3-1996

A Recombinant Inwardly Rectifying Potassium Channel Coupled to GTP-Binding Proteins

Kim W. Chan

M. Noelle Langan

Jin Liang Sui

J. Ashot Kozak

Wright State University - Main Campus, juliusz.kozak@wright.edu

Amanda Pabon

See next page for additional authors

Follow this and additional works at: https://corescholar.libraries.wright.edu/ncbp

Part of the Medical Cell Biology Commons, Medical Neurobiology Commons, Medical Physiology Commons, Neurosciences Commons, and the Physiological Processes Commons

Repository Citation


This Article is brought to you for free and open access by the Neuroscience, Cell Biology & Physiology at CORE Scholar. It has been accepted for inclusion in Neuroscience, Cell Biology & Physiology Faculty Publications by an authorized administrator of CORE Scholar. For more information, please contact corescholar@www.libraries.wright.edu, library-corescholar@wright.edu.
A Recombinant Inwardly Rectifying Potassium Channel Coupled to GTP-binding Proteins

KIM W. CHAN, M. NOELLE Langan, JIN LIANG SUI, J. ASHOT Kozak, AMANDA PABON, JOHN A.A. LADIAS,* and DIOMEDES E. LOGOTHETIS

From the Department of Physiology and Biophysics, Mount Sinai School of Medicine, City University of New York, New York, New York 10029; and *Department of Medicine, New England Deaconess Hospital, Harvard Medical School, Boston, Massachusetts 02215

ABSTRACT GTP-binding (G) proteins have been shown to mediate activation of inwardly rectifying potassium (K⁺) channels in cardiac, neuronal and neuroendocrine cells. Here, we report functional expression of a recombinant inwardly rectifying channel which we call KGP (or hpKir3.4), to signify that it is K⁺ selective, G-protein-gated and isolated from human pancreas. KGP expression in Xenopus oocytes resulted in sizeable basal (or agonist-independent) currents while coexpression with a G-protein-linked receptor, yielded additional agonist-induced currents. Coexpression of KGP and hGIRK1 (a human brain homolog of GIRK1/Kir3.1) produced much larger basal currents than those observed with KGP or hGIRK1 alone, and upon coexpression with receptor, similarly large agonist-induced currents could be obtained. Pertussis toxin treatment significantly diminished agonist-dependent currents due to either KGP or KGP/hGIRK1 expression. Interestingly, PTX also significantly reduced basal KGP or KGP/hGIRK1 currents, suggesting that basal activity is largely the result of G-protein gating as well. When the two channels were coexpressed with receptor, the relative increase in current elicited by agonist was similar whether KGP and hGIRK1 were expressed alone or together. When in vitro translated or when expressed in Xenopus oocytes or CHO mammalian cells, KGP gave rise to a nonglycosylated 45-kD protein. Antibodies directed against either KGP or hGIRK1 coprecipitated both proteins coexpressed in oocytes, providing evidence for the heteromeric assembly of the two channels and suggesting that the current potentiation seen with coexpression of the two channel subunits is due to specific interactions between them. An endogenous oocyte protein similar in size to KGP was also coprecipitated with hGIRK1.

INTRODUCTION

Channel activation by second messengers confined to the plasma membrane was first described with the gating of the atrial $K_{\text{ACb}}$ channels by pertussis toxin-sensitive G proteins (Soejima and Noma, 1984; Pfaffinger et al., 1985; Breitwieser and Szabo, 1985). Activation of cardiac $K_{\text{ACb}}$ channels underlies the rapid control of the heart rate by the vagus nerve (reviewed by Logothetis, 1987a). Several studies have focused on the mechanism of $K_{\text{ACb}}$ gating and have identified direct actions of specific G-protein subunits (Logothetis et al., 1987b, 1988; Codina et al., 1987; Ito et al., 1992; Wickman et al., 1994).

Pertussis toxin-sensitive, neurotransmitter-activated inwardly rectifying K⁺ currents have also been reported in tissues such as brain (Andrade et al., 1986; Andrade and Nicoll, 1987; North et al., 1987; Inoue et al., 1988; Williams et al., 1988; Pennington et al., 1993), pancreas (Fosset et al., 1988; Dunne et al., 1989; Rorsman et al., 1991) and pituitary (Bauer et al., 1990; Einhorn and Oxford, 1993; Takano et al., 1994). In such tissues, activation of G-protein coupled K⁺ channels is thought to underlie suppression of firing (e.g., Andrade et al., 1986).

Recently, related recombinant channels, belonging to the inwardly rectifying K⁺ channel family (Kir), have been shown to couple to G-protein-linked receptors and G-protein subunits and have been grouped in the Kir3.0 subfamily (Chandy and Gutman, 1993; Doupnik et al., 1995). Specifically, rat cardiac GIRK1 or KGA recombinant channels (rcKir3.1: Kubo et al., 1993; Dascal et al., 1993), when expressed in Xenopus oocytes, produced G-protein-gated membrane currents showing...
with GIRK1-specific antibodies coprecipitated a 45-kD heteromultimer of the two recombinant proteins, GIRK1 and CIR. These results led Krapivinsky and colleagues to conclude that the native atrial channel subunits to produce a heteromeric channel which displays properties (such as strong inward rectification, G-protein gating and PTX-sensitivity of both agonist-dependent and -independent currents) characteristic of the KGP subunit.

