Localization and Interaction of Epitope-tagged GIRK1 and CIR Inward Rectifier K⁺ Channel Subunits

M. E. KENNEDY, J. NEMEC and D. E. CLAPHAM

Mayo Foundation, Rochester, MN 55901, U.S.A.

(Accepted 14 August 1996)

Summary—GIRK1 and CIR are G-protein activated inward rectifier K⁺ channel subunits that combine to form the heteromultimer \( I_{K_{ACB}} \), the \( G_{\beta} \)-activated atrial K channel responsible for the vagal slowing of heart rate. Epitope-tagged channel subunits were constructed by the introduction of distinct six amino acid epitopes into the C-termini or putative extracellular domains of GIRK1 and CIR. Carboxyl-terminal tagged subunits were activated by purified \( G_{\beta} \) subunits in inside-out patches when expressed in Cos cells. Interestingly, insertion of three amino acids into the putative extracellular domain of GIRK1 resulted in an inactive subunit that acted as a dominant negative subunit when coexpressed with wild type GIRK1 and CIR in Xenopus oocytes. The epitope-tagged CIR-AU1 subunit coimmunoprecipitated GIRK1-AU5 from metabolically labeled Cos cells. Immunofluorescence labeling of Cos cells localized GIRK1-AU5 to internal cytoskeletal structures that co-stained with antibodies against the intermediate filament protein, vimentin. CIR-AU1 localized primarily to the plasma membrane. Double immunofluorescence labeling showed that GIRK1-AU5 plasma membrane staining was detectable only when coexpressed with CIR-AU1. Copyright © 1996 Elsevier Science Ltd

Keywords—G-protein-coupled inward-rectifier potassium channel, GIRK1, CIR, cytoskeleton, \( G_{\beta} \), AU5, AU1.

Inward rectifying potassium channels represent a genetically diverse group of proteins that function to regulate cell membrane potential (Doupnik et al., 1995; Hickman and Clapham, 1995). They play important roles in neurotransmission, vagal control of heart rate, and regulation of secretion (Jan and Jan, 1992; Clapham, 1994). Inward rectifiers such as, IRK1, ROMK1 and GIRK1/CIR conduct K⁺ more efficiently in the inward direction than the outward. These channels demonstrate small outward currents and varying degrees of inward rectification at membrane potentials above \( E_K \). In the pacemaker cells of the atria, acetylcholine released from the vagus nerve binds an M₂-muscarinic receptor that in turn activates a pertussis toxin-sensitive G-protein, liberating \( G_{\beta} \) subunits. \( G_{\beta} \)-subunits are able to directly bind and activate \( I_{K_{ACB}} \) channels (Logothetis et al., 1987; Wickman et al., 1994; Krapivinsky et al., 1995c). Activation of \( I_{K_{ACB}} \) ultimately leads to a slowing of the heart rate. Co-immunoprecipitation experiments identified that \( I_{K_{ACB}} \) is a heteromultimer of GIRK1/CIR subunits (Krapivinsky et al., 1995a). Coexpression of GIRK1/CIR in oocytes resulted in synergistically larger currents that more closely resembled native \( I_{K_{ACB}} \) compared to GIRK1-injected oocytes. We (and others) have consistently failed to obtain functional expression of GIRK1 alone in mammalian tissue culture cells such as CHO and HEK-293 cells, despite detectable levels of GIRK1 expression by Western blotting Krapivinsky et al., 1995a; Philipson et al., 1995; Velimirovic et al., 1995b; Navarro et al., 1996). Furthermore, antisense experiments demonstrated that functional expression of GIRK1 in oocytes is dependent on the presence of XIR, a Xenopus CIR homolog (Hedin et al., 1996). Channels resulting from CIR expression in mammalian cells lines display unusual electrophysiological properties consisting of low amplitude channels with brief open times ('spiky', Krapivinsky et al., 1995b). Thus, the expression of GIRK1 or CIR alone most likely does not mimic a normal physiological situation. 

\( I_{K_{ACB}} \)-like channels are also present in the brain (Inoue et al., 1988; Velimirovic et al., 1995a; Griff et al., 1996). The GIRK2 channel, a close relative of cardiac CIR, interacts with GIRK1 in the cerebellum to form a neuronal G-protein-activated inward rectifier (Kofuji et al., 1995; Velimirovic et al., 1995b; Lesage et al., 1995). A point mutation in the pore domain of GIRK2 resulted in a non-selective GIRK1/GIRK2 heteromultimeric channel that was no longer regulated by \( G_{\beta} \)-subunits (Navarro et al., 1996). Presumably the non-selective, unregulated channel caused aberrant cell death and cell
migration resulting in the weaver mouse phenotype of profound ataxia and cerebellar degeneration (Patil et al., 1995). Our current hypothesis is that native G-protein activated inward rectifiers are heteromultimers in heart and brain tissue.

