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Cloning of a Xenopus laevis Inwardly Rectifying K^+ Channel Subunit That Permits GIRK1 Expression of I_{KACh} Currents in Oocytes

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Summary

Xenopus oocytes injected with GIRK1 mRNA express inwardly rectifying K^+ channels resembling I_{KACh}. Yet I_{KACh}, the atrial G protein–regulated ion channel, is a heteromultimer of GIRK1 and CIR. Reasoning that an oocyte protein might be substituting for CIR, we cloned XIR, a CIR homolog endogenously expressed by Xenopus oocytes. Coinjecting XIR and GIRK1 mRNAs produced large, inwardly rectifying K^+ currents responsive to m2-muscarinic receptor stimulation. The m2-stimulated currents of oocytes expressing GIRK1 alone decreased 80% after injecting antisense oligonucleotides specific to the 5′ untranslated region of XIR, but GIRK1/CIR currents were unaffected. Thus, GIRK1 without XIR or CIR only ineffectively produces currents in oocytes. This result suggests that GIRK1 does not form native homomultimeric channels.

Introduction

I_{KACh} is a G protein–gated, inwardly rectifying K^+ channel defined by electrophysiological methods in the plasma membranes of atrial myocytes and pacemaker cells (Sakman, et al., 1983; Soejima and Noma, 1984; Breitwieser and Szabo, 1985; Pfaffinger et al., 1985; Kurachi et al., 1986; Logothetis et al., 1987). I_{KACh} participates in slowing the heart rate following vagal nerve stimulation by opening in response to extracellular acetylcholine (ACh). ACh binds to m2-muscarinic receptors, activating intracellular G_i-type GTP-binding G proteins and releasing G-protein $\beta\gamma$ subunits ($G\beta\gamma$). Purified or recombinant $G\beta\gamma$ stimulates I_{KACh} when applied to the intracellular side of atrial myocyte membrane patches (Logothetis et al., 1987; Wickman et al., 1994). Recent work confirms the direct regulation of I_{KACh} by $G\beta\gamma$ by demonstrating the specific binding of $G\beta\gamma$ to purified native and recombinant I_{KACh} subunits (Krapivinsky et al., 1995b). G proteins are used by virtually every cell type as a response mechanism for hundreds of different extracellular signaling molecules including many neurotransmitters and hormones (Hedin et al., 1993). I_{KACh} has therefore received intensive study, as the first example of an ion channel directly gated by a G protein, and also as the first known target for $G\beta\gamma$. I_{KACh} continues to be an important model system for addressing both ion channel function and G-protein signal transduction.

In 1993, a rat atrial mRNA encoding the GIRK1 (Kir 3.1) polypeptide, discovered via a homology approach, was found to produce I_{KACh}-like ion channels when expressed in Xenopus laevis oocytes (Kubo et al., 1993). GIRK1 was also cloned independently based on its ability to give I_{KACh}-like ion channels in Xenopus oocytes (Dascal et al., 1993). GIRK1 is homologous to other inwardly rectifying, K^+ ion channel subunits (Kir subunits), and is structurally distinct from voltage-gated K^+ channel proteins (Kv; reviewed in Doupnik et al., 1995). Atrial I_{KACh} channels were assumed to be composed of GIRK1 polypeptides alone, since injecting GIRK1 mRNA into oocytes yielded channels functionally similar to native I_{KACh} (Dascal et al., 1993; Kubo et al., 1993). However, biochemical analysis of native heart muscle GIRK1 polypeptides demonstrated their association with another Kir channel subunit, CIR (Kir 3.4; Krapivinsky et al., 1995a). Conditions in atrial myocytes were mimicked in Xenopus oocytes by injecting mRNA for both GIRK1 and CIR into each cell. Coinjection yielded much larger whole-cell I_{KACh}-like currents than did injecting either GIRK1 or CIR mRNA alone. This result, apparently reflecting a greater number of functional plasma membrane ion channels, strongly supports the biochemical evidence that the native atrial I_{KACh} channel is formed by both GIRK1 and CIR. Similarly, the large currents resulting from coinjecting GIRK1 and a CIR-related protein, GIRK2 (Kir 3.2) into oocytes (Duprat et al., 1995; Kofuji et al., 1995; B. M. Velimirovic et al., unpublished data), together with data indicating that these Kir subunits colocalize in brain structures (Karschin et al., 1994; Lesage et al., 1994; Kobayashi et al., 1995), support the idea that some brain I_{KACh}-like channels are GIRK1/GIRK2 heteromultimers.

