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

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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 $\underline{K}^+$ selective, $\underline{G}$-protein-gated and isolated from $\underline{h}$uman 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 $\underline{h}$GIRK1 (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_{ACh} channels by pertussis toxin-sensitive G proteins (Soejima and Noma, 1984; Pfaffinger et al., 1985; Breitwieser and Szabo, 1985). Activation of cardiac K_{ACh} 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_{ACh} 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 in-

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wardly 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

strong inward rectification, a ~42 pS single-channel conductance, and 1–2 ms mean open time. Although these characteristics provided evidence that GIRK1 represented the atrial K_{ACh}, heterologous expression in mammalian systems failed to produce K_{ACh} channel activity (Krapivinsky et al., 1995; Kofuji et al., 1995). This problem was overcome when GIRK1 was coexpressed in Chinese Hamster Ovary (CHO) cells with a highly homologous clone, CIR (rcKir3.4: Krapivinsky et al., 1995), which had been reported earlier to be the cDNA encoding cardiac K_{ATP} channels (Ashford et al., 1994). In oocytes, coexpressed channels resulted in eightfold greater currents than with either channel alone. In addition, immunoprecipitations from atrial membranes with GIRK1-specific antibodies coprecipitated a 45-kD protein, which upon microsequencing was found to correspond to CIR. These results led Krapivinsky and colleagues to conclude that the native atrial K_{ACh} is a heteromultimer of the two recombinant proteins, GIRK1/CIR (rcKir3.1/3.4). GIRK1 and CIR were shown to be 53% identical. Another G-protein–gated channel, GIRK2 (mbKir3.2, cloned from mouse brain), when expressed in *Xenopus* oocytes, exhibited opioid-receptor-evoked K^+ currents (Lesage et al., 1994). GIRK2 showed 69% identity to Kir3.4 and 54% identity to Kir3.1 channels. Coexpression of GIRK2 with GIRK1 in oocytes resulted in severalfold greater currents than those produced by either channel alone (Kofuji et al., 1995; Duprat et al., 1995), just as was the case with coexpression of CIR and GIRK1 (Krapivinsky et al., 1995). Another related channel, GIRK3 (mbKir3.3, also cloned from mouse brain), when injected alone, failed to functionally express in *Xenopus* oocytes (Lesage et al., 1995; Kofuji et al., 1995). GIRK3 showed 67% identity to Kir3.4 and 57% identity to Kir3.1 channels. Interestingly, coexpression of GIRK3 with GIRK1 resulted in severalfold greater currents in some studies (Kofuji et al., 1995), but not others (Lesage et al., 1995).

The present study reports characterization of a human Kir3.4 channel, KGP, heterologously expressed alone or together with hGIRK1 (a human brain homolog of the rat cardiac GIRK1/Kir3.1). The results obtained are in agreement with a recent report (Krapivinsky et al., 1995) and in addition provide a number of important new results. KGP produced strong inwardly rectifying K^+ currents both at the single-channel and whole-cell levels. This channel coupled efficiently to a G-protein-linked receptor via pertussis toxin (PTX)-sensitive G proteins. PTX treatment not only significantly reduced agonist-dependent but also basal (or agonist-independent) currents. Coexpression of KGP with hGIRK1 resulted in severalfold greater currents than with either channel alone. The agonist-dependent fraction of the total current response was comparable whether the channel subunits

were expressed alone or together. As with KGP alone, PTX treatment reduced both the agonist-dependent and -independent current responses of the coexpressed channels. Specific antibodies raised against either Kir3.4 or GIRK1 coprecipitated both proteins (KGP and hGIRK1) coexpressed in oocytes, providing evidence for heteromeric assembly of the two channels in the oocyte system. Moreover, immunoprecipitation experiments suggested the existence of an endogenous protein similar in size to Kir3.4 and capable of interacting with GIRK1.

The mechanism of G-protein gating of K_{ACh} has been under intense investigation in the last decade. The recent appreciation of the heteromeric nature of K_{ACh} requires a thorough characterization of the component subunits. Our study demonstrates assembly of recombinant 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-P-M2 region of GIRK1 (Kubo et al., 1993) was synthesized by PCR using GIRK1 as a template with the following primers: 5′-CTTTATCTTCAT-CCTCACCTACA (corresponds to the peptide FIFILTY in GIRK1) in the foward direction and 5′-GAACTGTTTGTACGTGGG-GTGGT (corresponds to the peptide LIGCMFIK) in the reverse direction. This probe was gel purified and labeled with α ^{32}P-dCTP by using random hexamer and Klenow before screening ~1 × 10^6 plaques from a human fetal brain λ-gt11 cDNA library (Clontech, Palo Alto, CA). The filters were prehybridized and hybridized in buffer containing 50% formamide, 5× SSPE, 5× Denhardt's solution, 0.1% SDS and 100 μg/ml denatured salmon sperm DNA at 37°C. 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 t857-c1218 of the KGP full length nucleotide sequence) was synthesized by PCR and was used to screen a human pancreatic λ-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 ^{32}P-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 guandinium 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 $CaCl_2$. 500 μl of 2× HeBS (HEPES-buffered saline) was placed in a sterile 15-ml conical tube. The DNA/$CaCl_2$ 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 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 T7 polymerase. hm2 in pSP72 was linearized with

BglII 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 nuclease-treated lysate translation system (Promega Corp.) KGP cRNA was added at 100 ng per translation reaction. ^{35}S-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 ^{35}S-methionine and ^{35}S-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°C. 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 with 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, CA). Microelectrodes were filled with 3 M KCl and had resistances of 0.5–1.0 MΩ. Bath solutions had the following composition: low potassium (LK) in millimolar: KCl 2, NaCl 91, MgCl$_2$ 1, NaOH/HEPES 5, CaCl$_2$ 1.8, pH 7.4; high potassium (HK, ND96K) in millimolar: KCl 91, NaCl 1, MgCl$_2$ 1, KOH/HEPES 5, CaCl$_2$ 1.8, pH 7.4. Bath solutions containing 26 or 50 mM K$^+$ had the NaCl concentration accordingly adjusted so that the osmolarity of the solutions was kept constant. Niflumic acid (300 μM) was routinely added to the bath solutions to block chloride currents (Sigma Chemical Co., St. Louis, MO). Single channel activity was recorded under voltage clamp with standard patch-clamp methods (Hamill et al., 1981; Methfessel et al., 1986) using an Axopatch 200A amplifier (Axon Instruments). For oocyte patch-clamp recordings, the pipette solution contained ND96K, while bath solutions contained EGTA as well (96 mM KCl, 5 mM EGTA, 1 mM MgCl$_2$, and 10 mM HEPES, pH 7.4). 5 μM ACh and 50 μM gadolinium were also included in the pipette solution. For recordings with CHO cells, solution K1 (120 mM KCl, 2 mM MgCl$_2$, 10 mM HEPES, and 5 mM EGTA, pH 7.35) was used in the bath, and KCA2 (K1 with 2 mM CaCl$_2$ and without EGTA) was used in the pipette. Most of the single-channel recordings were performed keeping the membrane voltage clamped at −100 mV. Some were recorded with voltage-ramp protocols (−100 to +100 mV) from a holding potential of 0 mV. Single-channel currents were sampled at 5-10 KHz and filtered at 2 KHz with the Axon Instruments interface DIGIDATA 1200. pCLAMP (version 6.01, Axon Instruments) was used for primary data analysis. All electrodes used were made from WPI-K borosilicate glass, using a Flaming-Brown micropipette puller (Sutter Instrument Co., Novato, CA). All experiments were performed at 20–25°C.

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 (t857-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 (rcK_{ATPi}; 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 K_{ATP} (hcK_{ATP}: Ashford et al., 1995; G388 is R in hcK_{ATP}), 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/RXXXGXXXLΦΦΦΦ: where X denotes any amino acid and Φ 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, S321, 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 NH$_2$ terminal 21 amino acids of rcKir3.4 (rcK_{ATPi}; 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
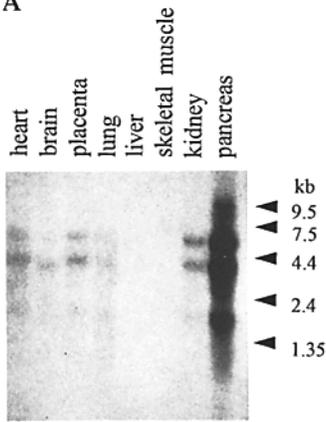
A

kb
9.5
7.5
4.4
2.4
1.35

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 rcK_ATP1 (25 differences with CIR; V188 is the same in both sequences, in contrast with rcK_ATP1). A putative nucleotide-binding site (Walker type B motif) is boxed.

