Inhibition of an Inward Rectifier Potassium Channel (Kir2.3) by G-protein βγ Subunits

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The molecular basis of G-protein inhibition of inward rectifier K⁺ currents was examined by co-expression of G-proteins and cloned Kir2 channel subunits in Xenopus oocytes. Channels encoded by Kir2.3 (HRK1/HIR/BIRK2/BIRK1) were completely suppressed by co-expression with G-protein βγ subunits, whereas channels encoded by Kir2.1 (IRK1), which shares 60% amino acid identity with Kir2.3, were unaffected. Co-expression of Goi and Gai subunits also partially suppressed Kir2.3 currents, but Goi, Gai, and a constitutively active mutant of Goi (Q204L) were ineffective. Gβγ and Kir2.3 subunits were co-immunoprecipitated using an anti-KR2.3 antibody. Direct binding of G-protein βγ subunits to fusion proteins containing Kir2.3 N terminus, but not to fusion proteins containing Kir2.1 N terminus, was also demonstrated. The results are consistent with suppression of Kir2.3 currents resulting from a direct protein-protein interaction between the channel and G-protein βγ subunits. When Kir2.1 and Kir2.3 subunits were coexpressed, the G-protein inhibitory phenotype of Kir2.3 was dominant, suggesting that co-expression of Kir2.3 with other Kir subunits might give rise to novel G-protein-inhibitable inward rectifier currents.

A large number of cDNAs encoding inwardly rectifying K⁺ channel subunits have now been cloned and exogenously expressed (1). A subfamily of these channels (Kir3) are activated by Gβγ proteins which bind directly to the channel (2, 3). This G-protein activation of Kir3 is probably responsible for vagal slowing of the heart and certain instances of synaptic inhibition in the brain (4). In numerous other processes, including thyrotrophin-releasing hormone-induced prolactin secretion in GH3 cells (5), microglia activation (6), and guard cell regulation of trophin-releasing hormone-induced prolactin secretion in GH3 cells (7), G-protein action is mediated via cells (5), microglia activation (6), and guard cell regulation of prolactin secretion in GH3 cells. We report here that one member, Kir2.3 (HRK1/HIR/BIRK2/BIRK1, see Refs. 8 and 9) is inhibited by co-expression with G-protein βγ subunits, whereas another member, Kir2.1 (IRK1, Ref. 10), which shares 60% amino acid identity, is unaffected. Co-immunoprecipitation of Gβγ and direct binding of G-protein βγ subunits to Kir2.3, but not Kir2.1, suggest that the inhibition of Kir2.3 currents results from a direct protein-protein interaction. When Kir2.1 and Kir2.3 subunits are coexpressed, the G-protein inhibitory phenotype of Kir2.3 is dominant. This suggests that, in native tissues, co-expression of Kir2.3 with different Kir subunits could give rise to numerous types of G-protein-inhibitable inward rectifier current.

EXPERIMENTAL PROCEDURES

Kir and G-protein expression in oocytes—cDNAs were subcloned into the transcription-competent vector pBlueScript SK(−) and propagated in Escherichia coli XL-1 blue. cRNA was transcribed in vitro using T7 or T3 RNA polymerase and capped from linearized cDNA. Stage V–VI Xenopus oocytes were isolated by partial ovariectomy under tricaine anesthesia and then defolliculated by treatment with 1 mg/ml collagenase (Type IA, Sigma) in 0 mM Ca²⁺ (ND96) (below) for 1 h. From 2 to 24 h after defolliculation, oocytes were pressure injected with ~50 nl of cRNA (1–100 ng/μl). Oocytes were maintained at room temperature in ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 5 mM Na-HEPES, pH 7.5) containing 2 mM CaCl₂ and supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml) for 1–2 days prior to recording. Kir currents were measured using the two-electrode voltage clamp technique in a small chamber (200 μl) mounted on the stage of a binocular microscope (SMZ-1, Nikon Instruments). The chamber was connected through agar bridges to the current sensing headstage of the voltage clamp amplifier (OC-725 Oocyte Clamp, Warner Instruments Corp.) and constantly perfused with a laminar flow of bathing solution supplied by one of five reservoirs connected to a manifold at the inlet to the chamber. Experiments were performed at room temperature (19–22°C). Intracellular electrodes were filled with 3 mM KCl and had tip resistances from 0.5 to 2 MΩ. Data were digitized on-line and stored on a 486 computer or were digitized at 22 kHz onto videotape (Neuro-corder DR-890, Neuro-Data Instruments) for off-line analysis. Currents were recorded in KD98 solution (98 mM KCl, 1 mM MgCl₂, 5 mM K-HEPES, pH 7.5).