**Materials and Methods**

**Cloning and Sequencing**

A 298-bp DNA probe corresponding to the M1-P2 region of GIRK1 (Kubo et al., 1993) was synthesized by PCR using GIRK1 as a template with the following primers: 5'CTTATCATCATCCTGACCTAGA (corresponds to the peptide BFIFILY in GIRK1) in the forward direction and 5'GAACCTGGTACGTGGGGTGTGT (corresponds to the peptide LIGCMFIK in the reverse direction). This probe was gel purified and labeled with α-32P-dCTP by using random hexamer and Klenow before screening (~1 × 10^6 plaques from a human fetal brain cDNA library (Clontech, Palo Alto, CA). The filters were washed twice with 2× SSC and 0.5% SDS at room temperature, followed by washing twice again with 1× SSC and 0.1% SDS at 37°C for 15 min. Five positive clones, including hGIRK1 and hKGB (a human brain homolog of KGP), were purified by secondary and tertiary screening. The inserts were subcloned into pGEM7zf (+) (Promega Corp., Madison, WI) and then sequenced by the dideoxy sequencing method (Sanger et al., 1977). Using Sequenase (United States Biochemical, Cleveland, OH). A DNA probe corresponding to the last 360 bp of hKGB (corresponding to 5367-1218 of the KGP full length nucleotide sequence) was synthesized by PCR and was used to screen a human pancreatic α-gt11 cDNA library (Clontech), under high stringency. Approximately two million plaques were screened. The prehybridization and hybridization buffers were the same as above, but the temperature was raised to 42°C. The filters were washed twice with 2× SSC and 0.5% SDS at room temperature and twice with 1× SSC and 0.1% SDS at 55°C for 20 min. 10 strong positive clones (out of the 51 primary positive clones) were purified and subcloned into pGEM7zf (+) before sequencing. The KGP clone (1.734 kb) was
completely sequenced in both directions by automated sequencing (Mount Sinai DNA Core Facility). The KGP cDNA was subcloned into the EcoRI site of pCDNA3 (Invitrogen Corp., San Diego, CA) and pGEM-HE (Liman, Tytgat, and Hess, 1992) for stable transfection into mammalian cells and injection into oocytes, respectively.

**Northern Blot Analysis**

(a) A multiple tissue Northern blot filter (Clontech) was probed with a 32P-labeled DNA probe corresponding to the last 360 bp (t857-c1218) of the hKGB clone (same probe used to screen the human pancreatic cDNA library). Prehybridization and hybridization were carried at 42°C in a buffer containing 5× SSPE, 10× Denhardt’s solution, 50% formamide, 2% SDS and 100 μg/ml denatured salmon sperm DNA. The filter was washed twice with 2× SSC and 0.05% SDS at room temperature and then washed twice with 0.1× SSC and 0.1% SDS at 50°C. (b) Transfected or control CHO cells were grown to 70% confluency and total RNA was extracted by the guanidinium method (Chomczynski and Sacchi, 1987). PolyA+ RNA was purified by using oligo-dT cellulose (Boehringer Mannheim Biochemicals, Indianapolis, IN). 3 μg polyA+ or 20 μg total RNA were loaded on a 1.2% formaldehyde agarose gel. RNA was blotted to a GeneScreen TM nylon membrane (New England Nuclear, Boston, MA) by capillary transfer (Sambrook et al., 1989). A DNA probe spanning the complete coding region of KGP cDNA was used and labeled by the random hexamer method. The condition for hybridization and washing were essentially the same as in part a, with the exception that the filter was washed with 0.1× SSC and 0.1% SDS solution for 1 h at 65°C.

**Stable Transfections**

CHO cells were split the day before transfection and were fed 4 h before transfection (Ausubel et al., 1993). 10–50 μg of DNA to be transfected was ethanol precipitated, washed twice with 70% ethanol and air dried. The pellet was resuspended in 500 μl of 250 mM CaCl2. 500 μl of 2× HeBS (HEPES-buffered saline) was placed in a sterile 15-ml conical tube. The DNA/CaCl2 solution was added dropwise while the 2× HeBS buffer was bubbled simultaneously into the tube. The formation of calcium phosphate precipitate was allowed to proceed for 20 min at room temperature. The calcium phosphate precipitate was then distributed simultaneously into the tube. The formation of calcium phosphate precipitate was then distributed evenly over the cells with gentle agitation. The cells were incubated under standard growth conditions for 4 h. Cells were washed twice with 5 ml 1× PBS, were fed, and allowed to grow for 3 d. The medium was switched to selective medium with G418 (800 μg/ml), and the cells were allowed to grow for 2–3 wk with change of new selective medium every 3 d. Colonies surviving after this period of time were considered stably transfected. Individual colonies were expanded to 80% confluency.

**Tissue Culture**

CHO-K1 cells were maintained in OPTI-MEM I supplemented with 1% BS. All cell lines transfected with KGP (CHO-K1/hp16 clones 1–6) were also maintained in Gentamicin (G418) at 800 μg/ml. Untransfected cells were killed at that concentration of G418.

**In vitro Transcriptions and Translations**

KGP and hGIRK1 in pGEM-HE was linearized with NheI and transcribed with SP6 polymerase. Transcriptions were performed using the Ambion megascript kit (Ambion, Austin, TX), as previously described (Logothetis et al., 1992). Cell-free translations were carried out in a nucleoside-treated lyase translation system (Promega Corp.) KGP cRNA was added at 100 ng per translation reaction. 35S-methionine was used in the reaction mixture in order to label the synthesized protein. In vitro translation products were analyzed by SDS-PAGE on 12% gels and detected by fluorography.

**Immunoprecipitation and Immunoblot Analyses**

(a) Immunoprecipitations. In vitro translated products were immunoprecipitated using the appropriate antibody (see text; Harlow and Lane, 1988). The samples were resuspended in 2% Triton X-100, 50 mM Tris-HCl, pH 7.4, SDS-deficient RIPA buffer for 2 h at 4°C. Antibody was added for an overnight incubation at 4°C. Protein A beads 50% slurry (Pierce Chemical Co., Rockford, IL) were added next. After PBS washes, the samples were run on a 12% acrylamide gel and were detected by fluorography. In vivo (i.e., in *Xenopus* oocytes) expressed channel proteins were immunoprecipitated using the following steps. Oocytes were injected with ~4 ng of channel cRNA (KGP, hGIRK1, or KGP/hGIRK1). After injection oocytes were incubated in ND96 solution supplemented with 1 μCi of 35S-methionine and 35S-cysteine (Dupont, Boston, MA). 3 d later, crude membrane preparations were resuspended in 2% Triton X-100, 50 mM Tris-HCl, pH 7.4. SDS-deficient RIPA Buffer and incubated for 2 h at 4°C. Antibodies were added for an overnight incubation at 4°C. Protein A beads 50% slurry (Pierce Chemical Co.) were added and incubated for 1 h. The beads were washed with PBS. 2× sample buffer was added to the pellets and the proteins were analyzed on 10% acrylamide gel and detected by fluorography.