B

```
                                *
    -195   aggatggcttaggtacctctgcccacaggaccccacaacagggagaggttccagctacagctcctccgtggggtcatggcaggggct

                             *                        *
    -108   ggggagtccctcaaaagccctgagcccccctgcaccgccgctaagggacaccccagaagttagcatcagtgggactcggaagctccgat

                  M  A  G  D  S  R  N  A  M  N  Q  D  M  E  I  G  V  T (P)(W) D (P) K  K      24
     -18   ctcaacaacatcccagctatggctggcgattctaggaatgccatgaaccaggacatggagattggagtcactcctgggacccaagaag

           I  P  K  Q  A  R  D  Y (V) P  I  A  T  D  R  T  R  L  L (A) E  G  K  K  P  R  Q  R  Y  M      54
      73   attccaaaacaggcccgcgattatgtccccattgccacagaccgtacgcgcctgctggccgagggcaagaagccacgccagcgctacatg

           E  K (S) G  K  C  N  V  H  H  G  N  V  Q  E  T  Y  R  Y  L  S  D  L  F  T  T  L  V  D  L      84
     163   gagaagagcggcaagtgcaacgtgcaccacgcgcaacgtccaggagacctaccggtacctgagtgacctcttcaccaccctggtggacctc

           K  W  R  F  N  L  L  V  F  T  M  V  Y  T (V) T  W  L  F  F  G  F  I  W  W  L  I  A  Y (I)     114
     253   aagtggcgcttcaacttgctcgtcttcaccatggtttacactgtcacctggctgttcttcggcttcatttggtggctcattgcttatatc

           R  G  D  L  D  H  V  G  D  Q  E  W  I  P  C  V  E  N  L  S  G  F  V  S  A  F  L  F  S  I      144
     343   cggggtgacctggaccatgttggcgaccaagagtggattccttgtgttgaaaacctcagtggcttcgtgtccgctttcctgttctccatt

           E  T  E  T  T  I  G  Y  G  F  R  V  I  T  E  |K  C  P  E  G  I  I  L  L  L  V  Q  A| I  L      174
     433   gagaccgaaacaaccattgggtatggcttccgagtcatcacagagaagtgtccagaggggattatactcctcttggtccaggccatcctg

           G  S  I  V  N  A  F  M  V  G  C  M  F (V) K  I  S  Q  P  K  K  R  A  E  T  L  M  F  S  N      204
     523   ggctccatcgtcaatgccttcatggtggggtgcatgtttgtcaagatcagccagcccaagaagagagcggagaccctcatgttttccaac

           N  A  V  I  S  M  R  D  E  K  L  C  L  M  F  P  R  V  G  D  L  R  N  S  H  I  V  E  A  S  I      234
     613   aacgcagtcatctccatgcgggacgagaagctgtgcctcatgttccgggtgggcgacctccgcaactcccacatcgtggaggcctccatc

           R  A  K  L  I  K  S  R  Q  T  K  E  G  E  F  I  P  L  N  Q  T  D  I  N  V  G  F  D  T  G      264
     703   cgggccaagctcatcaagtcccggcagaccaaagagggggagttcatcccctgaaccagacagacatcaacgtgggctttgacacgggc

           D  D  R  L  F  L  V  S  P  L  I  I  S  H  E  I  N  E  K  S  P  F  W  E  M  S (Q) A  Q  L      294
     793   gacgaccgcctcttcctggtgtctcctctgatcatctcccacgagatcaacgagaagagccctttctgggagatgtctcaggctcagctg

          (H) Q  E  E  F  E  V  V  V  I  L  E  G  M  V  E  A  T  G  M  T  C  Q  A  R  S  S  Y  M  D      324
     883   catcaggaagagtttgaagttgtggtcattctagaagggatggtggaagccacaggcatgacctgccaagcccggagctcctacatggat

           T  E  V  L  W  G  H  R  F  T  P  V  L  T  L  E  K  G  F  Y  E  V  D  Y  N  T  F  H  D  T      354
     973   acagaggtgctctggggccaccgattcacaccagtcctcaccttggaaaagggcttctatgaggtggactacaacaccttccatgatacc

           Y  E  T  N  T  P  S  C  C  A  K  E  L  A  E  M  K  R (E) G (R) L  L  Q (Y) L  P  S  P  P      384
    1063   tatgagaccaacacacccagctgctgtgccaaggagctggcagaaatgaagagggaaggccggctcctccagtacctccccagcccccca

           L  L  G  G  C  A  E  A (G)(L)(D) A  E  A  E (Q)(N) E  E (D) E  P (K) G  L (G)(G) S  R (E)     414
    1153   ctgctggggggctgtgctgaggcagggctggatgcagaggctgagcagaatgaagaagatgagcccaaggggctgggtgggtccagggag

          (A) R  G  S (V) *                                                                               419
    1243   gccaggggctcggtgtgaggggtgcagcctccctaagacctcctgtcactggcttcagtgaacacagacactgcagagcctgggagcagg

    1333   ggaggggaatagttgagtgtgctgtttgggggctcaggagccatcaagtctgtggggaggaaccataaacccagccctcacagctcccag

    1423   cacagggcctccctgagccagtggcatcctgcctgggccccccatggcagtgctgcctcttgtaggtgctggctaagggccaggccagga

    1513   tgagtttcc
```

A

B

C

D
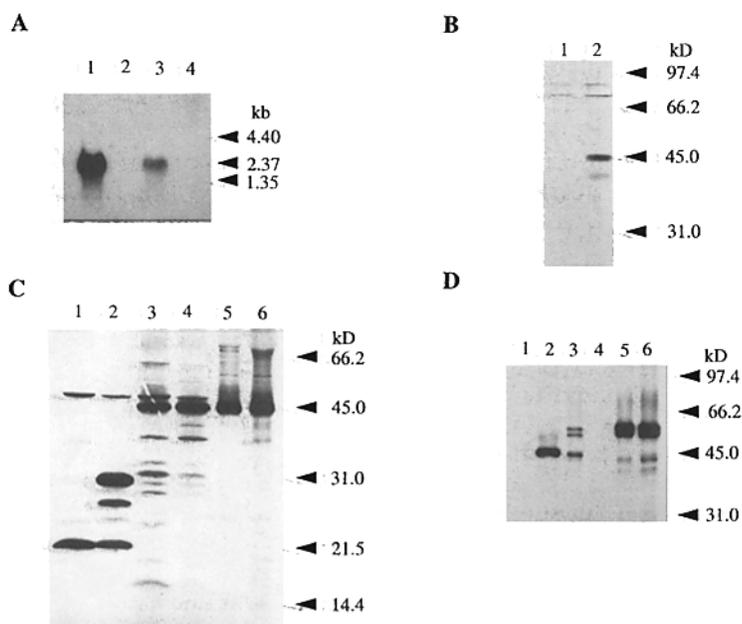
FIGURE 2. Detection of KGP cRNA and protein in cells and cell-free systems. (A) Northern blot analysis of total and poly A$^+$ RNA extracted from CHO cells either stably transfected with KGP (clone No. 6) or from untransfected controls. Hybridization signals were obtained with either poly A$^+$ or total 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 (^{35}S-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 *Saccharomyces 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 coimmunoprecipitation 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 coimmunoprecipitation 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.