Since at least two native I_{KACh}-like ion channels appear to be heteromultimers, GIRK1 may not be able to form homomultimeric channels. Our previous results support this idea. Two mammalian cell lines transfected with a cDNA expression vector for GIRK1 yielded no detectable novel ion channels, despite the fact that GIRK1 protein was clearly detectable and I_{KACh}-like single channels were routinely detected in both cell lines following transfection of cDNA expression vectors for both GIRK1 and CIR (Krapivinsky et al., 1995a). If GIRK1 cannot form homomultimeric channels, the channels observed in Xenopus oocytes injected with GIRK1 but not CIR mRNA might result from GIRK1 combining with an endogenous oocyte protein capable of substituting for CIR. Here, we demonstrate that heterologous expression of GIRK1 alone does not efficiently produce G protein–regulated inwardly rectifying K^+ channels in Xenopus oocytes in the absence of an endogenous Xenopus CIR-related polypeptide, XIR. These results suggest that native G protein–regulated inwardly rectifying K^+ channels that contain GIRK1 subunits are heteromultimers.

Results and Discussion

Cloning and Sequence of XIR

A Xenopus laevis ovary cDNA library was screened at low stringency with a probe derived from the rat atrial CIR coding sequence (Krapivinsky et al., 1995a). Nine independently isolated cDNA clones possessed the

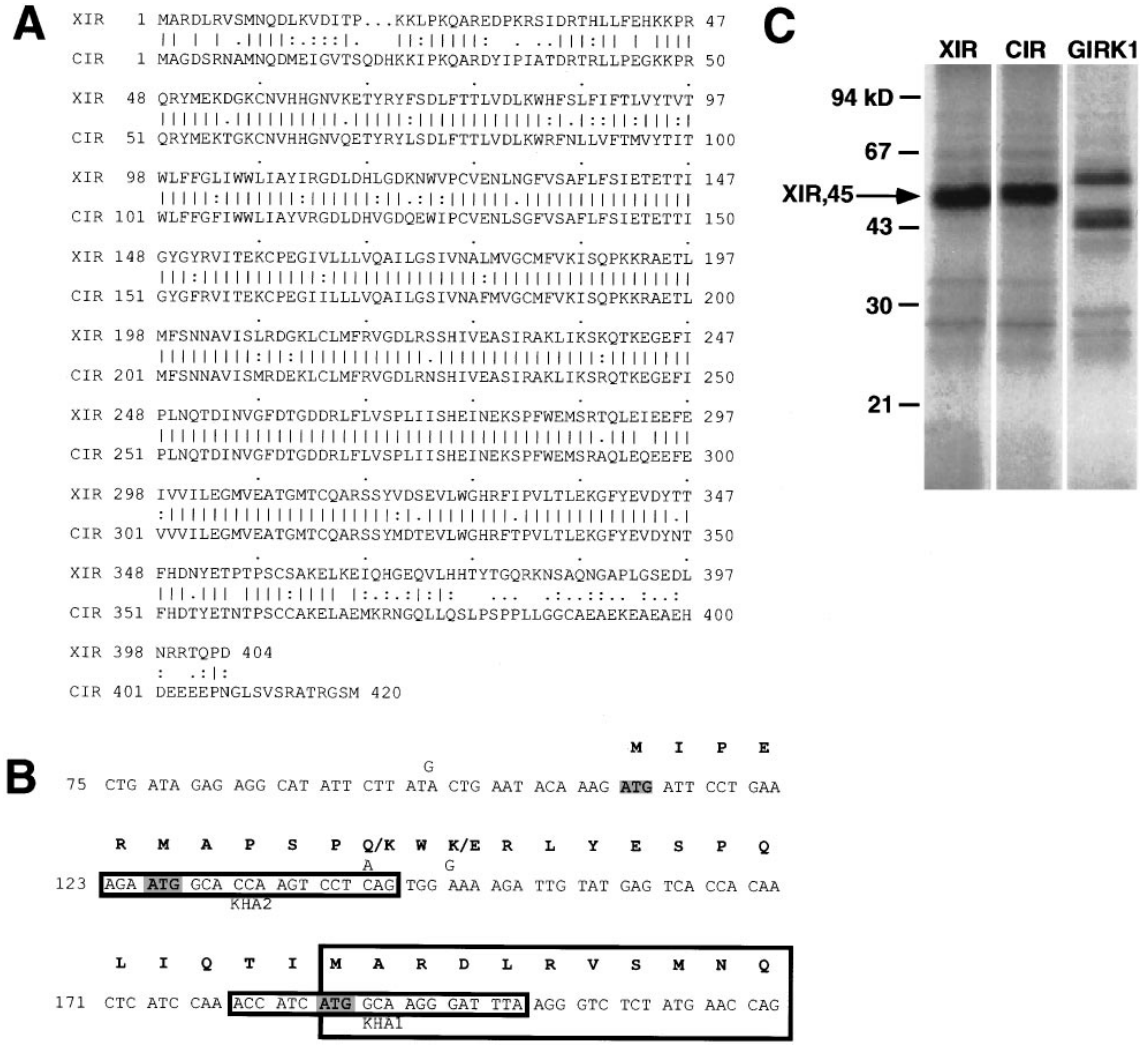

Figure 1. The XIR Gene Encodes a CIR-Related Protein

(A) The predicted amino acid sequence of XIR (beginning at ATG186; single-letter amino acid code) and comparison with CIR.