Here, we describe the functional characterization of epitope-tagged GIRK1 and CIR channels, including subunit interaction and localization when transiently expressed in COS cells. Introduction of a distinct six-amino acid epitope into the C-terminus of each subunit did not alter channel activity, but introduction of an epitope into the putative extracellular domain of GIRK1 resulted in a dominant negative form of GIRK1. The epitope-tagged subunits were successfully used in communoprecipitation reactions, and immunofluorescence detection of tagged inward rectifiers revealed an unusual localization pattern of GIRK1 to the cytoskeleton of COS cells.

**METHODS**

**Transient transfections, oocyte and mammalian cell culture**

*Xenopus* oocytes were isolated and defolliculated by collagenase treatment as described by Velimirovic et al., (1995a). Injections were performed in ND96 followed by incubation in ND96 supplemented with gentamicin, pyruvic acid, theophylline and 5% horse serum. Recordings were performed on day 2 after injection of cRNAs. COSM7 or COS-7 cells were cultured at 37°C in 5% CO₂ in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM glutamine and penicillin/streptomycin. COSM7 or COS-7 cells at 50–70% confluence (100 mm dishes), were transiently transfected with 5 µg of each pCDNA3 vector(s) (Invitrogen, San Diego, CA) containing the indicated channel cDNA using a 2:1 ratio of TransIT™LT-1 reagent (Pan Vera Corp, Madison, WI) to DNA according to the manufacturer’s protocol. A CD-4 containing expression plasmid, pBRG4, was included in transfections when cells were to be used for electrophysiological recordings, at a 1:10 ratio of pBRG4 to channel vector. This allowed identification of transfected cells with a fluorescently labeled anti-CD4 antibody.

**Construction of epitope-tagged channels**

Introduction of the epitope sequences was performed by the polymerase chain reaction using Vent polymerase (New England Biolabs, Beverly, MA). The six-amino acid (TDFYLK) AU5 epitope was introduced into the carboxyl terminus of GIRK1 (GIRK1-AU5), or into the putative extracellular domain by insertion of the sequence FYL between amino acids 153 and 154 (GIRK1-PAU5; p = pore). The AU1 epitope (DTYRY1) was introduced into the carboxyl terminus of CIR(CIR-AU1). The specific cDNA cassette containing the epitope-tagged region of GIRK1 or CIR was subcloned into the wild type GIRK1 or CIR pBSSK(-) backbone.

The sequence of the insert was verified byideoxy DNA sequencing. The entire cDNA sequence of CIR (Krapivinsky et al., 1995a) or the coding sequence of GIRK1 (Kubo et al., 1993) containing all 5′ untranslated, but no 3′ untranslated, region was subcloned into pCDNA3. Deletion of the 3′ untranslated region did not affect expression levels of GIRK1 (data not shown). cRNA synthesis was performed with linearized vectors containing channel subunit cDNAs using either T7 or T3 polymerases and the Megascript kit (Ambion, Austin, TX).

**Electrophysiological recordings**

Oocyte recordings were performed using a two-electrode voltage clamp in K96 buffer containing 96 mM KCl, 2 mM NaCl, 1.8 mM CaCl₂, 1 mM MgCl₂ and 10 mM HEPES, pH 7.6. A voltage protocol was applied from −120 to +60 mV in 20-mV steps for 500 msec before and after bath application of acetylcholine (5 µM final concentration). The holding potential was −70 mV. Single channel recordings were obtained from inside-out patches in symmetrical 140 mM K using K140 solution: 118.5 mM KCl, 5 mM KOH/EGTA, 2 mM MgCl₂ and 10 mM KOH/HEPES, pH 7.2, with HCl. Single-channel recordings were acquired using an Axopatch 200A amplifier (Axon Instruments) filtered at 2 kHz (8 pole Bessel filter) and digitally sampled at 5 kHz. Bovine brain Gβ1; subunits were purified as described by Sternweis and Robishaw (1984), resuspended in 0.2% (w/v) CHAPS buffer and used for electrophysiological recordings. The final [CHAPS] was less than 0.0025%.