when translated in the presence of canine microsomes (Fig. 2 *C*, Lns: 3, 4), in contrast with the α-factor of *Saccharomyces cerevisiae* (18-kD band is the nonglycosylated product; Fig. 2 *C*, Ln: 2) and in agreement with results obtained with CIR (Krapivinsky et al., 1995). Moreover, UB Ab was successful in immunoprecipitating the in vitro translated KGP protein (Fig. 2 *C*, Lns: 5, 6) both in the absence (Fig. 2 *C*, Ln: 5) or presence (Fig. 2 *C*, Ln: 6) of canine microsomes. An affinity purified C-terminal peptide antibody raised against the rat GIRK1 COOH-terminal end (GIRK1-Ab, Q375-T501; Ponce et al., 1995) was used in parallel with UB-Ab in immunoprecipitation experiments using metabolically labeled oocyte membranes expressing the KGP and hGIRK1 channels alone or together (Fig. 2 *D*). UB-Ab precipitated a 45-kD band from oocytes injected with KGP cRNA (Fig. 2 *D*, Ln: 2) but not from uninjected oocytes (Fig. 2 *D*, Ln: 1). Furthermore, in oocytes coinjected with KGP and GIRK1 cRNAs, UB-Ab coprecipitated along with the 45-kD band a doublet of ~56–58 kD corresponding to hGIRK1 (Fig. 2 *D*, Ln: 3). The 21 amino acid peptide that the UB-Ab was produced against was synthesized and used in identical immunoprecipitation experiments. In the presence of this peptide (18.8 μM) immunoprecipitation of KGP or coprecipitation of

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 Ba^{2+}-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 I

Oocytes	LK	HK	HK-PTX	ACh	ACh-PTX	Ba^{2+}	Ba^{2+}-PTX
Uninjected	0.13 ± 0.02 (9)	0.31 ± 0.03 (9)	—	—	—	0.03 ± 0.02 (9)	—
hm2	0.06 ± 0.02 (12)	0.38 ± 0.14 (12)	—	0.03 ± 0.02 (12)	—	0.00 ± 0.02 (5)	—
KGP	0.18 ± 0.03 (20)	1.60 ± 0.45 (20)	0.98 ± 0.14 (6)	0.04 ± 0.05 (13)	—	1.02 ± 0.29 (20)	0.30 + 0.07 (6)
KGP+hm2	0.25 ± 0.03 (23)	2.37 ± 1.00 (23)	1.04 ± 0.02 (7)	1.67 ± 0.22 (23)	0.44 ± 0.07 (7)	2.88 ± 0.51 (7)	0.82 + 0.10 (3)

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–HK without ACh). ACh-PTX: Same as (ACh) but oocytes incubated in 1 µg/ml pertussis toxin. Ba^{2+}: Currents blocked by Ba^{2+} (in HK solution). In KGP+hm2 experiments, Ba^{2+} applied in presence of ACh. Ba^{2+}-PTX: Same as in (Ba^{2+}). In both sets of experiments Ba^{2+} was applied in presence of ACh. ( ): Number of experiments performed.

ization-induced inward currents recorded in oocytes have been summarized in Table I. Uninjected oocytes bathed in LK solution gave currents similar to those of injected oocytes. To maximize inward K^+ currents, the bath solution was changed to a high K^+ (HK) containing solution ND96K, containing 96 mM KCl. Uninjected oocytes bathed in HK gave larger inward currents than when bathed in LK solutions, indicating the presence of endogenous inward K^+ currents. Application of 200 µM $BaCl_2$ in HK had no effect on the endogenous inward currents, as previously reported (Dascal et al., 1993b; Fig. 3, A and B). In contrast, as shown in Fig. 3 C, oocytes from the same frogs as the uninjected controls injected with KGP cRNA yielded significantly larger currents. Moreover, in the same oocytes, 200 µM $BaCl_2$ blocked a large component of the inward current (Fig. 3, C and D), suggesting that the KGP-generated current is blocked by Ba^{2+}. Current-voltage relationships of the Ba^{2+}-sensitive currents from uninjected (diamonds) vs injected (triangles) oocytes revealed the inwardly rectifying properties of KGP (Fig. 3 E). These results showed that KGP produces a Ba^{2+}-blocked, inwardly rectifying current.

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 Ba^{2+}, 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 Ba^{2+}-blocked currents (Table I). In contrast, oocytes coinjected with hm2 and KGP cRNAs exhibited significantly greater ACh-induced currents (Fig. 4 B). After ACh application, 200 µM Ba^{2+} (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 Ba^{2+}-sensitive currents were obtained. Similarly, small agonist-dependent currents were obtained from oocytes coinjected 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 Ba^{2+}-blocked currents. In oocytes coinjected 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 Ba^{2+}-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

A

1

2, 3

0.2 µA
20 ms

C

1

3

2

0.4 µA
20 ms

B

Ba

HK

LK

0.2 µA

2 min

D

Ba

HK

LK

E

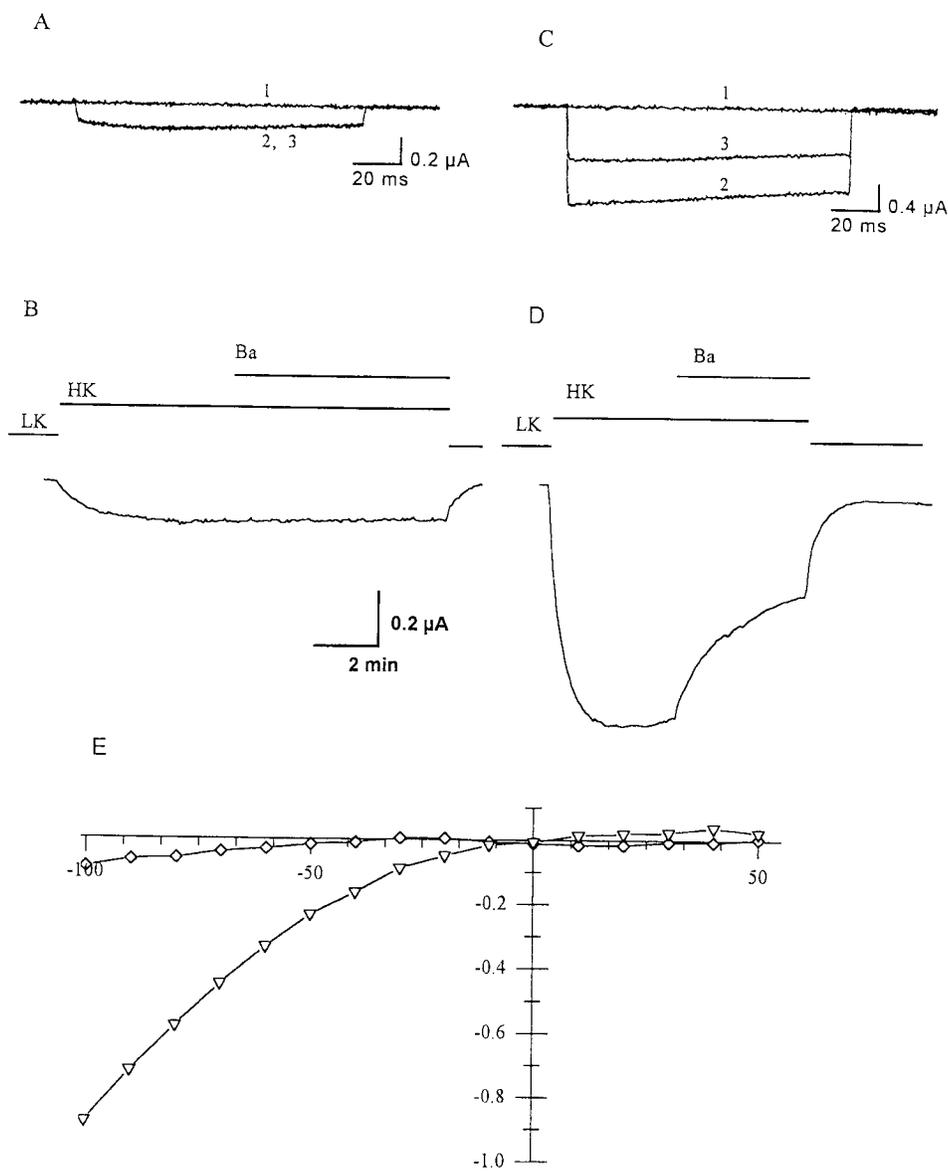-100   -50   50

-0.2

-0.4

-0.6

-0.8

-1.0

FIGURE 3. KGP gives rise to a Ba^{2+}-blocked inwardly rectifying current. Two electrode voltage-clamp recordings from *Xenopus* oocytes each injected with ~4 ng KGP cRNA. Hyperpolarization steps to -80 mV from a holding potential of 0 mV were given every 4 s. The current traces in LK solution have been subtracted from all current traces in subsequent conditions. Recordings were performed 2–4 d after injections. (*A*) Recordings from an uninjected oocyte. Trace *1* is the zero current in LK, *2* is the current in HK solution, *3* is the current in HK solution containing 200 µM $BaCl_2$. (*B*) The current measured at the end of each voltage pulse in the experiment in *A* is plotted as a function of time. (*C*) Recordings similar to those in *A* from an oocyte injected with ~4 ng of KGP cRNA. (*D*) Time course of currents from the experiment in *C*. (*E*) Representative current-voltage relationships of Ba^{2+}-blocked currents from an uninjected oocyte (*diamonds*) vs an oocyte injected with KGP cRNA (*triangles*).