(B) Three possible translation start sites in the XIR cDNA are shaded. The corresponding protein sequence is shown above. The large box delineates the protein sequence initated at the most probable start site. Three single bp differences found in a subset of XIR cDNAs would generate the two amino acid changes shown (see text). Small boxes delineate the sequences complementary to antisense oligonucleotides *KHA1* and *KHA2*.

(C) XIR, CIR, and GIRK1 cDNAs were in vitro–translated in the presence of ^{35}S-methionine and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The 56 kDa (upper) band in lane 3 is GIRK1; the lower band is probably from incorrect initiation (see Krapivinsky et al., 1995a).

same CIR-homologous sequence, encoding a novel protein we named XIR (for Xenopus laevis inwardly rectifying K^+ channel). No other Kir homologs were isolated from the Xenopus library. XIR Clone 9A contained an apparently full-length 1.2 kb open reading frame, predicting a polypeptide of 404 amino acids (Figure 1A). In vitro translation of Clone 9A produced a protein of the predicted M_r, 45 kDa, which comigrated with the product of in vitro translation of the rat atrial CIR cDNA (Figure 1C). XIR exhibits 78% amino acid identity to the rat atrial CIR protein (Kir 3.4). The XIR protein is next most identical to the CIR-related Kir polypeptides GIRK2 (Kir 3.2; 72%) and GIRK3 (Kir 3.3; 68%). XIR is only 56% identical to GIRK1 (Kir 3.1) and is even less similar to other Kir polypeptides (49% and 45% identical to IRK1 and ROMK1, respectively), clearly placing XIR in the Kir 3.0 subfamily. XIR probably represents a distinctive Kir 3.0 subunit, since the Xenopus XIR and the rat CIR coding sequence DNA exhibit only relatively low identity (74.9%). In addition, the extreme carboxy-terminal region of XIR shares little similarity with CIR or any other cloned Kir subunit and is unique in lacking regions of acidic residues (Figure 1A). We therefore propose that XIR be classified as Kir 3.5.

The most probable initiation codon of XIR is at position 186, the first appearance of an in-frame ATG within a strong Kozak consensus sequence (Figure 1B) (Kozak, 1986). Use of this ATG would provide XIR with an amino terminal length most similar to that of CIR, and was therefore used to predict the protein product. However, unlike CIR, the XIR cDNA exhibits two in-frame ATGs upstream of position 186 (Figure 1B). Utilization of these

upstream ATGs would produce XIR proteins with novel amino-terminal extensions of 20 or 25 amino acids. Interestingly, each of three independently isolated XIR cDNAs possessed 3 single bp differences in this region that would alter 2 amino acids in these amino-terminal extensions. These 3 bp differences were confirmed by sequencing both strands. These three clones exhibited no additional changes within their coding regions but possessed an additional single bp difference just following the termination codon (A1400T; data not shown). Certain mammalian Kir genes exhibit alternative splicing of entire segments of 5′ and 3′ untranslated and coding sequences (reviewed in Doupnik et al., 1995); however, no such large differences were found among the XIR clones. One XIR transcript of 3.1 kb was detected in mRNA from defolliculated Xenopus oocytes by Northern blot analysis (data not shown), confirming the endogenous transcription of XIR by Xenopus oocytes.