**Communoprecipitation assays**

Single 100-mm dishes of COSM7 cells expressing individual epitope-tagged GIRK1-AU5 or CIR-AU1 subunits or combinations of channel subunits were metabolically labeled with 50 µCi/ml of 38S-methionine (Amersham, Boston, MA) for 12 hr. Cells were washed twice with PBS (22 C) and lifted from the dish with PBS and 5 mM EDTA. The cells were then placed on ice to cool followed by centrifugation at 1200 g for 5 min. The cells were lysed at 4°C in 5 mM each Tris–HCl, EDTA, and EGTA at pH 8.0 (5/5/5) supplemented with 10−5 M phenylmethlysulfonylfluoride and 2 µg/ml each of leupeptin, pepstatin and aproitin protease inhibitor cocktail (PIC) by drawing the cell pellet through a 23-gauge needle 10 times. A crude particulate fraction was obtained by centrifugation at 150,000 g for 15 min at 4°C. This crude particulate fraction was then resuspended in 500 µl of 5/5/5 + PIC and centrifuged through a 20% sucrose cushion prepared in 5/5/5 + PIC at 150,000 g for 35 min at 4°C to remove contaminating cytosolic components. The resulting pellet was solubilized in 750 µl of 1% CHAPS, 10 mM HEPES, 300 mM NaCl, 5 mM EGTA, 5 mM EDTA at pH 8.0 + PIC by drawing the pellet through a 27-gauge needle 10 times. Insoluble material was removed by ultracentrifugation at 250,000 g
Untransfected CosM7 cells

30 nM G<sub>βγ</sub>

-80 +120 -80

24 s 5 pA

GIRK1-AU5/CIR-AU1

20 nM G<sub>βγ</sub>

+80 mV

10 ms 2 pA

24 s 5 pA

Fig. 1. GIRK1-AU5 and CIR-AU1 produce channels identical to I<sub>K<sub>ACII</sub></sub> when co-expressed in COSM7 cells. Top trace: inside-out patch recordings in symmetrical 140 K<sup>+</sup> from a control cell. The patch was excised into bathing solution containing 30 nM G<sub>βγ</sub>. The command potential is shown at the top. No inwardly rectifying, G<sub>βγ</sub> activated channels were observed in 11 out of 11 patches from untransfected COS cells. Bottom trace: representative inside-out patch from GIRK1-AU5, CIR-AU1 and CD4-transfected COSM7 cell. Arrow marks addition of G<sub>βγ</sub> 20 nM final concentration. Holding potential −80 mV. Inset: expansion of the basal channel activity just before G<sub>βγ</sub>-induced activation.

for 1 hr at 4°C. Samples were precleared for 1 hr at 4°C with 10 μl of Gammbind-G sepharose (Pharmacia, Sweden). Immunoprecipitations were performed by adding a 1:200 dilution of centrifuged ascites fluid for either the AU1 or AU5 monoclonal antibodies (BAbCO, Berkeley, CA) and 5 μl of Gammbind-G sepharose followed by incubation at 4°C for 4–10 hr. The Gammbind-G sepharose was washed at room temperature twice with 750 μl of solubilization buffer, twice for 3 min each with 750 μl of solubilization buffer containing 0.2% CHAPS, and once quickly with solubilization buffer containing 0.1% Triton-X100. Bound proteins were eluted from the Gammbind-G sepharose with 1 × SDS sample buffer at 55°C for 20 min. Samples were analyzed on SDS-PAGE followed by fluorography with Amplify (Amersham) and autoradiography.

Immunofluorescence assays

COS-7 cells were plated on glass coverslips and transfected as above with individual GIRK1-AU5, CIR-AU1 subunits or combinations thereof. Cells were fixed in 4% paraformaldehyde in PBS for 15 min followed by permeabilization and blocking with 0.2% Triton-X100, 3% BSA in PBS (PBSTB) for 45 min. Solutions containing BSA were filtered at 0.2 μm before use. Coverslips were incubated at room temperature for 1 hr with 500 μl of a 1:250 dilution of primary antibody in PBSTB followed by three 10-min washes in PBS supplemented with 0.1% Triton-X100 (PBST). A 1:250 dilution of FITC-conjugated or FITC-conjugated secondary antibody (Pierce, Rockford, IL) in PBSTB was incubated for 1 hr at room temperature followed by three 15-min washes in PBST. For double-labeling experiments, antibodies were incubated sequentially followed by washes in PBST (3 × 15 min at room temperature). Coverslips were mounted in Poly-aquamount (Polysciences) and viewed with a Zeiss Axiovert 100 fluorescence imaging microscope with a filter slider designed for imaging rhodamine, fluorescein or DAPI staining, or an Axiovert 135 microscope coupled to a
Zeiss LSM 410 confocal microscope using an external argon/krypton laser illumination beam at 488 nm (FITC) or 568 nm (RITC).