0 mV, were obtained from oocytes coinjected with KGP and hm2 receptor. Both the ACh-induced and Ba^{2+}-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 Ba^{2+}-blocked currents (Fig. 5, *B* and *C, triangles*) showed equivalent strong inward rectification, similar to that seen with Ba^{2+}-blocked currents of KGP alone, as previously shown in Fig. 3 *E* (Fig. 5 *C*; 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. 5 *D*).

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. 6 *A*). 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
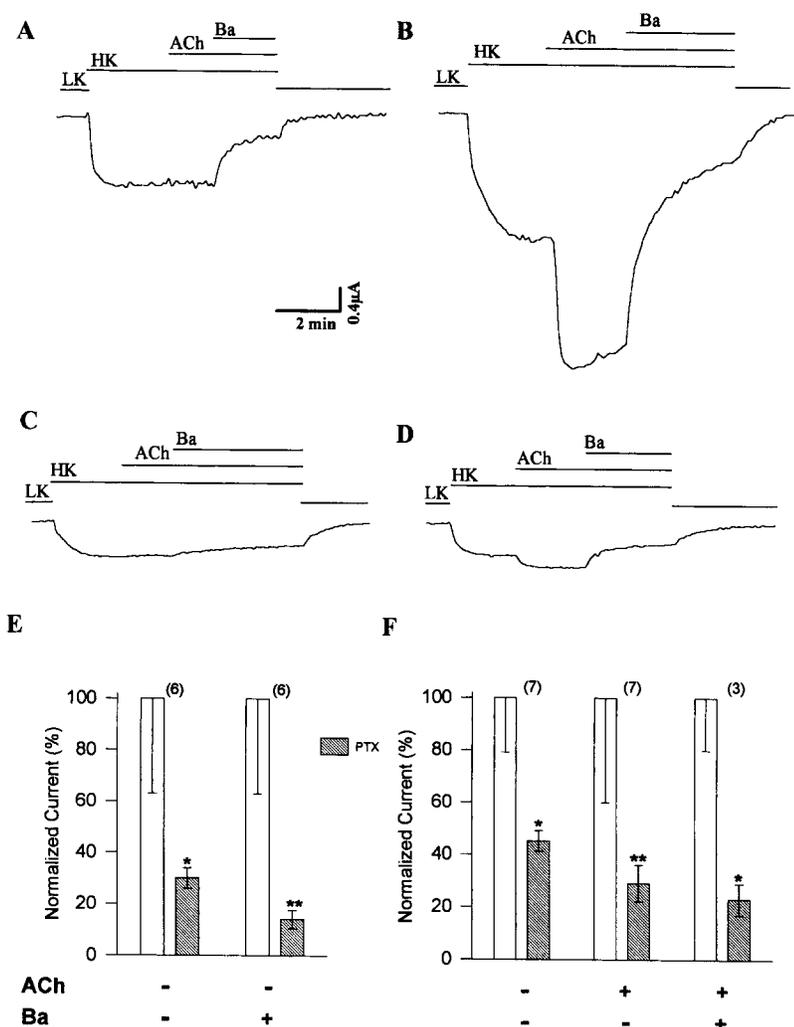
FIGURE 4. Receptor-mediated activation of KGP currents via PTX-sensitive G proteins. (A) Time course of currents from an oocyte injected with ~4 ng KGP and tested 2 d after injection (similar experiment as in Fig. 3 D but in addition testing for response to application of 100 μM ACh). (B) Time course of currents from an oocyte coinjected with hm2 (~1 ng) and KGP (~4 ng) and tested 3 d after injection. (C) Same as A but PTX treated for 2 d (1 μg/ml). (D) Same as (B) but PTX treated for 2 d (1 μg/ml). Oocytes were injected with KGP or coinjected with KGP and hm2 and divided into two groups, one of which was treated with 1 μg/ml PTX. 2 d later PTX-treated (*striped bars*) and untreated groups (*open bars*) were studied in parallel and currents elicited in response to 80-mV hyperpolarizations were measured, under the conditions indicated below. Mean currents of the PTX-treated group were plotted as percentages of the corresponding untreated controls in bar graph form. The numbers in parentheses indicate the numbers of paired experiments performed for each condition, while the asterisks indicate the significance level of the effect of the PTX treatment (paired t test: *, $P < 0.05$; **, $P < 0.01$). (E) Oocytes injected with KGP alone (no hm2) showed diminished HK-induced currents when PTX treated. Similarly, Ba^{2+}-blocked currents were significantly reduced. (F) In oocytes coinjected with KGP and hm2 currents induced in HK solution were significantly reduced (difference of the currents in LK from those in HK solutions). ACh-stimulated currents of these oocytes were also significantly reduced (difference of the currents recorded in HK solution in the absence from those recorded in the presence of ACh). Similarly, Ba^{2+}-blocked currents of the same oocytes showed a significant reduction (difference of the currents obtained in ACh-containing HK solution in the presence from those recorded in the absence of Ba^{2+}).

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 μM GTPγS, 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 τ_o = 0.4 ± 0.1 ms (mean ± 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 μM 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-
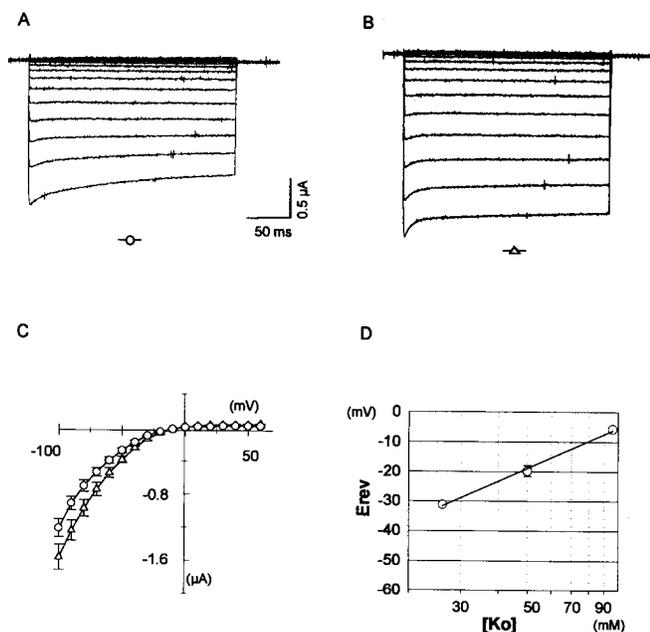
A

B

C

D

Figure 5. 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 Ba^{2+} 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 Ba^{2+}-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 extracellualr 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.

ceptors 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 μA; $n = 84$). 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 K_{ACh}, in agreement with previous results (Krapivinsky et al., 1995; Fig. 7 *D*).

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

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 Ba^{2+}-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 μA) and a small increase in the presence of ACh (−0.028 ± 0.009 μA). KGP produced HK currents which were threefold larger than hGIRK1 (−0.113 ± 0.023 μA) and were further increased with ACh (−0.176 ± 0.013 μA). 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 μA). ACh resulted in a further increase (−1.913 ± 0.206 μA). 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 Ba^{2+} were indicative of oocyte endogenous K^+ currents. The 5-d PTX treatment resulted in significantly diminished currents while ACh effects were abolished. Ba^{2+} 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.