Functional Homology of XIR and CIR Overexpressed in Xenopus Oocytes

We tested whether the CIR homolog, XIR, could associate with GIRK1 by overexpressing these subunits in oocytes via mRNA injection. All oocytes were also injected with mRNA encoding the m2-muscarinic receptor, to permit agonist stimulation of endogenous G proteins. Like CIR, XIR synergized with GIRK1 to produce large whole-cell inwardly rectifying K^+ currents in high-[K^+]-containing solutions following m2-receptor stimulation (Figure 2). For XIR and GIRK1 coinjected oocytes, the average peak current measured at −100 mV in the presence of ACh was 5.4 ± 0.5 μA, similar to currents of CIR and GIRK1 coinjected oocytes (7.2 ± 0.6 μA at −80 mV; Krapivinsky et al., 1995a), and significantly exceeding those of oocytes expressing GIRK1 alone (1.3 ± 0.1 μA) or XIR alone (0.2 ± 0.1 μA). Like I_{KACh} (Sakmann et al., 1983), ACh-induced currents from GIRK1-injected or GIRK1 and XIR coinjected oocytes showed strong inward rectification (Figure 2B). Oocytes overexpressing the XIR polypeptide in the absence of GIRK1 showed small ACh-stimulated currents that were significantly higher than the background currents of oocytes expressing m2 receptor alone (Figure 2C). Oocytes apparently lack an endogenous GIRK1-like partner for XIR, a result consistent with their small endogenous inwardly rectifying K^+ currents and with the fact that oocytes do not express mRNA transcripts similar to GIRK1 (Dascal et al., 1993). Xenopus oocytes contain many mRNAs and proteins required for embryonic development (Newport and Kirschner, 1982); thus, endogenous XIR is probably utilized to form an ion channel following subsequent expression of a GIRK1 homolog.

Expressing CIR alone in a mammalian cell line (CHO) produces distinctive, poorly resolved, fast (spiky), in-

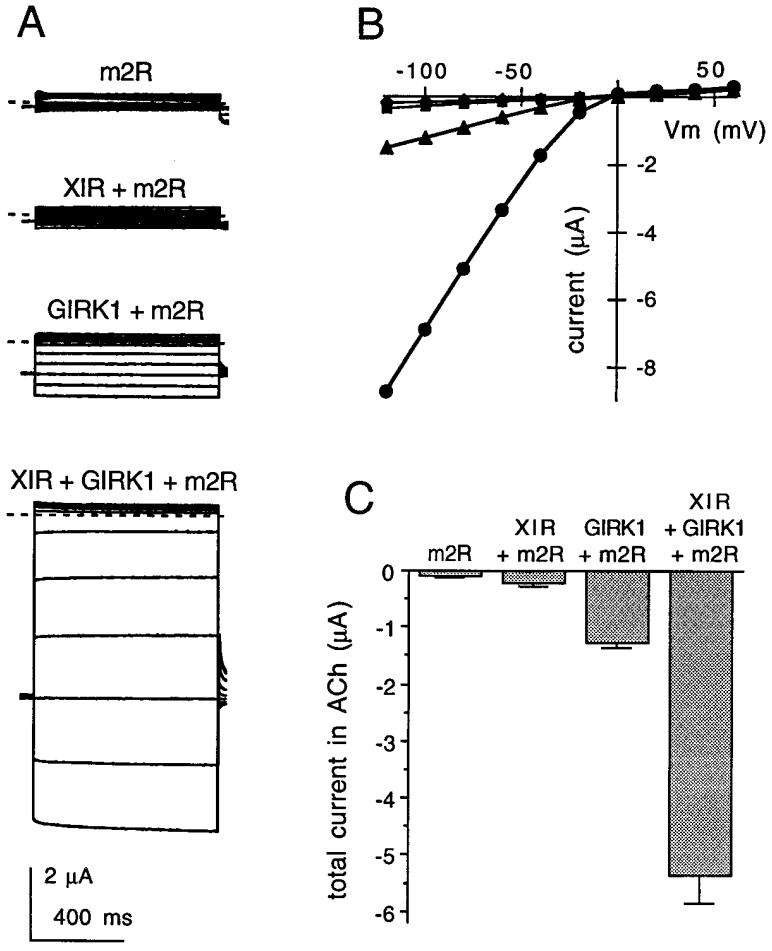

Figure 2. Coexpression of XIR and GIRK1 Produces Currents Much Larger than Expression of Either Subunit Alone

(A) Whole-cell currents measured in the presence of 5 μM ACh were recorded from oocytes injected with the indicated mRNAs. Voltage steps from −120 mV to +60 mV in 20 mV increments were applied from a holding potential of −80 mV.

(B) Current–voltage relations for the traces shown in A; m2R (diamonds), XIR + m2R (squares), GIRK1 + M2R (triangles), and XIR + GIRK1 + m2R (circles).

(C) Average current amplitudes were measured at −100 mV in 5 μM ACh for the indicated groups. Values shown in this and subsequent figures are mean ± SEM; n ranges from 9 to 18 for each column. The average current for each group is significantly different from the control m2R group.