Co-immunoprecipitation of G-proteins and Kir2.3 Subunits—A peptide corresponding to amino acids 424–436 of Kir2.3 (peptide B2C) was synthesized, coupled to bovine serum albumin, and used in the generation of rabbit polyclonal antibodies, which were affinity-purified before use for Western blotting (11). HEK293 cells were grown as described (12) and transfected by the CaPO₄ method with Kir2.3 or vector alone. Cells were lysed in buffer A (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors (2 μg/ml chymostatin, 2 μg/ml pepstatin, 2 μg/ml antipain, 4 μg/ml leupeptin)). Lysates were centrifuged and the supernatant was removed and dialuted 1:1 in 8 M urea. Protein sample buffer was added to a final concentration of 1% SDS, 1% β-mercaptoethanol, 1% glycerol. Samples were resolved by SDS-PAGE (10%)
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and transferred to Immobilon P (Millipore) in CAPSβ1 buffer (1 mM CAPS, 10% MeOH). Blots were processed for Western blotting and detection with the α-B2C antibody by the Renaissance system (DuPont NEN, Ref. 13). Transfected cells were metabolically labeled as described (14). Following labeling, cells were harvested and processed as described (15). Detergent-solubilized membrane fractions were pre-

cleared with 40 µl of protein A-agarose/ml and 20 µl/ml preimmune serum for 30 min, and 8 µg/ml α-B2C rabbit polyclonal antiserum was added and incubated for 2 h at 4 °C. Immune complexes were recovered with 15 µl of protein A-agarose (Oncogene Science catalogue no. IP06) and washed four times in Buffer A with 400 mM NaCl and 1% Triton X-100. Immunoprecipitates were resuspended in sample buffer, and urea was added to a final concentration of 4 M, boiled, and resolved by SDS-PAGE (10%). Gels were dried and opposed to film for 24 h. For co-immunoprecipitation experiments, HEK293 were transfected as described above with 5 µg of Kir 2.3 or vector alone, 2.5 µg of βγ subunit expression vector, and 2.5 µg of γ2 subunit expression vector. 14 h later, cells were harvested and washed twice in phosphate-buffered saline. Pelleted cells were lysed by resuspending in 1 ml of ice-cold Buffer A with 10% glycerol, 0.1% Triton X-100. Insoluble material was removed by centrifugation for 15 min, 16,000 × g at 4 °C. The supernatant was transferred to a fresh tube. A 15-µl aliquot was removed and mixed with sample buffer (5% SDS, 5% β-mercaptoethanol, 5% glycerol). To the remaining lysate, α-B2C rabbit polyclonal antiserum was added and incubated with mixing overnight at 4 °C. The following day, immune complexes were recovered with 15 µl of protein A-agarose (Oncogene Science catalogue no. IP06) and washed twice. The immunoprecipitate was resuspended in 15 µl of sample buffer, boiled, resolved by SDS-PAGE (12%), transferred, and processed for Western blotting as described above. The Gβ antibody (Santa Cruz Biotechnology, catalogue no. sc-378) was used at a 1:200 dilution. Autoradiographic exposure was 60 s.

Binding of G-proteins to Kir Fusion Proteins—aDNAs encoding the N and C termini of Kir2.1, -2.3, and -3.1 were generated by polymerase chain reaction and cloned in-frame to the GST coding sequence in pGEX-4T. The resulting polymerase chain reaction fragments coded for: Kir2.1 N-term, AA1–84; Kir2.1 C-term, AA182–428; Kir2.3 N-term, AA1–58; Kir2.3 C-term, AA171–442; Kir3.1 N-term, AA1–85; Kir3.1 C-term, AA183–501. Expression of fusion protein was performed as described (16), and quantification of bacterial lysates was performed by adding variable volumes of lysate to glutathione agarose. Fusion protein was eluted by addition of 15 µl of protein load buffer (5% SDS, 5% β-mercaptoethanol, 5% glycerol) and boiling for 2 min. Samples were resolved by SDS-PAGE (12%), and the gel was stained with Coomassie Blue. Bovine serum albumin of known concentration was loaded onto the same gel to quantitate fusion protein binding to the agarose. Lysate volumes yielding 500 ng for C-terminus and 2.5 µg for N-terminus fusion protein were incubated with 20 µl of glutathione agarose as described above. Following the wash, the affinity resin was equilibrated in βγ binding buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% polyoxy-ethylene 9 lauryl ether (Sigma P 9641)). Purified bovine brain Gβγ was added (17). Protein concentrations in the final assay volume of 40 µl were as follows: 60-kDa C-term fusion protein, 200 nM; 36-kDa N-term fusion protein, 1.2 µM; Gβγ subunits, 80 nM. The mixture was mixed every 3 min for 30 min on ice. The fusion protein-agarose was then washed four times in 1 ml of Gβγ binding buffer. Following the wash, samples were prepared and processed for Western blotting as described above. Autoradiographic exposure was 30 s. Following exposure, the blot was stripped and reprobed with an α-GST antibody (Santa Cruz Biotechnology, catalogue no. sc-4599) to ensure that equal quantities of fusion proteins were used. In experiments in which Gβγ was preincubated with GoGDP or Gαi,GTPγ (18) (1 µM Go1, preincubated with either 50 µM GTPγS or GDP for 90 min at 30 °C and incubated at 4 °C for 30 min.