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
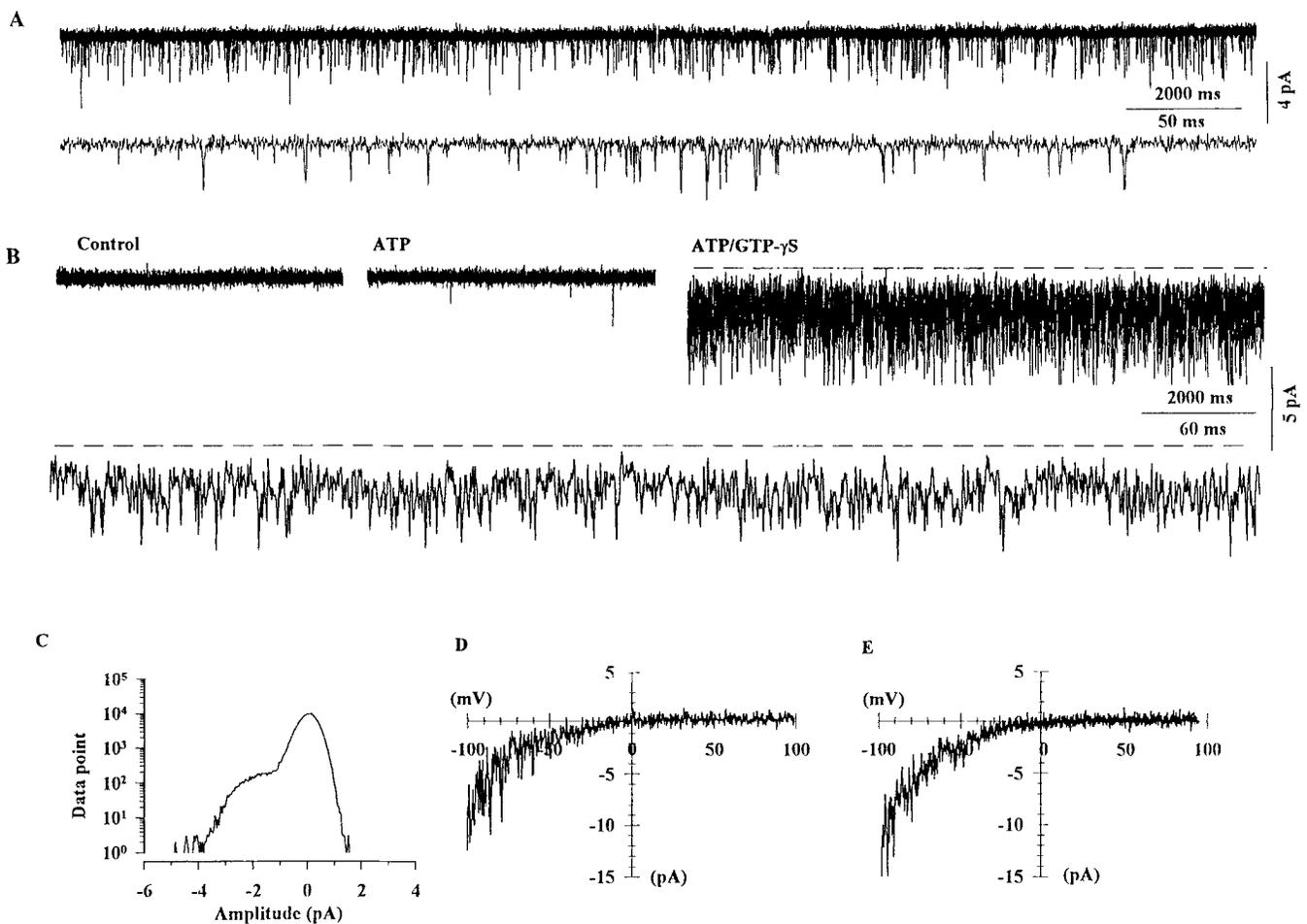
A

2000 ms

50 ms

4 pA

B   Control   ATP   ATP/GTP-γS

2000 ms

60 ms

5 pA

C

10⁵
10⁴
10³
10²
10¹
10⁰

Data point

-6   -4   -2   0   2   4
Amplitude (pA)

D

(mV)

5

-100   -50   0   50   100

-5

-10

-15   (pA)

E

(mV)

5

-100   0   50   100

-5

-10

-15   (pA)

FIGURE 6. Single-channel properties of KGP expressed in *Xenopus* oocytes and in CHO cells. (*A*) Single-channel activity in a cell-attached recording from *Xenopus* oocytes coinjected with KGP and hm2 cRNAs. The membrane was held at -100 mV. The lower trace was plotted on an expanded time scale. (*B*) Single-channel currents in an inside-out patch from CHO cells stably transfected with KGP cDNA. High channel activity resulted by coapplication of Mg-ATP (2 mM) and GTP-γS (50 μM) in the bath. The membrane was held at -100 mV. The lower trace was plotted on an expanded time scale. (*C*) All-point histogram plot of the expressed KGP channels from the record shown in *A*, showing the wide amplitude distribution of the open-channel currents, at -100 mV. (*D*) Current through single channels recorded from oocytes injected with KGP cRNA in the cell-attached configuration. The current trace shown (raw data) was obtained by employing a voltage ramp protocol (-100 to $+100$ mV from a holding potential of 0 mV). KGP ramps exhibited strong inwardly rectifying properties. (*E*) Current through single channels recorded from a CHO-cell membrane stably transfected with KGP, in the inside-out patch configuration. The voltage-ramp protocol was the same as in *D*. The channel was activated by the coapplication of Mg-ATP (2 mM) and GTP-γS (50 μM) in the bath solution.

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 Ba^{2+}, and able to couple to the G-protein-linked hm2 receptor. Both the whole-cell