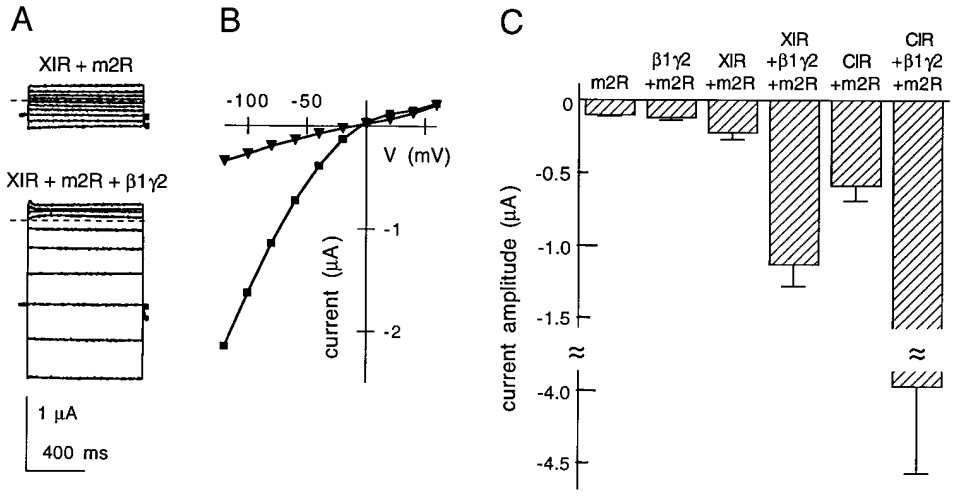

Figure 3. XIR Is Stimulated by Gβ1γ2

(A) Whole-cell currents measured in control (XIR + m2R) and in Gβ1γ2-coinjected oocytes (XIR + m2R + Gβ1γ2). Voltage protocol is as in Figure 2A.

(B) Current–voltage relations for the traces shown in (A).

(C) Average current amplitude at −100 mV. Currents for the m2R + XIR and m2R + CIR groups were measured in the presence of 5 μM ACh. Since application of ACh did not evoke any additional current in the m2R-alone group or in cells expressing Gβ1γ2 (see text), the means from these groups include some values measured with agonist and some without agonist; n ranges from 9 to 16 for each column.

wardly rectifying K^+ channels that are stimulated by application of Gβγ to the cytoplasmic side of inside-out patches (Krapivinsky et al., 1995a). Similarly, coinjecting mRNAs for G-protein Gβγ subunits (Gβ1γ2) stimulated the whole-cell currents of CIR-expressing oocytes (Figure 3C). To test whether XIR is also G protein–regulated, we coexpressed XIR and Gβγ. Like CIR, currents observed following injection of XIR mRNA were significantly enhanced by Gβγ coexpression (Figure 3). Although XIR/Gβγ expression yielded quantitatively smaller currents than did CIR/Gβγ (Figure 3C), in neither case did m2-receptor stimulation increase the currents further (data not shown). Clearly, both CIR and XIR can participate in forming G protein–stimulated K^+ channels in oocytes. Gβγ expression should also stimulate any endogenous XIR channels. Although we could not demonstrate statistical significance, oocytes expressing only Gβγ and m2 receptor consistently showed higher currents than oocytes expressing m2 receptor alone (Figure 3C). This result suggests that oocytes express only small numbers of endogenous XIR channels, consistent with our finding that endogenous XIR limits K^+ currents produced by GIRK1 mRNA injection (below).

Antisense Oligonucleotides to XIR Ablate K^+ Currents Produced by Expressing GIRK1 Alone

To test the effects of knocking out endogenous XIR, we synthesized two antisense phosphothioated oligonucleotides complementary to unique 5′ regions of the XIR cDNA (*KHA1* and *KHA2*; see Figure 1B). Both oligos interfere with XIR expression, since injecting either *KHA1* or *KHA2* together with XIR and GIRK1 mRNAs significantly decreased the resulting ACh-induced K^+ current (Figure 4). We next examined the effects of these oligonucleotides on K^+ currents resulting from expressing GIRK1 alone. When either *KHA1* or *KHA2* XIR antisense oligonucleotides were injected into oocytes along with GIRK1 mRNA, the resulting ACh-induced K^+ currents were significantly decreased (79% and 83% reduction, respectively). This did not result from a nonspecific