(b) Immunoblots. Cells were scraped and washed in PBS and collected in ice cold homogenization buffer (1 mM EDTA, 1 mM EGTA, 5 mM Tris, pH 8.0) containing protease inhibitors. They were then homogenized in a Dounce homogenizer and centrifuged at 1,000 g for 5 min. The supernatant was then centrifuged at 17,000 rpm for 1 h. The pellet was resuspended in the buffer and saved at ~80°F. Protein concentration was determined by the Lowry method (Bio-Rad Laboratories, Grand Island, NY). The homogenate was solubilized in SDS electrophoresis sample buffer and analyzed using 12.5% polyacrylamide SDS gel. Proteins were transferred to nitrocellulose membrane (Midland Scientific Inc., Omaha, NE) using the semidry transfer method. The membrane was blocked with 5% dry milk, 0.1% Tween-20 in TBS and incubated with the UB-Ab antibody (Upstate Biotechnology Incorporated, Lake Placid, NY). The blot was subsequently incubated with an anti-rabbit IgG conjugated to Biotin and Streptavidin-HRP conjugate (Amersham Corp., Arlington, IL).

**Oocyte Isolation and Injection**

Oocytes were isolated and microinjected as previously described (Logothetis et al., 1992, 1993).

**Electrophysiological Studies**

Two-electrode voltage clamp on *Xenopus* oocytes was performed as previously described (Stühmer, 1992; Logothetis et al., 1992), using a Gene Clamp 500 amplifier (Axon Instruments, Foster City, IL).
RESULTS

Cloning of Two Human Inwardly Rectifying Potassium Channels

A PCR-generated cDNA probe, spanning the M1 through M2 regions (298 bp long) of GIRK1 (Kubo et al., 1993), was used to screen a human fetal brain cDNA library (Clontech). Under low stringency conditions, two positive clones (one strongly, hGIRK1, and one weakly hybridizing, hKGB, where h, human; B, brain) were identified and isolated. Sequencing of hGIRK1 revealed only six amino acid differences from the corresponding rat clone. These were (human, position, rat): D32G, T64N, I295V, T396S, A481P and A482T (Accession No. U39196). Sequencing of hKGB revealed a partial length cDNA clone of 1.173 kb which showed 69% identity to the rat GIRK1 nucleotide sequence (data not shown). A DNA probe corresponding to the last 360 bp of the 3' end of hKGB (h857-c1218) was generated by PCR and was used under high stringency conditions in Northern blot analysis of poly A+ RNA from eight different human tissues (Clontech, Fig. 1 A). The tissue distribution in order of relative abundance of hKGB cRNA was: pancreas, kidney, heart, placenta, lung, and brain. Under these conditions, hKGB cRNA was not detected in skeletal muscle and liver. Due to the relatively high abundance of the hKGB cRNA in pancreas, a full-length clone was obtained by screening a human pancreatic cDNA library with the same DNA probe used in the Northern blot. Under high stringency conditions, 51 strongly hybridizing clones were detected. Ten cDNA clones were analyzed and a 1.734 kb clone (hP16) was sequenced completely. 1.257 kb of this clone comprised an open reading frame (KGP; Fig. 1 B; accession No. U39195) with the position of the initiating methionine being suggested by the existence of three in frame stop codons in the 5' noncoding sequence. Nucleotide comparison of KGP with hKGB showed 99.6% identity and revealed that the brain partial length clone was missing 33 and 51 bp from the coding 5' and 3' ends, respectively. Comparison of KGP with recently reported recombinant and functionally characterized rat cardiac channels rcKir3.4 (rcKATP; Ashford et al., 1995; CIR: Krapivinsky et al., 1995) revealed as many as 26 amino acid differences (circled amino acids in Fig. 1 B). KGP, however, was virtually identical to a clone referred to as human cardiac KATP (hcKATP; Ashford et al., 1995; G388 is R in hcKATP), which has not yet been functionally characterized. A Walker type-B motif (Walker et al., 1982) was identified in both the pancreatic and cardiac sequences (K/RXXXGXXXL: where X denotes any amino acid and L any hydrophobic amino acid) at position 160, which represents a single putative nucleotide-binding site (Fig. 1 B, boxed amino acids). Additionally, six potential protein kinase C phosphorylation sites (T37, S/T57, T70, T158, S209, and S233) and six potential casein kinase II phosphorylation sites (T18, T80, S209, T263, S231, T350), common to both KGP and the cardiac Kir3.4 channels, were identified.

