCR product of the expected size was observed to be most abundant in insulinoma and cerebellum RNA, while lower levels of expression were detected in all other tissues examined, including human islet and pancreatic exocrine tissue (Fig. 3). The fact that RT-PCR product signals were only slightly enhanced in islets relative to exocrine samples could reflect significant cross-contamination of the exocrine and islet preparations and/or the existence of GIRK2 mRNA at lower levels in exocrine tissue.

Segregation analysis of a panel of human-Chinese hamster ovary/mouse somatic cell hybrids with specific oligonucleotides that amplified a 130-bp fragment from the 3'-untranslated region of the hi-GIRK2 gene allowed unequivocal localization of this gene to chromosome 21 (data not shown). This confirms data reported by others during revision of this manuscript, which indicated that this gene maps to chromosome 21q22 (28).

Isolation and Characterization of ri-CIR and Chromosomal Localization of hi-CIR—Cloned hi-CIR.pcr, the RT-PCR product originating from a human β-cell tumor which resembled the rat cardiac cDNA known as CIR or KATP-1, was used to screen islet cDNA libraries. This led to the isolation of two copies of a 3736-bp cDNA homologous to rat atrial CIR from an oligo(dT)-primed rat islet library. CIR was first reported as the cardiac ATP-sensitive channel and was hence initially designated KATP-1 (15). The results of experiments presented below, as well as those recently reported by others (26), failed to show that expression of this cDNA in oocytes results in functional ATP-sensitive currents, and we have accordingly designated our islet cDNA variant ri-CIR, rather than KATP-1 (26). The islet cDNA contained a 1260-bp open reading frame encoding a 47.8-kDa protein that was identical with the rat atrial CIR amino acid sequence (Fig. 2) (26), except for three silent single base changes, along with significant variation in the untranslated segments. Of note, both our sequence and the atrial CIR differ from that reported for KATP-1 in two conserved residues.
The 5'-untranslated region contained a 133-bp segment 100% identical to that reported for CIR, plus a novel intervening sequence of 387 bp inserted two bases 5' of the putative initiation methionine codon. The surrounding translation initiator sequence was thus CCATCTATGG, as opposed to ACGACTATGG reported in the cardiac forms. The possible implications of this finding, if any, on the efficiency of translation of the protein are presently unknown. This additional 5'-untranslated segment had several AUG triplets, none of which were followed by a long open reading frame. Considerable sequence divergence with CIR was also observed throughout the 3'-untranslated region, including the presence of 16 single bp substitutions, 4 insertions of 1–10 bp, and an additional 3' extension of 219 bp consistent with an alternative polyadenylation site in the islet cDNA (data not shown).

When these studies were initiated, only the rat CIR nucleic acid sequence was known. To identify the human CIR sequence, an oligonucleotide was synthesized based on the human b-cell tumor PCR product (hi-CIR.pcr) sequence and rapid amplification of cDNA was performed to obtain further human sequence from human islet mRNA (data not shown). Partial sequence analysis, which eventually proved to be identical to that entered in public nucleic acid data bases with the designation of human cardiac KATP-1, allowed us to design oligonucleotide primers for chromosomal localization studies. The 116-bp PCR product that was thus generated was shown to segregate with chromosome 11 in human-rodent somatic cell hybrids. A panel of chromosome 11 deletion hybrids was utilized for further physical mapping of the human CIR gene to chromosome 11q23-ter (Fig. 4). Given the role of islet cell K⁺ channels in the regulation insulin secretion, CIR may be regarded as a candidate gene for any form of diabetes mapping to this chromosomal region. Interestingly, a 45,X male with a translocation (Y;11)(q11.2;q24) has been reported, and the phenotype includes hypoglycemia along with multiple dysmorphic features (29).

Northern blot analysis, using a radiolabeled 0.7-kb human islet CIR PCR fragment as a probe, disclosed the presence of three major transcripts of 6.8, 5.4, and 2.4 kb, which appeared to migrate more slowly than the corresponding mRNAs. mRNA levels in several human tissues were assessed by reverse transcription-PCR analysis. Total RNA from human tissues was treated with RNase-free DNase, reverse-transcribed, and cDNA corresponding to 80, 20, 5, and 1.25 ng of RNA was amplified for 25 or 28 cycles using primers specific for human islet GIRK2 and CIR, respectively. INS, insulinoma; HI, pancreatic islets; EXO, exocrine; LIV, liver; CER, cerebellum; MUS, muscle; VEN, left ventricle; and DUO, duodenum.
to be abundantly expressed in both islet and exocrine pancreas RNA, but very weakly expressed in insulinoma (Fig. 3). Hybridization of a multiple human tissue Northern blot (Clontech, Palo Alto, CA) with the same probe disclosed the same pattern of transcript sizes in total pancreas and, with lower intensity, in heart. No clear signal was detected in the remaining tissues (data not shown). Using RT-PCR analysis, a unique band of the expected size was seen in all human tissues examined, although human pancreatic islet and pancreatic exocrine samples showed the highest intensity (Fig. 3). Interestingly, the relative intensity of hybridization and RT-PCR signals was not indicative of specific expression in either endocrine or exocrine pancreatic cells, suggesting that CIR mRNA is present in both fractions.