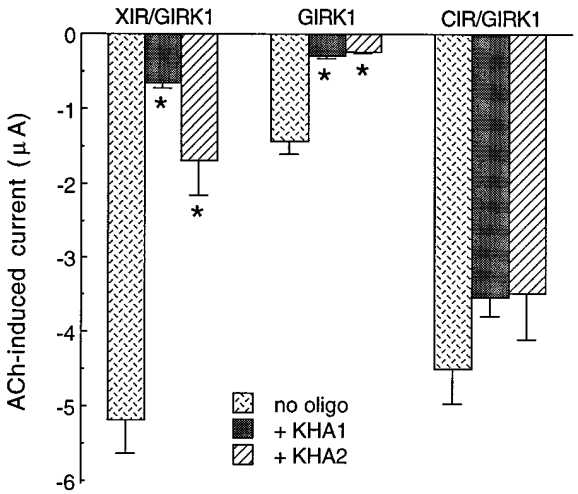

Figure 4. Antisense Knockout of XIR Inhibits GIRK1 Current

The average current at −100 mV was measured in oocytes injected with the indicated channel mRNAs plus the m2R and with or without 13 ng of antisense oligonucleotide. To eliminate the contribution of endogenous and leak components, the ACh-induced current was calculated by subtracting current without agonist from current in the presence of 5 μM ACh; n ranges from 4 to 7 for each column. Asterisks indicate mean values that are significantly different from control (no oligo) values.

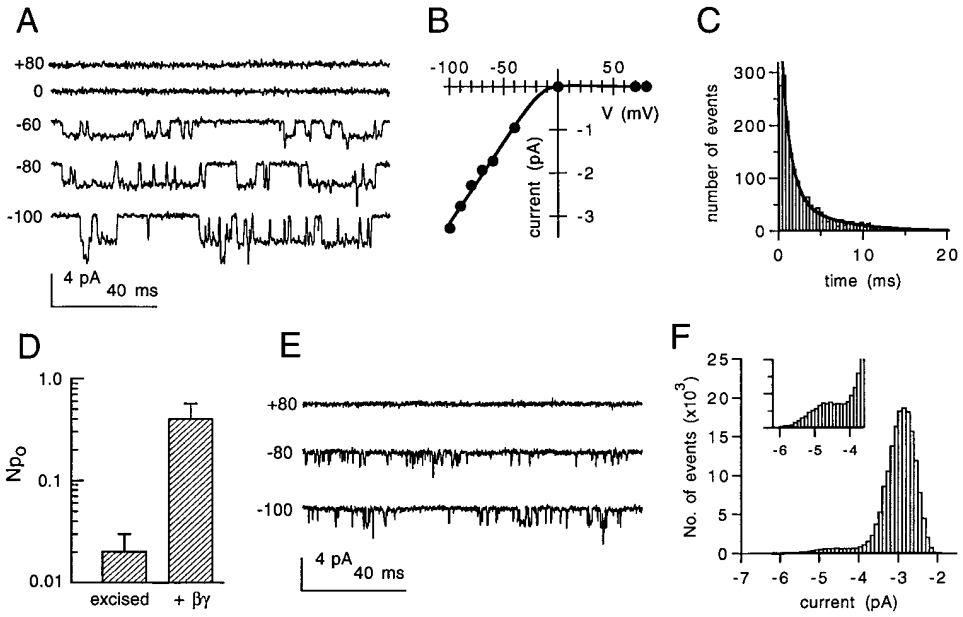

Figure 5. Single-Channel Characteristics of XIR/GIRK1 and XIR

(A) Examples of single-channel activity in an excised patch from an oocyte expressing XIR, GIRK1, m2R, and Gβ1γ2. The potential applied to the patch is indicated to the left of each trace in mV.

(B) Current–voltage relationship for the channel events observed in the patch shown in (A). Since no openings were observed at or above 0 mV, a current of 0 pA was assigned to these voltages. A least squares fit to the linear portion of the curve (−100 mV to −40 mV) yielded a single-channel conductance of 38 pS.

(C) Histogram of channel open times at −80 mV for the patch in (A). The smooth line is a double exponential fit for this patch with time constants 1.0 ms and 5.4 ms.

(D) Channel activity (Npo) at −80 mV is shown for excised patches from oocytes expressing XIR, GIRK1, and m2R before and after the addition of 20 nM purified bovine brain Gβγ subunits to the bath. Values shown are mean ± SEM for three patches.

(E) Single channels in an excised patch from an oocyte expressing XIR and Gβ1γ2, but not GIRK1.

(F) All-points amplitude histogram at −100 mV for the patch shown in (E). Inset: same data shown on expanded Y scale.

decrease in GIRK1 protein levels, since currents resulting from CIR and GIRK1 coexpression were unaffected by antisense coinjection. The availability of endogenous XIR therefore limits the ability of GIRK1 alone to form K^+ channels in oocytes.