Heterologously Expressed KGP Associates with hGIRK1

KGP was stably transfected in CHO cells. PolyA+ as well as total RNA were isolated from one such clonal cell line (CHO No. 6), as well as from untransfected cells, and were probed with the 1.257-kb coding sequence of KGP. A signal corresponding to KGP was obtained only with the RNA from the stably transfected cell line (Fig. 2 A, Ln1: polyA+, Ln3: total RNA). An antibody raised against the rat AH1 terminal 21 amino acids of rcKir3.4 (rcKATP; Upstate Biotechnology; UB-Ab), which shares a contiguous identity with KGP in 16 residues, was used in immunoblot and immunoprecipitation experiments. In Western immunoblots, a 45-kD band was identified, using UB-Ab in membranes from stably transfected CHO cells (CHO No. 6; Fig. 2 B, Ln: 2), but was absent in untransfected CHO control cells (Fig. 2 B, Ln: 1). Fluorographic detection of the in vitro translated KGP protein is shown in Fig. 2 C. The 45-kD protein corresponding to KGP showed no evidence for glycosylation.
FIGURE 1. Tissue distribution of KGP cRNA and its nucleotide and deduced amino acid sequences. (A) Northern blot analysis of multiple human tissue polyA⁺ RNA (2 µg/lane) showed greatest abundance in pancreas. At least three strongly hybridizing bands (~2.19, 4.17 and 5.75 kb) could be distinguished in the various tissues. (B) The nucleotide sequence of KGP is shown (nucleotide count on the left) with the deduced amino acid sequence (amino acid count on the right) indicated on top at the middle of each codon. Bars mark putative transmembrane domains representing the M1 (92-113), P (139-155) and M2 (164-185) regions, respectively. 5’ and 3’ untranslated sequences are also shown. Stop codons are marked by asterisks. Circled amino acids denote 26 differences between KGP and rcK₄Tm (25 differences with CIR; V188 is the same in both sequences, in contrast with rcK₄ATm). A putative nucleotide-binding site (Walker type B motif) is boxed.
Figure 2. Detection of KGP cRNA and protein in cells and cell-free systems. (A) Northern blot analysis of total and poly A+ RNA extracted from CHO cells either stably transfected with KGP (clone No. 6) or from untransfected controls. Hybridization signals were obtained with either poly A+ or total RNA from transfected cells (lanes 1 and 3) but not from untransfected controls (lanes 2 and 4). 3 μg of polyA+ RNA was loaded in each of lanes 1 and 2, while 20 μg of total RNA was loaded in each of lanes 3 and 4. (B) Western immunoblots of membrane proteins from stably transfected CHO cells identify a 45-kD band corresponding to KGP. Membrane proteins from control and transfected cells were solubilized and electrophoresed, transferred to nitrocellulose, treated with UB-Ab and visualized by chemiluminescence (see experimental procedures). In this blot, a specific 45-kD band was detected in KGP transfected CHO cells (lane 2) but not in the corresponding controls (lane 1). (C) Fluorographic detection (35S, methionine). of in vitro translated KGP in the absence (lanes 1, 3, 5) or presence (lanes 2, 4, 6) of canine microsomes. The in vitro translated KGP product gave a 45-kD band, which in the presence of canine microsomes showed no evidence for glycosylation (lanes 3-6). In contrast, under similar conditions, the S. cerevisiae α-factor (18-kD band, lane 1) was efficiently glycosylated in the presence of canine microsomes (lane 2). The in vitro translated products (as shown in lanes 3 and 4) were immunoprecipitated with UB-Ab (see text and experimental procedures for details) and detected by fluorography (lanes 5 and 6). The 45-kD band was the major immunoprecipitated protein. (D) Coimmunoprecipitations of heteromeric subunits from Xenopus oocyte membranes. UB-Ab was used in lanes 1-3 in immunoprecipitation experiments using crude membranes from uninjected oocytes (lane 1), oocytes injected with KGP cRNA (lane 2), and oocytes coinjected with KGP and hGIRK1 (lane 3). GIRK1-Ab was used in lanes 4-6 in immunoprecipitation experiments using crude membranes from uninjected oocytes (lane 4), oocytes injected with hGIRK1 cRNA (lane 5), and oocytes coinjected with hGIRK1 and KGP (lane 6). The bands at 45 kD indicate immunoprecipitated KGP protein while the doublet at 56-58 kD of canine microsomes. The in vitro translated KGP product gave a 45-kD band, which in the presence of canine microsomes showed no evidence for glycosylation (lanes 3-6). In contrast, under similar conditions, the S. cerevisiae α-factor (18-kD band, lane 1) was efficiently glycosylated in the presence of canine microsomes (lane 2). The in vitro translated products (as shown in lanes 3 and 4) were immunoprecipitated with UB-Ab (see text and experimental procedures for details) and detected by fluorography (lanes 5 and 6). The 45-kD band was the major immunoprecipitated protein. (D) Coimmunoprecipitations of heteromeric subunits from Xenopus oocyte membranes. UB-Ab was used in lanes 1-3 in immunoprecipitation experiments using crude membranes from uninjected oocytes (lane 1), oocytes injected with KGP cRNA (lane 2), and oocytes coinjected with KGP and hGIRK1 (lane 3). GIRK1-Ab was used in lanes 4-6 in immunoprecipitation experiments using crude membranes from uninjected oocytes (lane 4), oocytes injected with hGIRK1 cRNA (lane 5), and oocytes coinjected with hGIRK1 and KGP (lane 6). The bands at 45 kD indicate immunoprecipitated KGP protein while the doublet at 56-58 kD indicates immunoprecipitated hGIRK1 protein.

KGP and hGIRK1 were inhibited (data not shown). Similarly, GIRK1-Ab precipitated a ~56-58-kD doublet from oocytes injected with hGIRK1 cRNA (Fig. 2 D, Ln: 5) but not from uninjected oocytes (Fig. 2 D, Ln: 4). In oocytes coinjected with KGP and hGIRK1 cRNAs, GIRK1-Ab coprecipitated along with the ~56-58-kD bands a 45-kD band (Fig. 2 D, Ln: 6). These results strongly suggest that in oocytes KGP and hGIRK1 associate in a heteromeric complex. Interestingly, in oocytes injected with hGIRK1 alone, GIRK1-Ab coprecipitated along with hGIRK1 an endogenous protein of similar molecular weight as KGP, suggesting that Xenopus oocytes express an endogenous protein capable of associating with hGIRK1.

