The Cardiac Inward Rectifier K⁺ Channel Subunit, CIR, Does Not Comprise the ATP-sensitive K⁺ Channel, IKATP*

Grigory Krapivinsky, Luba Krapivinsky, Bratislav Velimirovic, Kevin Wickman, Betsy Navarro, and David E. Clapham‡

From the Department of Pharmacology, Mayo Foundation, Rochester, Minnesota 55905

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Cardiac IKACH is comprised of two inwardly rectifying K⁺ channel subunits, CIR and GIRK1 (Krapivinsky, G., Gordon, E. G., Wickman, K., Velimirovic, B., Krapivinsky, L., and Clapham, D. E. (1995) Nature 374, 135–141). A cardiac protein virtually identical to CIR, termed rcKATP-1 (Ashford, M. L. J., Bond, C. T., Blair, T. A., and Adelman, J. P. (1994) Nature 370, 456–459), was reported to form an ATP-sensitive inwardly rectifying K⁺ channel, IKATP. We attempted to determine whether CIR alone or together with an unknown protein(s) participated in the formation of cardiac IKATP. Expression of CIR in insect, oocyte, and mammalian cell systems did not increase the appearance of ATP-sensitive currents, but rather gave rise to unique strongly inwardly rectifying, G protein-regulated K⁺ currents. CIR protein is found exclusively in atria, in contrast to the predominance of IKATP functional activity in ventricle. Also, CIR was completely depleted from heart membrane after immunodepletion of GIRK1. We conclude that CIR/rcKATP-1 is not a subunit of cardiac IKATP and that GIRK1 is the only channel protein coassociating with CIR in heart.

Inwardly rectifying K⁺ channels (IRKs) maintain resting membrane potential and control cell excitability. Two of the most highly regulated IRK channels are the cardiac atrial muscarinic-gated channel, IKACH, and the ATP-sensitive channel, IKATP (3–6). IKACH is in part responsible for the vagally mediated slowing of heart rate. Characteristic features of IKACH are its gating by the G protein βγ dimer (Gβγ), single channel conductance of 35–40 picoamperes in symmetrical 140 mM K⁺, sharp inward rectification in the presence of internal Mg²⁺, and a mean single channel open time of ~1 ms (3, 7–11). Recently, it was shown that cardiac IKACH was comprised of two homologous subunits, termed GIRK1 and CIR (1). GIRK1 and CIR are both members of a subfamily of IRKs regulated by G proteins (GIRKs or Kir 3.0 (12)). In brain, GIRK1 may combine with a CIR homolog, GIRK2, to form a channel with properties similar to IKACH (13).

IKATP is a K⁺-selective, inward rectifier found in pancreatic β cells, heart, smooth muscle, skeletal muscle, brain, and kidney. Evidence suggests that IKATP channels play roles in the early repolarization of ischemic cardiac cells and insulin secretion. Cardiac IKATP is blocked by intracellular ATP and sulfonamides, is modulated by intracellular nucleotide diphosphates and activated α subunits of G proteins (Gαs), rectifies weakly, and has a characteristic single channel bursting profile (4–6). A recently cloned cDNA, termed rcKATP-1, was reported to form IKATP when expressed in the human embryonic kidney cell line HEK293 (2). rcKATP is virtually identical to CIR. The sequence for rcKATP-1 reported in GenBank™ differs by only two amino acids from CIR (I188V, a conserved substitution, and Q375E). Each difference can be explained by a single base polymorphism. To reconcile the demonstration that CIR is part of cardiac IKACH (1) and the demonstration that CIR expression generates IKATP (2), it was suggested that CIR does not form IKATP alone, but may coassemble with an unknown subunit(s) expressed in cardiac and HEK293 cells to form IKATP. In this report we demonstrate that CIR is not part of cardiac IKATP and appears to be wholly associated with GIRK1 in heart.