The close sequence homology shared by CIR and GIRK2 (Fig. 2) was employed to assess their relative abundance in pancreatic islet cell subpopulations using competitive RT-PCR. To circumvent the fact that minor differences in the efficiency of PCR amplification of different sets of primers can greatly affect the rate of accumulation of two different PCR products, one pair of PCR primers expected to amplify both cDNAs was used. As predicted from the sequences of GIRK2 and CIR mRNAs, RT-PCR from total RNA derived from fluorescence-activated sorted purified pancreatic islet β-cells and non-β islet cells yielded a single 213-bp band after 28 cycles (Fig. 5). After Sau3AI digestion, which was expected to cleave CIR but not GIRK2, a major component of the amplified product appeared to be cleaved, while a 213-bp band of lower intensity remained (Fig. 5). Conversely, after selective cleavage of GIRK2 sequence with HindIII, a predominant component of the 213-bp PCR product was left intact. Cleavage with both enzymes resulted in complete digestion of the 213-bp band. An analogous restriction enzyme pattern was observed using an independent pair of primers and sequence-specific restriction enzyme digestion (data not shown). The restriction pattern in all cases was consistent with that expected if the major products of amplification were only CIR and GIRK2, rather than additional closely related known KIR genes such as GIRK1 and GIRK3 (12, 27). The relative accumulation of GIRK2 and CIR PCR products was independent of cycle number between 25 and 35 cycles, suggesting that both templates were amplified with a similar efficiency. Thus, both CIR and GIRK2 mRNA were expressed in β-cell and non-β-cell enriched preparations, while in the the two primary islet cell preparations the relative abundance of CIR mRNA clearly exceeded that of GIRK2. This finding is in apparent contrast to the report of Ashford et al. (15), that CIR (KATP-1) did not hybridize to poly(A)⁺ RNA from a rat insulinoma cell line. However, our Northern blot data also suggest that GIRK2 is more abundant than CIR in a human insulinoma specimen, but not in native islets. One likely explanation for this observation is that tumor β-cell lines often either do not express the characteristic markers of the native β-cell phenotype or do so at abnormal levels (30).

Expression of hi-GIRK2 and ri-CIR in Xenopus Oocytes—When we expressed hi-GIRK2 or ri-CIR in Xenopus oocytes, alone or in combination with one another, or with the cloned sulfonylurea receptor (18), no K⁺ currents were detectable above the levels observed in uninjected oocytes (not shown). Procedures that were expected to activate KATP channels in other cell types (10 μM diazoxide, 1 μM carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone, 10 mM de-oxyglucose), failed to activate KATP Channels. This remained true when oocytes were co-injected with sulfonylurea receptor cRNA along with KIR subunits (not shown). However, strong inwardly rectifying K⁺ currents could be activated by carbachol (CCh) in oocytes expressing M2 muscarinic receptors plus GIRK2 and CIR (Fig. 6). Oocytes injected with M2 receptor and only one of hi-GIRK2, or ri-CIR, expressed negligible CCh-activated currents (Fig. 6). These results suggest that CIR and GIRK2 are capable of co-assembling to form G-protein-activated heteromultimer pore-forming units. This finding is strongly reminiscent of the recent demonstration that CIR and GIRK1 are physically associated in rat atrial plasma membranes and together form part of native pertussis toxin-sensitive KIR channels (26). In the context of the superfamily of KIR genes, GIRK1, GIRK2, GIRK3, and CIR form a distinct group defined by a close degree of sequence homology, whereas GIRK2 and CIR are most closely related, with only approximately 30% overall divergence (Fig. 2) (12, 15, 26, 27). Diversity among G-protein-activated currents in different cell types may thus result from heteromultimeric association between different members of this subfamily of KIR proteins, in analogy to what occurs within the superfamily of voltage-dependent potassium channels, where a variety of functionally distinct phenotypes can result from the association of subunits derived from different members of the same gene subfamily, though not among subunits from different subfamilies (14).

Although CIR was initially reported as the cardiac ATP-sensitive K⁺ channel (KATP-1) (15), inhibition of ATP production failed to activate K⁺ currents in oocytes injected with CIR and/or GIRK2. This was also true when the sulfonylurea receptor (18), a candidate site for endowment of ATP sensitivity to islet KATP channels, was co-expressed. The inward rectification of the expressed receptor-activated K⁺ channels, which is likely to be determined by the primary structure of the pore forming subunit itself, is much stronger than that of native
**K<sub>ATP</sub>** channels (31) (Fig. 6). The studies presented here thus provide evidence that oocytes injected with GIRK2 do not express K<sub>ATP</sub> channels and confirms data recently presented by others that CIR expression in multiple cell systems does not result in K<sub>ATP</sub> channels (26). A role for these molecules in K<sub>ATP</sub> channel complexes in association with as yet unidentified subunits nevertheless remains possible.

The precise relationships between the molecular components of human islet K<sub>IR</sub> channels identified in the present study and native β-cell G-protein-activated K<sup>+</sup> currents remain to be defined. In addition to K<sub>ATP</sub> channels, which are known to be gated through G-proteins, small conductance channels that are activated through G-protein-coupled receptors and that are insensitive to ATP and sulfonoureas have been recorded (4). A role for G-protein-activated K<sub>IR</sub> channels in islet β-cell physiology can be anticipated given that multiple hormones and neurotransmitters cause hyperpolarization and inhibit insulin secretion via G-protein-coupled receptors and K<sup>+</sup> channels (2, 4, 16).

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