KGP Gives Rise to a Ba2+-blocked, Inwardly Rectifying Current

Xenopus oocytes were injected with in vitro transcribed KGP cRNA and membrane currents were recorded two or more days later using two-electrode voltage clamp. In most experiments, oocytes were initially perfused with low potassium (LK) solution ND96 containing 2 mM KCl. Voltage steps to ~80 mV were given from a holding level of 0 mV. The magnitudes of hyperpolar-


<table>
<thead>
<tr>
<th>Oocytes</th>
<th>LK</th>
<th>HK</th>
<th>HK-PTX</th>
<th>ACh</th>
<th>ACh-PTX</th>
<th>Ba²⁺</th>
<th>Ba²⁺-PTX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninjected</td>
<td>0.13 ± 0.02</td>
<td>0.31 ± 0.03</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.03 ± 0.02</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(9)</td>
<td>(9)</td>
<td></td>
<td></td>
<td></td>
<td>(9)</td>
<td></td>
</tr>
<tr>
<td>hm2</td>
<td>0.06 ± 0.02</td>
<td>0.38 ± 0.14</td>
<td>0.31 ± 0.02</td>
<td>0.03 ± 0.02</td>
<td>0.00 ± 0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(12)</td>
<td>(12)</td>
<td>(12)</td>
<td>(12)</td>
<td>(5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KGP</td>
<td>0.18 ± 0.03</td>
<td>1.60 ± 0.45</td>
<td>0.98 ± 0.14</td>
<td>0.04 ± 0.05</td>
<td>1.02 ± 0.29</td>
<td>0.30 ± 0.07</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(20)</td>
<td>(20)</td>
<td>(6)</td>
<td>(13)</td>
<td>(20)</td>
<td>(6)</td>
<td></td>
</tr>
<tr>
<td>KGP+hm2</td>
<td>0.25 ± 0.03</td>
<td>2.37 ± 1.00</td>
<td>1.04 ± 0.02</td>
<td>1.67 ± 0.22</td>
<td>0.44 ± 0.07</td>
<td>2.88 ± 0.51</td>
<td>0.82 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>(23)</td>
<td>(23)</td>
<td>(7)</td>
<td>(23)</td>
<td>(7)</td>
<td>(7)</td>
<td>(3)</td>
</tr>
</tbody>
</table>

Values: Mean ± SEM of magnitude of currents in microamperes. LK: Hyperpolarization-activated currents in low potassium solution. HK: Hyperpolarization-activated currents in high potassium solution. HK-PTX: Same as (HK) but oocytes incubated in 1 μg/ml pertussis toxin immediately following cRNA injections. ACh: ACh-induced currents (currents in HK containing ACh). ACh-PTX: Same as (ACh) but oocytes incubated in 1 μg/ml pertussis toxin. Ba²⁺: Currents blocked by Ba²⁺ (in HK solution). Ba²⁺-PTX: Same as in (Ba²⁺). In both sets of experiments Ba²⁺ was applied in presence of ACh. (): Number of experiments performed.

Receptor-mediated Activation of KGP Currents via PTX-sensitive G Proteins

Oocytes injected with KGP channel cRNA alone did not show significant ACh-induced currents. 200 μM Ba²⁺, as shown previously in Fig. 3 D, blocked part of the current seen in the presence of HK (Fig. 4 A). Oocytes injected with hm2 receptor cRNA alone showed no significant ACh-induced or Ba²⁺-blocked currents (Table I). In contrast, oocytes co-injected with hm2 and KGP cRNAs exhibited significantly greater ACh-induced currents (Fig. 4 B). After ACh application, 200 μM Ba²⁺ (in the continued presence of ACh), not only abolished the ACh-induced current but also blocked part of the agonist-independent current, as was shown earlier in Figs. 3 D and 4 A (Fig. 4 B). Oocytes from the same batch as those in Fig. 4, A and B, were treated in the same manner but were also incubated in pertussis toxin (PTX), immediately after injection. Fig. 4 C shows the effect of a 2-d PTX treatment on the currents of an oocyte injected with KGP cRNA alone. Small basal and Ba²⁺-sensitive currents were obtained. Similarly, small agonist-dependent currents were obtained from oocytes co-injected with KGP and hm2 cRNAs (Fig. 4 D). The results from several experiments similar to those shown in Fig. 4, A–D, have been summarized and compared in bar graph form in Fig. 4, E and F. Fig. 4 E plots the percent effect of pertussis toxin (PTX) treatment (crossed bars) on currents from oocytes injected with KGP cRNA alone, as compared with the same number of PTX-untreated controls (open bars, 100%). PTX treatment caused a significant reduction in HK currents as well as in the magnitude of the Ba²⁺-blocked currents. In oocytes co-injected with KGP and hm2 cRNAs (Fig. 4 F), PTX treatment significantly diminished currents in HK (in the absence of ACh), a result consistent with that obtained with KGP expression alone (Fig. 4 C). Similarly, PTX significantly diminished the magnitudes of ACh-induced and Ba²⁺-blocked currents (Fig. 4 D). Oocytes injected with the voltage-gated channel Kv1.1 showed currents which were not significantly different in the presence or absence of PTX, arguing against nonspecific PTX effects (n = 6; data not shown). These results strongly suggest that KGP channels can couple to PTX-sensitive G proteins not only to elicit agonist-dependent but also basal activity.

KGP Gives Rise to Strong Inwardly Rectifying K⁺ Currents

Families of currents elicited at voltage steps ranging from −100 to +60 mV, from a holding potential of
0 mV, were obtained from oocytes coinjected with KGP and hm2 receptor. Both the ACh-induced and Ba2+-blocked (in the presence of ACh) currents exhibited strong inwardly rectifying properties (Fig. 5, A–C). ACh-induced (Fig. 5, A and C, circles) and Ba2+-blocked currents (Fig. 5, B and C, triangles) showed equivalent strong inward rectification, similar to that seen with Ba2+-blocked currents of KGP alone, as previously shown in Fig. 3E (mean ± SEM, n = 13). The reversal potentials of the ACh-induced currents showed a 53 mV change per 10-fold change in external K+ concentration, similar to that expected of a potassium-selective channel (Fig. 5D).