Single-Channel Analysis Shows that XIR/GIRK1 Channels Resemble I_{KACh}

The simplest explanation for the above results is that GIRK1 more efficiently forms heteromultimeric, rather than homomultimeric, ion channels. This hypothesis predicts that the channels derived from expressing GIRK1 alone in oocytes are actually XIR/GIRK1 heteromultimers. We therefore measured the single-channel characteristics of XIR/GIRK1 channels and compared them with published values derived from expressing GIRK1 alone. Oocytes were injected with mRNA encoding XIR, GIRK1, and the m2 receptor. Gβ1γ2 subunits were also sometimes expressed to increase agonist-independent channel activity in patches. XIR/GIRK1 single channels were inwardly rectifying and had a conductance of 38 pS in symmetrical 140 mM K^+ in the range of −100 to −40 mV (Figures 5A and 5B). Figure 5C shows a histogram of XIR/GIRK1 channel open times best fit by two exponentials. The mean time constants for three patches were 1.0 ± 0.1 and 4.1 ± 0.8 ms at −80 mV (the fractional amplitudes were 58% and 42%, respectively). These values are similar to those reported following expression of GIRK1 alone in oocytes (Dascal et al., 1993; Kubo et al., 1993). Single XIR/GIRK1 channels in excised patches were activated 30-fold by addition of 20 nM purified bovine brain Gβγ to the bath (Figure 5D), consistent with G protein activation of channels resulting from expressing GIRK1 alone in oocytes (Dascal et al., 1993; Kubo et al., 1993; Reuveny et al., 1994; Lim et al., 1995). The appearance of channels with these characteristics required both XIR and GIRK1 expression, since channels derived from overexpressing XIR alone had shorter open times (Figure 5E) and poorly resolved single-channel amplitudes (Figure 5F), similar to channels resulting from expressing CIR alone (Krapivinsky et al., 1995a).

In summary, we find that GIRK1 cannot efficiently form K^+ channels in Xenopus oocytes in the absence of coexpressed CIR or an endogenous CIR homolog, XIR. Injecting XIR antisense oligonucleotides significantly decreased oocyte currents arising from expressing GIRK1 alone, while leaving CIR/GIRK currents unaffected. The fact that GIRK1-derived currents increase significantly upon expression of CIR (Krapivinsky et al., 1995a) or additional XIR (above) additionally supports this result. We also found that channels resulting from oocyte expression of GIRK1 alone are indistinguishable at the single-channel level from channels resulting from coex-

pressing GIRK1 and XIR. We conclude that expressing GIRK1 alone leads primarily to GIRK1/XIR channels on the oocyte plasma membrane. The heteromultimeric nature of these channels resembles native atrial I_{KACh}, which is a complex of GIRK1 and CIR (Krapivinsky et al., 1995a). Together with our failure to detect I_{KACh} in two mammalian cell lines expressing GIRK1 alone (Krapivinsky et al., 1995a), these results indicate that GIRK1 cannot form a functional homomultimeric G protein–regulated inwardly rectifying K^+ channel. The I_{KACh} channels recently described in an insulinoma cell line made to express GIRK1 (Philipson et al., 1995) most likely result from GIRK1 combining with endogenous CIR, since we have documented CIR expression by two other insulinoma cell lines and by pancreatic islets (G. Krapivinsky, unpublished data).

In the Kir 3.0 family, GIRK1/Kir 3.1 appears unique in requiring another subunit (XIR/Kir 3.5, GIRK2/Kir 3.2, or CIR/Kir 3.4) in order to form inwardly rectifying K^+ channels. In contrast, the distinctive spiky channels readily observed after expressing XIR, CIR, or GIRK2 alone in oocytes or cell lines suggest that these subunits can form homomultimeric channels (above; Duprat et al., 1995; Kofuji et al., 1995; Krapivinsky et al., 1995a; B. M. Velimirovic et al., unpublished data), even though native channels of this type have not been reported. Both the GIRK1 heteromultimeric channels and the XIR, CIR, or GIRK2 homomultimeric channels are constitutively activated by coexpression of G protein $\beta1\gamma2$ subunits (above; Kofuji et al., 1995; B. M. Velimirovic et al., unpublished data). Apparently, all of these members of the Kir 3.0 family, not just GIRK1, form G protein–regulated inwardly rectifying K^+ channels. The function of GIRK3/Kir 3.3 is even less clear, but it may bind and inhibit GIRK2 functions (Kofuji et al., 1995). Future work will delineate the interdependent and heteromultimeric nature of the G protein–regulated family of Kir 3.0 subunits. It will be essential, however, to take into account the tendency of GIRK1 to form I_{KACh}-like channels by combining with endogenous proteins, as demonstrated here for GIRK1 expressed in Xenopus oocytes.