ACh-sensitive and Ba^{2+}-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 Mg^{2+}-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
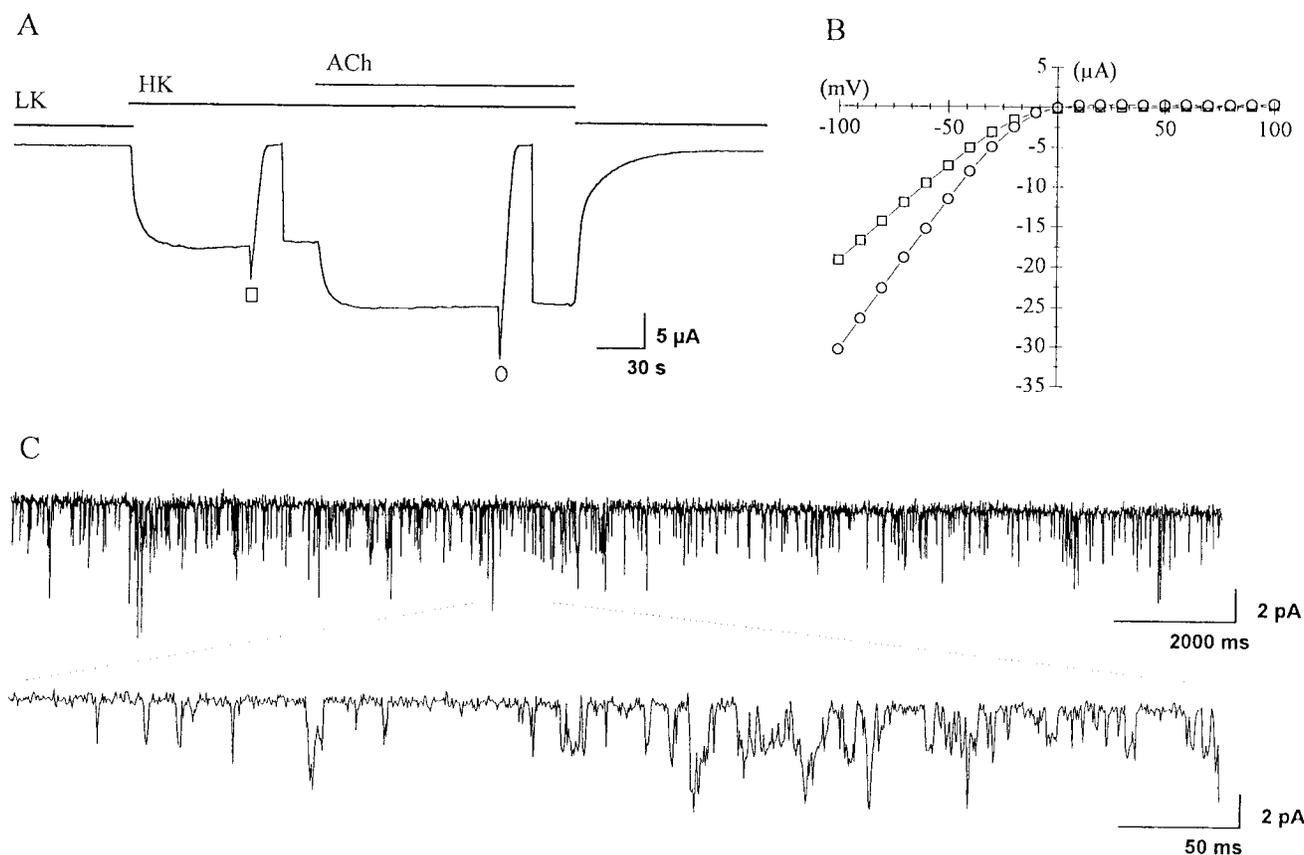
FIGURE 7. Co-expression of KGP and hGIRK1 in *Xenopus* oocytes yields large inwardly rectifying currents. Each oocyte was coinjected with KGP (∼8 ng), hGIRK1 (∼8 ng) and hm2 receptor (∼1 ng) cRNAs and electrical recordings were performed 2–3 d later. (*A*) Time course of currents recorded by two electrode 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 K_{ACh}-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.
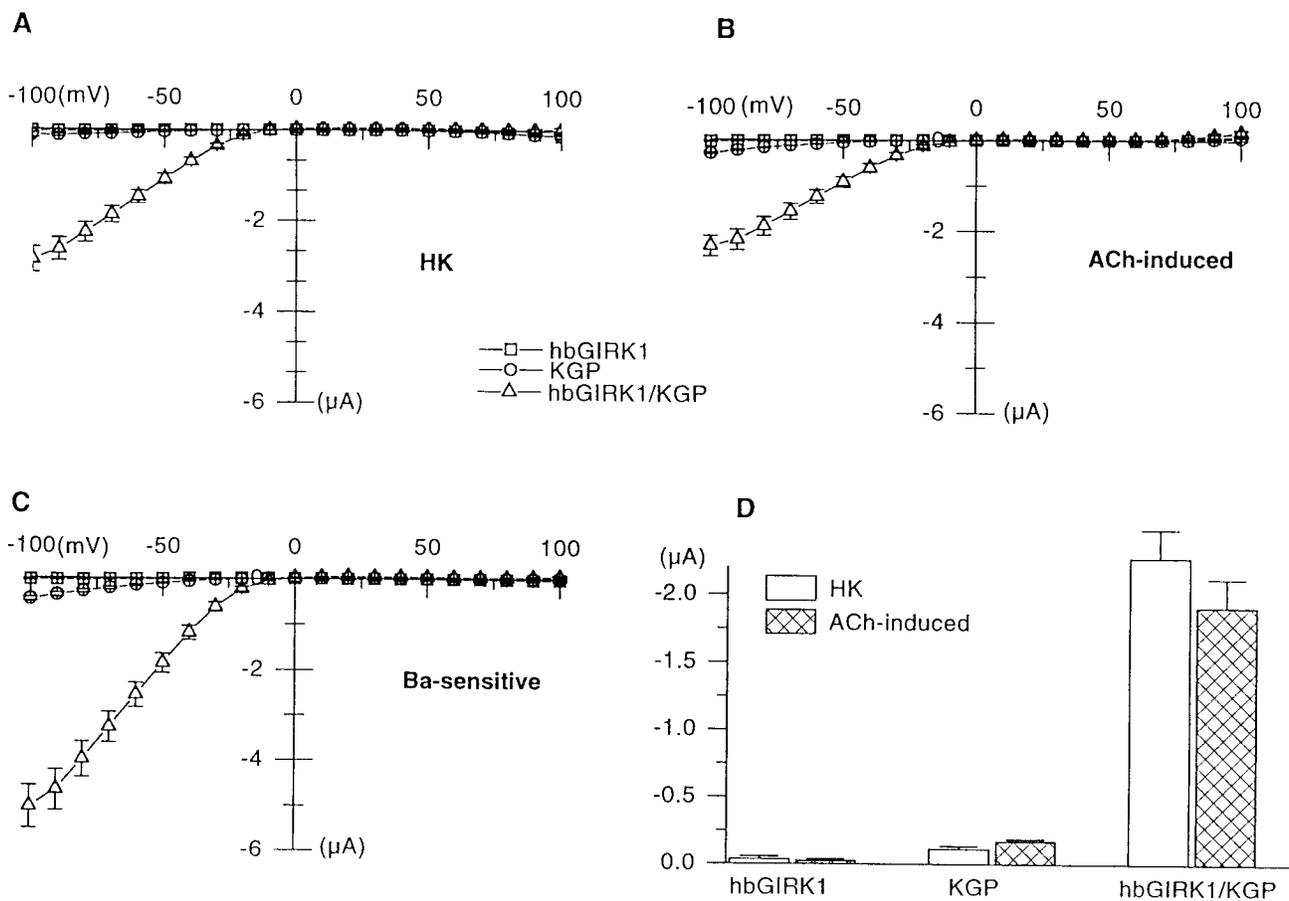
Single-Channel Properties of KGP

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 K_{ATP} Currents

The first report of rcKir3.4 cloning and functional expression presented evidence that this channel comprised a cardiac K_{ATP} conductance (Ashford et al., 1995). Subsequently, it was reported that the same recombinant cDNA (independently cloned) did not represent a K_{ATP} channel since it displayed single-channel properties unlike those expected of a K_{ATP} channel (Krapivinsky et al., 1995). Our results with the highly related KGP clone (25 amino acid differences from CIR and 26 from rK_{ATP1}) offer further insight into this

A

-100(mV) -50   0   50   100

-2

-4

HK

—□— hbGIRK1
—○— KGP
—△— hbGIRK1/KGP

-6   (µA)

B

-100(mV) -50   0   50   100

-2

-4

ACh-induced

-6   (µA)

C

-100(mV) -50   0   50   100

-2

-4

Ba-sensitive

-6   (µA)

D

(µA)
-2.0

-1.5

-1.0

-0.5

0.0

☐ HK
▨ ACh-induced

hbGIRK1   KGP   hbGIRK1/KGP

FIGURE 8. Similar relative increases in currents elicited by agonist whether KGP and hGIRK1 are expressed alone or together. Oocytes from the same batch were each injected with hm2, hGIRK1 and hm2, KGP and hm2, or hGIRK1, KGP and hm2 cR-NAs (hm2: ~1 ng, hGIRK1: ~4 ng, KGP: ~ 4 ng). Currents were recorded 4 d after injection in different bath solutions, namely control (HK alone), 100 µM ACh or 200 µM Ba^{2+} (added in HK solution). Mean values (± SEM) were calculated from currents from 6–8 experiments in the voltage range from −100 to +100 mV. (A) The mean values obtained from oocytes injected with hm2 receptor were subtracted from equivalent conditions of oocytes injected with channel subunits and current-voltage relationships in HK for all three groups injected with channel subunits were plotted. (B) The ACh-induced currents signify the difference currents in the absence from those in the presence of ACh. Current-voltage relationships for all three groups injected with channel subunits were plotted. (C) The Ba^{2+}-sensitive currents signify the difference currents in the presence from those in the absence of 200 µM Ba^{2+} but in the presence of 100 µM ACh. Current-voltage relationships for all three groups injected with channel subunits were plotted. (D) Currents at −80 mV were plotted in bar graph form to compare the relative magnitude of the basal (HK) and agonist-dependent (ACh-induced) currents for all three groups.