Single-Channel Characteristics of KGP

Single-channel activity of KGP could be detected in either oocytes (Fig. 6, A, C, D) or CHO cells expressing the KGP channel (Fig. 6, B and E). Oocytes injected with KGP cDNA displayed characteristic channel activity (55 out of 103 patches or 53%, data not shown) which was absent from uninjected controls (n = 35). Similarly, oocytes coinjected with KGP and hm2 often displayed similar characteristic channel activity in cell-attached recordings (27 out of 33 patches or 82%; 5 μM acetylcholine present in the pipette; Fig. 6A). Upon patch excision channel activity was abolished. Coapplication of 2 mM MgATP and 50 μM GTPγS was capable of stimulating KGP activity in patches from oocytes expressing KGP (2 out of 17 patches or 12%) but not in uninjected oocytes (n > 35). Stably transfected CHO cells (with KGP cDNA alone) showed rare and low basal KGP channel activity (7 out of 35 patches or 20%), presumably due to either lower expression levels or less optimal environment than in oocytes. Control, untransfected CHO cells showed no KGP activity.
channel activity \((n = 68)\). Although cell-attached recordings from transfected CHO cells exhibited low channel activity (data not shown), inside-out patches sometimes responded to bath co-applications of 2 mM MgATP and 50 \(\mu\)M GTP\(\gamma\)S, in a manner similar to that reported for CIR (Krapivinsky et al., 1995; 2 out of 10 patches or 20%; Fig. 6 B).

The brief, poorly resolved channel openings of KGP made an accurate determination of single-channel conductance and open-time kinetics difficult. As shown in the amplitude histogram of Fig. 6 C (at -100 mV), single-channel currents were in the range of 1.5-3 pA, suggesting a value in conductance between 15-30 pS. The average open time at -80 mV was \(\tau_o = 0.4 \pm 0.1\) ms (mean \(\pm\) SEM; \(n = 7\); sampled at 10 KHz). The strong inwardly rectifying properties of KGP were demonstrated with voltage ramps from -100 to +100 mV in either injected oocytes or transfected CHO cells (Fig. 6, D and E, respectively).

**Lack of Functional Expression of GIRK1 in Stably Transfected CHO Cells**

CHO control untransfected as well as cells stably cotransfected with rat GIRK1 and hm2 receptor cDNAs were tested electrophysiologically. Cell-attached recordings with 5 \(\mu\)M ACh in the pipette, showed no novel single-channel activity in the transfected \((n = 42)\) as compared to the untransfected controls \((n > 100;\) data not shown).

**Coexpression of KGP and hGIRK1 Yields Large Inwardly Rectifying Currents**

It was recently shown that coexpression of the rat cardiac CIR and GIRK1 channels resulted in severalfold larger currents than those obtained from expression of either recombinant channel alone (Krapivinsky et al., 1995). We tested coexpression of our homologous recombinant channels, KGP and hGIRK1, with hm2 re-
The receptor-activated KGP currents are strongly inward rectifying and potassium selective. Two electrode voltage-clamp recordings from *Xenopus* oocytes coinjected with KGP (4 ng) and hm2 receptor (~1 ng). (A) Currents elicited by ACh (total current in the presence of ACh minus HK-induced current) in response to a family of voltage steps ranging from -100 to +60 mV, from a holding potential of 0 mV, every 2 s. (B) Currents blocked by Ba2+ in response to the same voltage protocol as in A. (C) Current-voltage relationships of ACh-sensitive (circles, as in A; mean ± SEM; n = 13) and Ba2+-blocked currents (triangles; as in B; mean ± SEM; n = 13) show similar strong inwardly rectifying currents. (D) Reversal potentials of ACh-induced KGP currents as a function of extracellular potassium concentration. The slope obtained, 53-mV change in reversal potential per 10-fold change in external K concentration, is similar to the value of 58 predicted by the Nernst equation for a potassium selective current.

Receptors in *Xenopus* oocytes. Hyperpolarizations in HK bath solutions induced large magnitude currents, while in the same oocytes ACh caused a further large increase (Fig. 7 A; range of total currents 3–50 pA; n = 84). The current-voltage relationship of the high potassium-induced current in the absence or presence of ACh showed strong inwardly rectifying properties (Fig. 7 B). Cell-attached recordings revealed prominent single-channel activity with kinetics and conductance characteristics similar to KAC, in agreement with previous results (Krapivinsky et al., 1995; Fig. 7 D).

**KGP Abundance in Pancreas**

We have cloned a human Kir3.4 (KGP) channel which is expressed in a number of tissues. The predominant abundance of the Kir3.4 message in pancreas (multiple

**DISCUSSION**

**KGP Abundance in Pancreas**

We have cloned a human Kir3.4 (KGP) channel which is expressed in a number of tissues. The predominant abundance of the Kir3.4 message in pancreas (multiple

from control oocytes injected with hm2 receptor were subtracted from those coinjected with hm2 and channel subunit(s). Fig. 8 A shows current-voltage relationships from oocytes bathed in HK solution. Comparable ACh-induced currents were obtained, as shown in Fig. 8 B. The Ba2+-sensitive currents constituted the sum of the ACh-induced and HK currents and showed a similar pattern of potentiated currents. The relative currents resulting from the channel subunits injected alone or together were compared in bar graph form. The hyperpolarization-induced currents (at -80 mV) in the presence of HK (open bars) and the ACh-induced currents (cross-hatched bars) are shown in Fig. 8 D. hGIRK1 produced small HK currents (-0.035 ± 0.023 pA) and a small increase in the presence of ACh (-0.028 ± 0.009 pA). KGP produced HK currents which were threefold larger than hGIRK1 (-0.113 ± 0.023 pA) and were further increased with ACh (-0.176 ± 0.013 pA). Oocytes coinjected with both channels (hGIRK1/KGP) produced HK currents which were 20-fold greater than KGP and 65-fold greater than hGIRK1 (-2.275 ± 0.214 pA). ACh resulted in a further increase (-1.913 ± 0.206 pA). Thus, both basal (agonist-independent) and agonist-dependent currents were comparable in magnitude whether the two channels were expressed alone or together.