RESULTS AND DISCUSSION

To examine the effects of G-proteins on Kir2 channels, we co-expressed cloned Kir2.1 (10) or Kir2.3 (8, 9, 19) subunits with G-protein α, β, and γ subunits in Xenopus oocytes. Neither Go1 nor Gβγ subunits affected currents through Kir2.1 channels (Fig. 1). In contrast, 24 h after RNA injection, Gβγ expression completely suppressed Kir2.3 currents, and a par-

Fig. 1. G-protein βγ subunits inhibit Kir2.3, but not Kir2.1 currents. a, representative 2-microelectrode voltage-clamped currents through Kir2.3 and Kir2.1 channels, expressed alone or with Gβγ,γ2 subunits. In each case, the same amount of Kir cRNA was injected (25 ng). Scale bars represent 1 µA and 100 ms. b, mean steady-state current-voltage relationships for experiments of the type shown in a (n = 5–7 oocytes, all experiments performed on the same batch of oocytes), normalized to the mean current at −140 mV in oocytes expressed without G-protein subunits. The bars indicate S.E. where larger than the symbol size. c, averaged currents at −140 mV from oocytes expressing Kir2.1 or Kir2.3 subunits alone or with G-protein subunits (relative to current without G-protein co-expression). Bars indicate S.E. (n = 5–7 oocytes). At −140 mV, the average currents from oocytes expressing Kir2.1 or Kir2.3 alone were −42.1 ± 10.33 µA, respectively.
subunits gave rise to a Kir current that was similar in voltage dependence and kinetic properties to the individual Kir2 currents. Given that Kir channels form as tetramers (21, 22), and assuming that Kir2.1 and 2.3 subunits coassemble randomly, only 6% (1/24) of the expressed channels will be homomeric (relative to current without G-protein co-expression). In a 1:1 mixture of Kir2.1 and Kir2.3, the simplest interpretation of these results is that not only can Kir2.1 and Kir2.3 subunits form homomultimeric channels, but probably only one Kir2.3 subunit is required for channels to show sensitivity to Gβγ inhibition.

The striking difference between the effects of G-proteins on the structurally related Kir2.1 and Kir2.3 channels suggests a specific interaction between the G-proteins and Kir2.3 channel subunits. When Kir2.3, Gβγ, and Gγ2 were co-expressed in HEK293 cells, immunoprecipitation of Kir2.3 with anti-Kir2.3 antibodies also precipitated Gβ (Fig. 3). In vitro assays further establish direct interactions between Gβγ subunits and Kir2.3 (Fig. 4). There was no measurable binding of Gγ subunits to glutathione (GST) itself, nor to GST-fusion proteins containing the C-terminal domains of Kir2.1 or -2.3 (not shown). However, Gβ bound to the N-terminal domain of Kir2.3, at levels comparable to the binding of Gγ subunits to the N-terminal domain of Kir3.1 (Fig. 2 and Refs. 23 and 24). No binding of Gβ to Kir2.1 was detected, consistent with the finding that Kir2.1 channel activity was unaffected by co-expression with Gβγ subunits.