controversy. The single-channel properties, such as conductance and open time kinetics, were different than those of K_{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 K_{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 K_{ATP} channel. Finally, several control cell lines used in previous studies claiming heterologous expression of putative K_{ATP} recombinant clones (Ashford et al., 1995; Inagaki et al., 1995; Chan et al., 1995; Kozak et al., 1995) appear to possess

endogenous K_{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 K_{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 µA. Antibodies
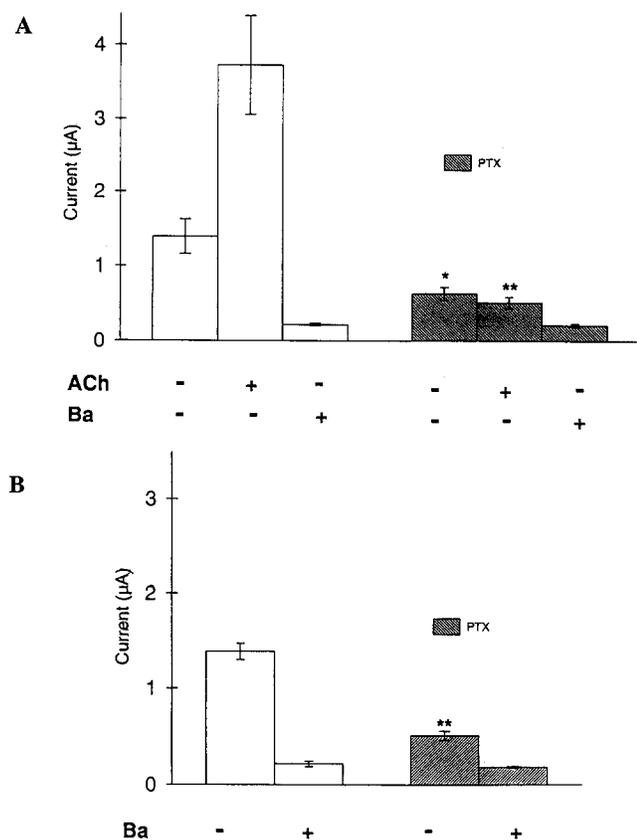
FIGURE 9. PTX effects on heteromeric channel currents. (A) Oocytes from the same batch were each coinjected with hGIRK1 (~4 ng), KGP (~4 ng) and hm2 (~1 ng) cRNAs. (B) Oocytes from the same batch as in A were each coinjected with hGIRK1 (~4 ng) and KGP (~4 ng). For both A and B, half of the injected oocytes were incubated in ND96 solution containing 1 μg/ml PTX, whereas the remaining half were incubated in PTX-free ND96 solution (control). Currents for both control and PTX treated oocytes were recorded 5 d after injection in different bath solutions, namely control (HK alone), 100 μm ACh (added in HK solution) or in 200 μM Ba^{2+}. Total currents at -80 mV were plotted in bar graph form to compare the effect of PTX treatment for basal currents (ACh $-$, Ba^{2+} $-$), currents in the presence of ACh ($+$) and currents in the presence of Ba^{2+} ($+$).

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 of the NH$_2$- 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. Coexpres-

sion 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 G_α 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 K_{ACh} 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 G_α 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.

REFERENCES

Andrade, R., R.C. Malenka, and R.A. Nicoll. 1986. A G protein couples serotonin and GABA$_B$ receptors to the same channels in hippocampus. *Science (Wash. DC)*. 234:1261–1265.

Andrade, R., and R.A. Nicoll. 1987. Pharmacologically distinct actions of serotonin on single pyramidal neurones of the rat hippocampus recorded *in vitro.'J. Physiol. (Lond.)*. 394:99–124.

Ashford, M.L.J., C.T. Bond, T.A. Blair, and J.P. Adelman. 1994. Cloning and functional expression of a rat heart K_{ATP} channel. *Nature (Lond.)*. 370:456–459.

Ausubel, M.F., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl. 1993. Current Protocols in Molecular Biology. John Wiley and Sons, Inc., New York, NY.

Bauer, C.K., W. Meyerhof, and J.R. Schwartz. 1990. An inward-rectifying K$^+$ current in clonal rat pituitary cells and its modulation by thyrotrophin-releasing hormone. *J. Physiol. (Lond.)*. 429:169–189.

Blitzer, R.D., G. Omri, M. De Vivo, D.J. Carty, R.T. Premont, J. Codina, L. Birnbaumer, S. Cotecchia, M.G. Caron, R.J. Lefkowitz, E.M. Landau, and R. Iyengar. 1993. Coupling of the expressed α_{1B}-adrenergic receptor to the phospholipase C pathway in *Xenopus* oocytes. *J. Biol. Chem.* 268:7532–7537.

Breitwieser, G.E., and G. Szabo. 1985. Uncoupling of cardiac muscarinic and β-adrenergic receptors from ion channels by a guanine nucleotide analogue. *Nature (Lond.)*. 317:538–540.

Chan, K.W., J. Sui, J.A.A. Ladias, and D.E. Logothetis. 1995. Cloning and functional expression of human pancreatic and brain ATP-sensitive potassium channels. *Biophys. J.* 68:A353. (Abstr.)

Chandy, K.G., and G.A. Gutman. 1993. Nomenclature for mammalian potassium channel genes. *Trends Pharmacol. Sci.* 14:434.

Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156–159.

Codina, J., A. Yatani, D. Grenet, A.M. Brown, and L. Birnbaumer.

1987. The α Subunit of the GTP binding protein G_k opens atrial potassium channels. *Science (Wash. DC)*. 236:442–445.

Dascal, N., W. Schreibmayer, N.F. Lim, W. Wang, C. Chavkin, L. DiMagno, C. Labarca, B.L. Kieffer, C. Gaveriaux-Ruff, D. Trollinger, H.A. Lester, and N. Davidson. 1993a. Atrial G protein-activated K$^+$ channel: expression cloning and molecular properties. *Proc. Natl. Acad. Sci. USA.* 90:10235–10239.

Dascal, N., N.F. Lim, W. Schreibmayer, W. Wang, N. Davidson, and H. A. Lester. 1993b. Expression of an atrial G-protein activated potassium channel in *Xenopus* oocytes. *Proc. Natl. Acad. Sci. USA.* 90:6596–6600.

Doupnik, C.A., N. Davidson, and H.A. Lester. 1995. The inward rectifier potassium channel family. *Curr. Opin. Neurobiol.* 5:268–277.

Dunne, M.J., M.J. Bullett, G. Li, C.B. Wollheim, and O.H. Petersen. 1989. Galanin activates nucleotide-dependent K$^+$ channels in insulin-secreting cells via a pertussis toxin-sensitive G-protein. *EMBO J.* 8:413–420.

Duprat, F., F. Lesage, E. Guillemare, M. Fink, J.P. Hugnot, J. Bigay, M. Lazdunski, G. Romey, and J. Barhanin. 1995. Heterologous multimeric assembly is essential for K$^+$ channel activity of neuronal and cardiac G-protein-activated inward rectifiers. *Biochem. Biophys. Res. Commun.* 212:657–663.

Einhorn, L.C., and G.S. Oxford. 1993. Guanine nucleotide binding proteins mediate D_2 dopamine receptor activation of a potassium channel in rat lactotrophs. *J. Physiol. (Lond.)*. 462:563–578.

Fosset, M., H. Schmid-Antomarchi, J.R. de Weille, and M. Lazdunski. 1988. Somatostatin activates glibenclamide-sensitive and ATP-regulated K$^+$ channels in insulinoma cells via a G-protein. *FEBS Lett.* 242:94–96.

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. *Pflügers Archiv. (Eur. J. Physiol.)*. 391:85–100.

Harlow, E., and D. Lane. 1988. Antibodies: A Laboratory Manual. Cold Spring Harbor Press, Cold Spring Harbor, NY.

Inagaki, N., Y. Tsuura, N. Namba, K. Masuda, T. Gonoi, M. Horie, Y. Seino, M. Mizuta, and S. Seino. 1995. Cloning and functional characterization of a novel ATP-sensitive potassium channel ubiquitously expressed in rat tissues, including pancreatic islets, pituitary, skeletal muscle, and heart. *J. Biol. Chem.* 270:5691–5694.

Inoue, M., S. Nakajima, and Y. Nakajima. 1988. Somatostatin induces an inward rectification in rat locus coeruleus neurones through a pertussis toxin-sensitive mechanism. *J. Physiol. (Lond.)*. 407:177–198.

Ito, H., T. Sugimoto, I. Kobayashi, K. Takahashi, T. Katada, M. Ui, and Y. Kurachi. 1991. On the mechanism of basal and agonist-induced activation of the G protein-gated muscarinic K$^+$ channel in atrial myocytes of guinea pig heart. *J. Gen. Physiol.* 98:517–533.

Ito, H., R.T. Tung, T. Sugimoto, I. Kobayashi, K. Takahashi, T. Katada, M. Ui, and Y. Kurachi. 1992. On the mechanism of G protein βγ subunit activation of the muscarinic K$^+$ channel in guinea pig atrial cell membrane. *J. Gen. Physiol.* 99:961–983.

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. *J. Biol. Chem.* 257: 7210–7216.

Katada, T., and M. Ui. 1982b. Direct modification of the membrane adenylate cyclase system by islet-activating protein due to ADP-ribosylation of a membrane protein. *Proc. Natl. Acad. Sci. USA.* 79: 3129–3133.