**PTX Effects on Heteromeric Channel Currents**

Since PTX reduced both basal and agonist-dependent KGP currents, its effects were also tested on the heteromeric channel currents. Oocytes were coinjected with hm2 receptor and the hGIRK1 and KGP subunits, and 5 d later gave basal currents that were more than doubled in the presence of ACh (Figure 9 A). The currents remaining after application of 200 μM Ba2+ were indicative of oocyte endogenous K+ currents. The 5-d PTX treatment resulted in significantly diminished currents while ACh effects were abolished. Ba2+ blocked a small PTX-insensitive component of the current. In oocytes coinjected with the channel subunits alone, PTX caused a significant reduction in basal currents (Fig. 9 B), similar in magnitude to that obtained when hm2 receptor was also coexpressed (see Fig. 9 A). The effects of PTX on the currents of the coinjected channels were therefore similar to those of the KGP channel, suggesting that the G-protein gating and PTX sensitivity of the heteromer can be attributed at least in part to the KGP subunit.

**Coexpression of KGP and hGIRK1 Does Not Preferentially Enhance the Agonist-dependent Fraction of the Total Potentiated Current**

Current measurements comparing the relative functional expression of hm2 receptor with the hGIRK1 or KGP subunits alone, or of coexpression with the heteromer hGIRK1/KGP were obtained. Currents resulting

**DISCUSSION**

**KGP Abundance in Pancreas**

We have cloned a human Kir3.4 (KGP) channel which is expressed in a number of tissues. The predominant abundance of the Kir3.4 message in pancreas (multiple
tissue Northern blot and numerous positive clones isolated from cDNA library) suggests that this channel could form a major component of inwardly rectifying currents found in this tissue with a role in the hormonal regulation of secretion.

**Inward Rectification**

KGP was functionally expressed in *Xenopus* oocytes and CHO cells and characterized both at the whole-cell and single-channel levels. It was shown to be K⁺ selective, to be blocked by external Ba²⁺, and able to couple to the G-protein-linked hm2 receptor. Both the whole-cell ACh-sensitive and Ba²⁺-blocked currents showed strong inwardly rectifying properties. Similarly, single-channel activity in both KGP expressing oocytes and CHO cells exhibited strong inward rectification. Interestingly, the single residue in the M2 region, N179, which for members of other Kir subfamilies has been shown to be partly responsible for the Mg²⁺-dependent inward rectification (weak rectification when it is neutral and strong when it is acidic: Lu and Mackinnon, 1994; Stanfield et al., 1994; Wible et al., 1994), does not appear to function similarly in KGP. Alternatively E231, which corresponds to a residue in the hydrophilic COOH-terminal domain previously shown to also control in part...
FIGURE 7. Co-expression of KGP and hGIRK1 in *Xenopus* oocytes yields large inwardly rectifying currents. Each oocyte was coinjected with KGP (~8 ng), hGIRK1 (~8 ng) and hm2 receptor (~1 ng) cRNAs and electrical recordings were performed 2–3 d later. (A) Time course of currents recorded by two electrode voltage clamp applying 80 mV hyperpolarizing voltage steps (400 ms long and every 2 s) from a holding potential of 0 mV. Currents from oocytes coinjected with both channels, induced in HK solution in the absence or presence of ACh, were several fold larger than those produced in oocytes injected with either of the two channels separately. Breaks in the time course record indicate current responses of voltage steps from −100 to +100 mV, plotted in B. (B) Current-voltage relationships from the experiment shown in A in the absence (squares) or presence of ACh (circles). The strong inwardly rectifying properties of the currents resulting from the coinjection are evident. (C) Single channel activity recorded from an oocyte coinjected with KGP, hGIRK1 and hm2 cRNAs in the cell-attached configuration. The membrane was held at −100 mV. The lower trace was plotted on an expanded time scale. The longer single-channel openings show KATP-like kinetic properties, in agreement with the equivalent cardiac clones (Krapivinsky et al., 1995).

**Mg$^{2+}$-dependent inward rectification in IRK1 (Kir2.1, Yang et al., 1995), may be involved in producing the strong inward rectification seen with KGP.**

**Single-Channel Properties of KGP**

Single-channel openings of KGP were brief and difficult to fully resolve, showing a mean open time of ~0.4 ms. Probably due to the limited resolution, single-channel conductance ranged between 15–30 pS. Patch excision abolished channel activity, suggesting that a cytoplasmic component was necessary to keep the channel active. Neither 2 mM MgATP nor 50 μM GTPγS alone were capable of efficiently re-activating the channel. However, when coapplied, 2 mM MgATP and 50 μM GTPγS could sometimes re-activate KGP channels. These single-channel characteristics are in agreement with those previously reported for CIR (Krapivinsky et al., 1995). In the context of the MgATP requirement for channel activation it is interesting to note that the putative nucleotide binding site which we have identified (Walker type B motif) is intact in GIRK2 and Kir3.4 channels, but not in GIRK1 and GIRK3 channels.

**KGP Channel Expression Does Not Produce KATP Currents**

The first report of rKir3.4 cloning and functional expression presented evidence that this channel comprised a cardiac KATP conductance (Ashford et al., 1995). Subsequently, it was reported that the same recombinant cDNA (independently cloned) did not represent a KATP channel since it displayed single-channel properties unlike those expected of a KATP channel (Krapivinsky et al., 1995). Our results with the highly related KGP clone (25 amino acid differences from CIR and 26 from rKATP) offer further insight into this
controversy. The single-channel properties, such as conductance and open time kinetics, were different than those of \( \kappa_{\text{ATP}} \) channels and in agreement with those presented by Krapivinsky and colleagues (Krapivinsky et al., 1995). Similarly, intracellular ATP did not inhibit channel activity as would be expected with a \( \kappa_{\text{ATP}} \) channel. Moreover, our pancreatic clone exhibited strong inwardly rectifying characteristics, both at the single-channel and whole-cell levels, unlike the weak rectification expected of a \( \kappa_{\text{ATP}} \) channel. Finally, several control cell lines used in previous studies claiming heterologous expression of putative \( \kappa_{\text{ATP}} \) recombinant clones (Ashford et al., 1995; Inagaki et al., 1995; Chan et al., 1995; Kozak et al., 1995) appear to possess endogenous \( \kappa_{\text{ATP}} \) channels (Sui et al., manuscript in preparation), raising concerns regarding proper controls in previous studies. Thus, our data support other reports (Krapivinsky et al., 1995; Duprat et al., 1995) claiming that independent expression of Kir3.4 channels is not representative of a \( \kappa_{\text{ATP}} \) conductance.