The results presented thus far suggest that Kir2.3 channels provide a novel inward rectifier target for inhibition by G-proteins, with Gβγ having opposite effects on Kir3 channels and on Kir2.3 channels. These findings may explain the numerous instances of G-protein activation leading to Kir channel inhibition in native tissues. However, inhibition by both Ga and Gβγ is not readily explained by current paradigms of G-protein action, wherein the actions of Gβγ are often independent of Ga, or antagonized by Ga subunits (25–28). To gain further insight into the nature of the Go binding to and inhibition of Kir2.3 channels, we examined the effects of activated Ga subunits on Kir subunits (Fig. 2). We co-expressed Kir2.3 with a constitutively activated Ga1 mutant (Fig. 2a) in which a glutamine at residue 204 is replaced by a leucine (GaQ204L) (29). Kir2.3 currents were unaffected by co-expression with...
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GaQ204L. The simplest interpretation of these results is that activated Ga does not interact with Kir2.3 and suggests that the inhibitory effect of co-expression of Kir2.3 with Ga arises from GaGDP interaction with the channel or through an indirect mechanism. It is not known whether overexpressed Ga in oocytes is likely to be in a GTP- or GDP-bound form, and thus it is not possible to directly predict what interactions should occur with co-expressed Kir3.3 subunits. In vitro, GaQ1 bound only weakly to a GST-fusion protein containing the N-terminal domain of Kir2.3 (not shown). In addition, preincubation of GDP-bound GaQ1, but not GTPγS-bound GaQ1, with Gβγ prevents Gβγ binding to the N-terminal domain of Kir2.3 (Fig. 4b), suggesting that the heterotrimeric G-protein does not associate with the channel terminus, in parallel with G-protein interactions with Kir3.1 (24). Given the variability of Ga effects on the channel (partial inhibition by GaQ1 and GaQ4, no inhibition by GaQ3 and GaQ4) and the ubiquity of Gβγ isoforms in effector action (27), we predict that G-protein activation is likely to cause inhibition of inward rectifier current by released Gβγ in Kir2.3 expressing cells.

The activation of Kir3.1 channels by G-proteins has been clarified at a molecular level (16, 23–25, 28). It is hypothesized that Kir3.1 is closed in the basal state with Gβγ coupled to Ga and bound to the Kir3.1 N terminus (24). Ligand binding to the G-protein coupled receptor leads to dissociation of Ga from Gβγ, translocation of Gβγ subunits to the C-terminal region of the channel, and channel opening (16). The N-terminal region of Kir3.1 is not required for channel activation by G-proteins (16), but may play a role in maintenance of the closed state (23). The present results provide evidence for the following model of Kir2.3 channel function. In the basal state, Kir2.3 channels are open and unassociated with a G-protein. Assuming some degree of evolutionary conservation of gating mechanisms, binding of liberated Gβγ to the N-terminal cytoplasmic region would then close the channel.

Kir3 subfamily members couple to muscarinic M2 receptors, and activation of Kir3 channels by muscarinic receptor stimulation probably accounts for vagal-induced slowing of the heart rate (4). Activation of M1 receptors strongly suppresses inward rectifier currents in sympathetic neurons (30), and preliminary evidence suggests that cloned Kir2.3 channels, but not Kir2.1 channels, can be inhibited by M1 receptor activation or protein kinase C stimulation (31, 32), although the coupling mechanisms are unknown. The present results provide the first demonstration of Gβγ inhibiting a cloned Kir channel, and we hypothesize that the interaction of Kir2.3 subunits with G-proteins couples activation of receptors to K+ channel inhibition. There is a large, and unexplained, number of circumstances in which G-protein activation leads to inhibition of an inward rectifier current with properties like Kir2.3. In developing skeletal muscle, capillary endothelium, a mast cell line, and in guinea-pig enterocytes, injection of GTPγS completely suppresses strong inward rectifier currents (33–36). In pituitary GH2 cells, thyrotrophin-releasing hormone induces prolactin secretion by G-protein-mediated inhibition of Kir2-like inward rectifier current (5). In renal juxtaglomerular cells, angiotensin II is suggested to block inward rectifier K+ channels through a G-protein activation (37), and direct evidence for a membrane-delimited pathway of G-protein inhibition of inward rectifier current has been provided for Vicia guard cells (7). An unidentified G-protein has been implicated in inhibition of a strong inward rectifier in nucleus basalis neurons (38), and, again, GTPγS injection completely suppresses inward rectifier currents in brain microglia (6) and in frog sympathetic ganglia (39).

Our model suggests that Kir2.3 is not bound to a G-protein in the basal state and, by inference, should not be associated with any specific neurotransmitter receptor. Kir2.3 channels are expressed in numerous tissues (9). In neuronal tissues, G-protein activation of Kir3 channels would lead to synaptic inhibition, whereas G-protein inhibition of Kir2.3 channels would lead to synaptic excitation. Kir2.3 is localized throughout the brain and is particularly concentrated in the hippocampus and forebrain (19, 40). The demonstration (Fig. 2) that Gβγ inhibition of Kir channels containing Kir3 subunits is dominant in heteromultimers with another Kir2 subunit provides a molecular basis for G-protein inhibition of inward rectifier currents in multiple tissues.

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