Experimental Procedures

cDNA Cloning and In Vitro Translation

We screened 10^6 pfu of a Xenopus laevis ovary cDNA library (λ-ZAPII; Stratagene) with a 1.8 kb XhoI–PstI fragment containing most of the rat atrial CIR coding sequence. Rinsing was performed under conditions of low stringency (2X SSC, 0.1% SDS, 56°C). DNA sequencing identified one hybridizing clone as a CIR homolog. This clone was used to screen 2×10^6 additional pfu at higher stringency (rinsing at 0.1X SSC, 0.1% SDS, 65°C), generating a total of nine XIR cDNA clones. DNA sequencing revealed that each was a partial or full-length cDNA with the same open reading frame. One full-length cDNA (Clone 9A) was completely sequenced in both directions using an oligonucleotide-directed approach. Sequencing of other clones provided sequence confirmation and additional 3′ untranslated information. The coding sequence of XIR has been submitted to GenBank (Accession # U42207). Sequence analyses and GenBank comparisons were run on a VAX computer using the Genetics Computer Group, Inc., Wisconsin Package software, version 8.0. For Figure 1C, 0.5 μg of Clone 9A plasmid (XIR) was transcribed from the T3 promoter and translated for 1.5 hr at 30°C in the presence of ^{35}S-methionine, using a TNT in vitro translation kit (Invitrogen).

Oocyte Expression

Xenopus oocyte preparation and handling were as described (Dascal and Lotan, 1992; Quick and Lester, 1994). In brief, stage V and VI oocytes were defolliculated by treatment with 2 mg/ml collagenase (Worthington Biochemical Corp.) for 2–3 hr, and injected with mRNA 12–24 hr later. The incubation medium was 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, 2.5 mM Na-pyruvate, 50 μg/ml gentamycin, and 500 μM theophylline (pH 7.4), sometimes supplemented with 2%–5% horse serum (GIBCO BRL). mRNA injected per oocyte was 10–20 ng XIR, 1–2 ng GIRK1, 2 ng CIR, 200–400 pg m2 receptor, 2.5 ng β1, and 1.25 ng γ2. mRNAs were transcribed in vitro from cDNA clones using either mCAP (Stratagene) or Megascript (Ambion) kits. Transcription templates for Gβ1 and Gγ2 were, as in Kubo et al., 1993, created by subcloning Gβ1 (human) and Gγ2 (bovine) cDNAs into a vector (pFROGY) derived from pCDM6 (A. Connolly, UCSF), which contains an SP6 promoter and Xenopus β-globin 5′ and 3′ untranslated regions. GIRK1 was in pBluescriptII KS(−), and the m2 receptor (human) was in pGEM3z. XIR mRNA was transcribed using the T3 promoter of Clone 9A in the form of an excised library λ phage Bluescript plasmid, and contained 187 bp 5′ and 2512 bp 3′ untranslated sequences in addition to the XIR coding sequence. For antisense experiments, 13 ng of each phosphothioated oligonucleotide (see Figure 1B for sequences) was injected per oocyte at the same time as the mRNAs.

Electrophysiology

Oocyte currents were recorded 2–4 days postinjection at 22°C–25°C by the two-electrode voltage-clamp method using a Turbo-tech 01C amplifier and filtered at 300 Hz with an 8-pole Bessel filter. Inwardly rectifying K^+ channel currents were recorded in a high [K^+] solution containing 96 mM KCl, 2 mM NaCl, 1.0 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM Na-HEPES (pH 7.4). When indicated, 5 μM ACh (Sigma) was added to the bath. Microelectrodes contained 3.0 M KCl and were pulled to 1–2 MΩ resistance. Statistical significance was determined using Student's t test and $p < .05$.

Single-channel currents were measured with an Axopatch 200A patch-clamp amplifier (Axon Instruments), filtered at 10 kHz, and stored on magnetic tape. For single XIR/GIRK1 channel analysis, currents were replayed from tape, filtered at 2.5 kHz, and sampled at 12.5 kHz. For single XIR-alone channel analysis, currents were filtered at 5 kHz and sampled at 20 kHz. For display, filtering was 1.5 kHz for Figure 5A and 3 kHz for Figure 5E. The bath and pipette solutions contained 140 mM KCl, 2 mM $MgCl_2$, 5 mM HEPES, 5 mM EGTA (pH 7.2). Stretch-activated channels, which have a conductance nearly identical to I_{KACh} but do not exhibit inward rectification, were often present in patches. To suppress stretch channels, 10–100 μM $GdCl_3$ was sometimes included, and EGTA omitted, from the pipette solution. However, since $GdCl_3$ did not always completely suppress stretch channels, we excluded from analysis any patches that exhibited channel activity in the outward direction. To determine channel activity (represented as Npo), continuous single-channel currents were integrated over a fixed time interval following baseline subtraction to obtain the total current, I. Npo was then calculated by dividing I by the single-channel current amplitude, i. Bovine brain G-protein Gβγ subunits used for single-channel stimulation were purified as described (Wickman et al., 1994).

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