**Heteromeric Subunit Association Yields Potentiated Currents**

A striking result, which has been reproduced with our recombinant channels, has been that coexpression of Kir3.1 with Kir3.4 channels or their close relative (GIRK2) greatly potentiated the resulting currents. In our case, we recorded currents up to 50 \( \mu \text{A} \). Antibodies
specific for one or the other channel coprecipitated both proteins providing strong evidence for the association of the two channels into a heteromeric complex in *Xenopus* oocytes. Homology comparisons between hGIRK1 and KGP showed divergent sequences at both the NH2- and COOH-terminal regions of the two cDNAs. It is possible that favorable interactions between distinct regions of the two subunits are responsible for the effect of the current potentiation. Since GIRK2 was also capable of producing large currents when coexpressed with GIRK1, it is possible that common structural elements among GIRK2 and Kir3.4 channels could be responsible for the interactions with GIRK1 which lead to current potentiation. Coexpression resulted in large basal currents. The agonist-dependent component was proportionately the same regardless of whether the channel subunits were expressed alone or together. This result suggests that the current potentiation observed with coexpression did not just alter the ability of the heteromeric channel to couple to G proteins via the G-protein-linked receptor.

### G-Protein Gating and PTX Sensitivity of KGP Subunits and Heteromeric Channels

Heterologously expressed KGP efficiently coupled to a G-protein-linked receptor producing microampere size currents, unlike its cardiac counterpart, CIR (at least 10-fold smaller currents; Krapivinsky et al., 1995). Moreover, sizable basal currents exhibited by KGP allowed identification of its independent functional expression, unlike GIRK1 channels which in our hands produced much smaller currents than KGP (see Fig. 8). Pertussis toxin, which ADP ribosylates Gα subunits and functionally uncouples them from receptor (Katada and Ui, 1982a, b), not only significantly reduced agonist-dependent but also basal KGP currents (2 d treatment). This result suggests that the high level of agonist-independent, basal activity of this recombinant channel is related to G-protein gating. This finding is in agreement with previous studies with Kα2; suggesting that basal activity was the result of G-protein gating (Ito et al., 1991; Okabe et al., 1991). The precise mechanism by which PTX decreased basal currents, however, remains unclear.

The PTX effects were reproduced for the heteromeric channel. PTX abolished agonist-dependent currents while it greatly reduced basal currents (5 d treatment). The increase in the PTX effect with the length of treatment is consistent with previous reports using different G-protein-linked proteins expressed in oocytes (e.g., agonist-dependent currents; Blitzer et al., 1993). The sensitivity of basal currents to PTX is consistent with the hypothesis that there is a receptor-G protein-channel complex where interactions among members of the complex are always present but can be enhanced by agonist-receptor interactions (where G-protein would dissociate and alter gating of the channel). In such a scheme, ADP-ribosylation of the Gα subunits would affect both basal as well as agonist-induced interactions by virtue of altering both the receptor-G protein coupling as well as the G protein-channel interactions.

### An Endogenous Oocyte Kir3.4-like Protein Associates with hGIRK1

It has been suggested, from preliminary evidence in low-stringency Northern blots, that there may be endogenous Kir3.4 homolog in *Xenopus* oocytes (Krapivinsky et al., 1995). In our immunoprecipitation experiments, GIRK1-Ab coprecipitated a band similar in size...
to KGP. This ~45 kD band could represent an endogenous oocyte protein capable of associating with GIRK1. The existence of an oocyte protein which can associate with GIRK1 (possibly a Kir3.4 homolog) cautions against what was thought to be independent GIRK1 expression which in fact may be indicative of heteromeric GIRK1/Kir3.4 expression. In such a case, knockout of endogenous Kir3.4 expression would be necessary to validate independent GIRK1 expression in oocytes (Kubo et al., 1993; Dascal et al., 1993a) and effects by G-protein subunit coexpression on independent GIRK1 activity (Reuveny et al., 1994; Takao et al., 1994; Lim et al., 1995). The precise contribution of each channel subunit of the heteromeric complex in properties such as G-protein gating, open-time kinetics, and precise gating interactions responsible for the current potentiation, remain to be elucidated.

We thank Drs. Tom Bonner for the hm2 receptor cDNA, Angeliki Buku for synthesis of the antigenic peptide identical to the one against which the UB-Ab was generated, Lily Jan for the rat GIRK1 cDNA, the late Peter Hess for pGEM-HE, and William B. Thornhill for the COOH-terminal peptide antibody to GIRK1 and the CHO cells stably transfected with GIRK1. We also thank Aime Buro for technical assistance with tissue culture. In addition, we are grateful to Drs. Neil Castle, Joseph Margiotta, William B. Thornhill, and Michel Vivaudou for critical comments on the manuscript.

This work was supported by NIH grant (AI34270) and AHA grant (93014380) to J.A.A. Ladis and by grants from NIH (HL46383), AHA/New York City (Grant-In-Aid) and by support from the Lucille P. Markey Charitable Trust to D. E. Logothetis.

Original version received 4 October 1995 and accepted version received 30 November 1995.

R E F E R E N C E S


Hamill, O.P., A. Marty, E. Neher, B. Sakmann, and F.J. Sigworth. 1981. Improved patch-clamp techniques for high-resolution cur-
rent recording from cells and cell-free membrane patches. 


Katada, T., and M. Ui. 1982a. ADP ribosylation of the specific membrane protein of C6 cells by islet-activating protein associated with modification of adenylate cyclase activity. 


Katada, T., and M. Ui. 1982b. Direct modification of the membrane protein of C6 cells by islet-activating protein associated with the specific membrane protein of C6 cells. 


Neuron. 8:531–540.


Neuron. 10:1121–1129.


North, A.J.T. Williams, A. Suprenant, and M.J. Christie. 1987. μ and δ receptors belong to a family of receptors that are coupled to potassium channels. 


Nature (Lond.). 349:77–79.