MATERIALS AND METHODS

Plasma membranes from bovine atria and ventricle were isolated as described (15). Two to 10 μg of membrane protein was separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (Millipore) film for Western blots. A CIRN2 antibody raised against an N-terminal peptide (amino acids 19–32) was affinity-purified as described (1). A CIRN2 specifically recognized CIR expressed in SF9 cells and immunoprecipitated in vitro translated CIR (data not shown). Western blots were developed with anti-rabbit peroxidase (Pierce) and enhanced chemiluminescence (ECL; Amersham Corp.).

For coimmunoprecipitation experiments, plasma membrane proteins were biotinylated (16) and solubilized in a buffer containing 10 mM HEPES, pH 7.5, 300 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM dithiothreitol, protease inhibitors (2 μg/ml aprotonin, leupeptin, and pepstatin, 0.2 mM phenylmethylsulfonyl fluoride) and immunoprecipitated with a CIRN2 (7 μg/ml of solubilized protein) using 10 μl of protein A-Sepharose FF (Pharmacia Biotech Inc.) for 2 h at 4 °C. After washing of the immunoprecipitate with the same buffer, specifically bound proteins were eluted with 2 × 25 μl of 100 μM CIRN2 peptide. Proteins were visualized by Western blotting with streptavidin-peroxidase (Pierce) and ECL and probed with an anti-GIRK1 antibody, aCsh (1). The CIR coding region was subcloned into pcDNA1/Amp (Invitrogen) downstream from the cytomegalovirus promoter for use in mammalian transfection experiments. To maximize the chance of assaying HEK293 and CHO cells transiently expressing CIR, we included a construct driving expression of murine CD4, a T cell-specific cell-surface antigen, in all transfections (1). CHO and HEK293 cells plated on 10-cm Petri dishes at 50% confluence were transfected via calcium phosphate precipitation with CIR or rcKATP-1 (10 μg) and 5 μg of the CD4 construct. 24 h post-transfection, CHO cells were detached, concentrated, and incubated at 4 °C for 10–15 min with 5 μl of (R)-phycocerythrin-conjugated anti-mouse CD4 monoclonal antibodies (Pharmingen, 0.4 μg/ml). Unbound antibody was removed by a 5-ml wash with phosphate-buffered saline. Cells were replated on coverslips in their respective media and allowed to reattach for 1–2 h prior to electrophysiological experi-
Fig. 1. Heterologous expression of CIR or rcKATP-1 in Sf9, CHO, and HEK293 expression systems gives similar channel currents. Recordings are from excised patches at the voltages shown. An "all-point" amplitude histogram was constructed for channels appearing upon infection of Sf9 cells with a recombinant CIR baculovirus (a), following transfection of CHO cells with CIR (b), or rcKATP-1 in HEK293 cells (c). Holding membrane potential at positive voltages reveals the strong inward rectification of these channels. With the addition of 5 mM EDTA, which chelates the 2 mM bath Mg²⁺, inward rectification was abolished.

In all three systems, amplitude histograms were generated from inside-out patches held at −80 mV, stimulated by GTPγS (Sf9 cells) or ATP and GTPγS (HEK293 and CHO cells).

In control cells a channel resembling that described as I_KATP by Ashford et al. (2) was recorded. The channel was present in 1–3% (CHO; n > 100) to 18% (HEK293; n = 78) of control or mock-transfected cells. These endogenous channels were not noted by Ashford et al. (2).

I_KATP activity is higher in ventricular than atrial cells (4, 11, 17, 18), while I_KACh is found exclusively in atria. We used antibodies specific for CIR to examine the distribution of this inward rectifier subunit in bovine ventricle and atrial tissues. A unique peptide corresponding to amino acids S19-Y32 of CIR (identical in rcKATP-1) was synthesized to raise a CIR/rcKATP-1-specific polyclonal antibody. In contrast to the functional distribution of I_KATP, CIR (rcKATP-1) was not detected in ventricular plasma membranes. CIR, however, was readily apparent in atria, consistent with I_KACh expression (Fig. 2).