Kofuji, P., N. Davidson, and H.A. Lester. 1995. Evidence that neuronal G-protein-gated inwardly rectifying K$^+$ are activated by Gβγ subunits and function as heteromultimers. *Proc. Natl. Acad. Sci. USA.* 92:6542–6546.

Kozak, J.A., K.W. Chan, J. Sui, and D.E. Logothetis. 1995. Heterologous expression of a potassium channel in clonal mammalian cells by direct microinjection of cDNA. *Biophys. J.* 68:A267 (Abstr.)

Krapivinsky, G., E.A. Gordon, K. Wickman, B. Velimirovic, L. Krapivinsky, and D.E. Clapham. 1995. The G-protein-gated atrial K$^+$ channel I_{KACh} is a heteromultimer of two inwardly rectifying K$^+$-channel proteins. *Nature (Lond.)*. 374:135–141.

Kubo, Y., E. Reuveny, P.A. Slesinger, Y. N. Jan, and L. Y. Jan. 1993. Primary structure and functional expression of a rat G-protein-coupled muscarinic potassium channel. *Nature (Lond.)*. 364:802–806.

Lesage, F., F. Duprat, M. Fink, E. Guillemare, T. Coppola, M. Lazdunski, and J.-P. Hugnot. 1994. Cloning provides evidence for a family of inward rectifier and G-protein coupled K$^-$ channels in the brain. *FEBS Lett.* 353:37–42.

Lesage, F., E. Guillemare, M. Fink, F. Duprat, C. Heurteaux, M. Fosset, G. Romey, J. Barhanin, and M. Lazdunski. 1995. Molecular properties of neuronal G-protein-activated inwardly rectifying K$^+$ channels. *J. Biol. Chem.* 270:28660–28667.

Lim, F.N., N. Dascal, C. Labarca, N. Davidson, and H.A. Lester. 1995. A G-protein-gated K channel is activated via β2-adrenergic receptors and Gβγ subunits in *Xenopus* oocytes. *J. Gen. Physiol.* 105:421–439.

Liman, E.R., J. Tytgat, and P. Hess. 1992. Subunit stoichiometry of a mammalian K$^+$ channel determined by construction of multimeric cDNAs. *Neuron.* 9:861–871.

Logothetis, D.E. 1987a. On the molecular regulation of the cardiac muscarinic K$^+$ channel by the subunits of GTP-binding proteins. Dissertation, Harvard University, Cambridge, MA.

Logothetis, D.E., Y. Kurachi, J. Galper, E. Neer, and D. Clapham. 1987b. The βγ subunits of GTP-binding proteins activate the muscarinic K$^+$ channel in heart. *Nature (Lond.)*. 325:321–326.

Logothetis, D.E., D. Kim, J.K. Northup, E. Neer, and D. Clapham. 1988. Specificity of action of guanine nucleotide-binding regulatory protein subunits on the cardiac muscarinic K$^+$ channel. *Proc. Natl. Acad. Sci. USA.* 85:5814–5818.

Logothesis, D.E., S. Movahedi, C. Satler, K. Lindpaintner, and B. Nadal-Ginard. 1992. Incremental reductions of positive charge within the S4 region of a voltage-gated K$^+$ channel result in corresponding decreases in gating charge. *Neuron.* 8:531–540.

Logothetis, D.E., B.F. Kammen, K. Lindpaintner, D. Bisbas, and B. Nadal-Ginard. 1993. Gating charge differences between two voltage-gated K$^+$ channels are due to the specific charge content of their respective S4 regions. *Neuron.* 10:1121–1129.

Lu, Z., and R. Mackinnon. 1994. Electrostatic tuning of Mg^{2+} affinity in an inward-rectifier K$^+$ channel. *Nature (Lond.)*. 371:243–245.

Methfessel, C.,V. Witzemann, T. Takahashi, M. Mishina, S. Numa, and B. Sakmann. 1986. Patch clamp measurements on *Xenopus laevis* oocytes: currents through endogenous channels and implanted acetylcholine receptor and sodium channels. *Pflügers Arch. (Eur. J. Physiol.)*. 407:577–588.

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. *Proc. Natl. Acad. Sci. USA.* 84:5487–5491.

Okabe, K., A. Yatani, and A.M. Brown. 1991. The nature and origin of spontaneous noise in G protein-gated ion channels. *J. Gen. Physiol.* 97:1279–1293.

Pennington, N.J., J. S. Kelly, and A.P. Fox. 1993. Unitary properties of potassium channels activated by 5-HT in acutely isolated rat dorsal raphe neurones. *J. Physiol. (Lond.)*. 469:407–426.

Pfaffinger, P.J., J. M. Martin, D.D. Hunter, N. M. Nathanson, and B. Hille. 1985. GTP-binding proteins couple cardiac muscarinic receptors to a K channel. *Nature (Lond.)*. 317:536–538.

Ponce, A., E. Bueno, E. Vega-Saenz de Miera, L. Chow, D. Hillman, S. Chen, M.B. Wu, X. Wu, L. Zhu, B. Rudy, and W.B. Thornhill. 1995. G protein-gated inward rectifying K channel proteins (GIRK1) are present in the soma and dendrites as well as nerve terminals of specific neurons in the brain. *J. Neurosci.* In press.

Reuveny, E., P.A. Slesinger, J. Inglese, J.M. Morales, J.A. Iniguez-Lluhi, R.J. Lefkowitz, H.R. Bourne, Y.N. Jan, and L.Y. Jan. 1994. Activation of the cloned muscarinic potassium channel by G protein βγ subunits. *Nature (Lond.)*. 370:143–146.

Rorsman, P., K. Bokvist, C. Ammala, P. Arkhammar, P.-O. Berggren, O. Larsson, and K. Wahlander. 1991. Activation by adrenaline of a low-conductance G protein-dependent K$^+$ channel in mouse pancreatic B cells. *Nature (Lond.)*. 349:77–79.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanger, F., S. Nicklen, and A.R. Coulson. 1977. DNA sequencing with chain-termination inhibitors. *Proc. Natl. Acad. Sci. USA.* 74: 5463–5467.

Soejima, M., and A. Noma. 1984. Mode of regulation of the ACh-sensitive K-channel by the muscarinic receptor in rabbit atrial cells. *Pflügers Arch. (Eur. J. Physiol.)*. 400:424–431.

Stanfield, P.R., N.W. Davies, P.A. Shelton, M.J. Sutcliffe, I.A. Khan, W.J. Brammar, and E.C. Conley. 1994. A single aspartate residue is involved in both intrinsic gating and blockage by Mg^{2+} of the inward rectifier, IRK1. *J. Physiol. (Lond.)*. 478:1–6.

Stühmer, W. 1992. Electrophysiological recording from *Xenopus* oocytes. *Methods Enzymol.* 207:319–339.

Takano, K., S. Asano, and N. Yamashita. 1994. Activation of G protein-coupled K$^+$ channels by dopamine in human GH-producing cells. *Am. J. Physiol.* 266:E318–E325.

Takao, K., M. Yosii, A. Kanda, S. Kokubun, and T. Nukada. 1994. A region of the muscarinic-gated atrial K$^+$ channel critical for acti-

vation by G protein βγ subunits. *Neuron.* 13:747–755.

Walker, J.E., M. Saraste, M.J. Runswick, and N.J. Gay. 1982. Distantly related sequences in the α- and β- subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J.* 1:945–951.

Wible, B.A., M. Taglialetela, E. Ficker, and A.M. Brown. 1994. Gating of inwardly rectifying K⁺ channels localized to a single negatively charged residue. *Nature (Lond.).* 371:246–249.

Wickman, K.D., J.A. Iniguez-Lluhi, P.A. Davenport, R. Taussig, G. B. Krapivinsky, M.E. Linder, A.G. Gilman, and D.E. Chapham. 1994. Recombinant G-protein βγ-subunits activate the muscarinic-gated atrial potassium channel. *Nature (Lond.).* 368:255–257.

Williams, J.T., W.F. Colmers, and Z.Z. Pan. 1988. Voltage- and ligand-activated inwardly rectifying currents in dorsal raphe neurons *in vitro. J. Neurosci.* 8:3499–3506.

Yang, J., Y.N. Jan, and L.Y. Jan. 1995. Control of rectification and permeation by residues in two distinct domains in an inward rectifier K⁺ channel. *Neuron.* 14:1047–1054.