CIR was shown to communoprecipitate with GIRK1 in atria (1). To determine whether CIR associates with another protein(s) in heart tissue, we immunoprecipitated CIR directly. aCIRN2 immunoprecipitated the 45-kDa CIR as well as 58- and 72-kDa proteins from atrial membranes, but none were immunoprecipitated from ventricular plasma membranes (Fig. 3).

In control experiments, aCIRN2 did not communoprecipitate in vitro translated GIRK1 (data not shown). The proteins migrating at 72 and 58 kDa were identified as glycosylated and unglycosylated forms, respectively, of GIRK1 by the specific anti-GIRK1 antibody, aCsh (Fig. 3, see also Ref. 1). Thus, we did not find novel proteins interacting with CIR in atria. However, we could not rule out the possibility that other proteins comigrated with, and therefore could not be discriminated from, CIR and GIRK1 polypeptides.

To address the possibility that other proteins comigrated with CIR and GIRK1 polypeptides, we immunoprecipitated...
FIG. 2. CIR (rcKATP-1) protein is expressed in atrial but not ventricular tissues. Plasma membranes were isolated from bovine heart atrial (A) and ventricular (V) tissues and Western blotted with 1 μg/ml anti-CIR antibody (aCIRN2, left panel). The right panel shows a Western blot with the same antibody in the presence of 20 μM antigenic peptide.

FIG. 3. aCIR antibody coimmunoprecipitates GIRK1 from bovine atrial plasma membranes. aCIRN2 immunoprecipitated three polypeptides from atrial plasma membranes and none from ventricular membranes. The 45-kDa polypeptide has an electrophoretic mobility identical to atrial CIR on Western blots. The other polypeptides (58 and 72 kDa) were recognized with anti-GIRK1 antibody (aCsh). In control experiments, aCIRN2 did not immunoprecipitate in vitro translated GIRK1.

GIRK1 and measured depletion of CIR. If other proteins interacted with CIR, full depletion of GIRK1 would not eliminate all detectable CIR. GIRK1 was immunoprecipitated with an increasing amount of aCsh. The relative amounts of GIRK1 and CIR remaining in the supernatant and after immunoprecipitation were determined by Western blot with aCsh and aCIRN2 antibodies. As GIRK1 was depleted, CIR was exhausted; no CIR remained in the supernatant after removal of GIRK1/CIR complexes (Fig. 4). Therefore, we conclude that GIRK1 is the only channel protein associated with CIR in heart.

CONCLUSION

We have shown that the inward rectifier subunit CIR (rcKATP-1) initially proposed by Ashford et al. (2) to comprise I_{KATP} does not participate in formation of cardiac I_{KATP}. Our evidence is summarized as follows. 1) Expression of CIR alone in SF9, CHO, and HEK293 expression systems did not yield channels with properties similar to I_{KATP}. Interestingly, CIR channels rectify strongly, but have an asparagine, not an aspartic acid residue, in their putative second transmembrane segment. This contradicts the hypothesis that asparagine is a common feature of weak inward rectifiers(19). 2) CIR was present only in cardiac atria, not ventricle, whereas I_{KATP} is abundant in both tissues. 3) CIR protein does not associate with inward rectifier subunits other than GIRK1 in heart. We speculate that the channels reported by Ashford et al. (2) in HEK293 cell were actually endogenous channels, not novel currents appearing as a result of CIR or rcKATP-1 expression. It seems likely that an as yet undefined but homologous inward rectifier subunits may form cardiac I_{KATP}, perhaps in conjunction with the sulfanylurea receptor (20). In fact, an IRK (uKATP-1) from a rat pancreatic cDNA library with only 43–46% identity to any of ROMK1, GIRK1, CIR/rcKATP-1, and IRK1 has been reported to constitute I_{KATP} in HEK293 cells (14).

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