RESULTS

Functional characterization of epitope-tagged GIRK1-AU5 and CIR-AU1 subunits

In untransfected COSM7 cells, no inwardly rectifying K⁺-selective channels similar to I_{KAC} were observed, even after perfusion with 30 nM G_{p_{12}} (n = 11, Fig. 1 top trace). In contrast, patches from COSM7 cells cotransfected with GIRK1-AU5 and CIR-AU1 (GIRK1-AU5/CIR-AU1) exhibited the characteristic 35 pS inwardly-rectifying I_{KAC} channel sensitive to purified bovine brain G_{p_{12}}, with a mean open time of ~ 1.1 msec (n = 10 of 11 CD-4 positive cells; Fig. 1 bottom trace). GIRK1-AU5/CIR-AU1 subunits coexpressed in oocytes with the m2 subtype of muscarinic receptor were activated by 5 μM acetylcholine. Alternatively, they were constitutively activated by coexpression of G_{p_{12}} subunit cRNAs (data not shown). Together, these results indicate that the epitope-tagged GIRK1-AU5/CIR-AU1 channels behave indistinguishably from native I_{KAC} (Logothetis et al., 1987; Krapivinsky et al., 1995a). The high basal activity observed for GIRK1-AU5/CIR-AU1 expressed in COSM7 cells has been seen previously in both oocytes and CHO cells for GIRK1/CIR (Krapivinsky et al., 1995a).

Coimmunoprecipitation of GIRK1-AU5 and CIR-AU1

One of the reasons for creating epitope-tagged GIRK1 and CIR K⁺ channels was to use the epitope-selective monoclonal antibodies in biochemical assays of subunit interaction and synthesis. The AU5 and AU1 monoclonal antibodies selectively immunoprecipitated GIRK1-AU5 or CIR-AU1, respectively. There was no detectable cross-reactivity of the AU5 antibody with CIR-AU1 or of the AU1 antibody against GIRK1-AU5 [Fig. 2(A)]. Immunoprecipitations from 35S-methionine-labeled COSM7 cells identified a 55-kDa doublet and a smaller amount of a higher 75-kDa band for GIRK1-AU5-expressing COS cells or a single 45-kDa band for CIR-AU1-expressing cells. These bands were not present in control pCDNA3 vector-transfected cells [Fig. 2(B); lanes 1, 2 and 3). The doublet migrating at 55 kDa in GIRK1-AU5-expressing COSM7 cells is consistent with both Xenopus oocyte expressed GIRK1 and native GIRK1 in bovine atria (Krapivinsky et al., 1995a,b). The small amount of ~75-kDa band most likely represents glycosylated GIRK1-AU5 that was present in much smaller quantities in COSM7 cells compared to native GIRK1 in bovine atria (Krapivinsky et al., 1995a,b). Coexpressed GIRK1-AU5/CIR-AU1 were coimmunoprecipitated by the AU1 antibody [Fig. 2(B)].
The additional 55-kDa and ~75-kDa proteins present in AU1 immunoprecipitates most likely represented GIRQ1-AU5 associated with CIR-AU5 since: (a) they were not present in CIR-AU1-only transfected cells or in pCDNA3 control cells; (b) both bands co-migrated with expressed GIRQ1-AU5 in COSM7 cells; and (c) GIRQ1-AU5 was not directly immunoprecipitated by the AU1 antibody.

A dominant negative form of GIRQ1

Insertion of three amino acids (FYL) between amino acids 153 and 154 of the putative extracellular domain of GIRQ1 was used to form the AU5 epitope (GIRQ1-PAU5), but the channels were apparently non-functional even when co-injected with CIR (in Xenopus oocytes). The AU5 epitope was not recognized in either immunoprecipitation or immunofluorescence assays. The GIRQ1-PAU5 construct did produce full length GIRQ1-PAU5 protein in an in-vitro translation assay. Immunofluorescence of GIRQ1-PAU5 that also contained the AU5 epitope on the C-terminus was similar to GIRQ1-AU5 immunofluorescence (data not shown). To determine if GIRQ1-PAU5 channels could inhibit the activity of wild type GIRQ1/CIR heteromultimers, a two-fold greater amount of GIRQ1-PAU5 was co-injected with wild type GIRQ1/CIR cRNA in Xenopus oocytes. GIRQ1-PAU5 coexpression resulted in a significant decrease in acetylcholine stimulated current in K96 [Fig. 3(A,B)]. The level of acetylcholine-induced current at ~100 mV for GIRQ1/CIR/GIRQ1-PAU5 (~1.6 ± 0.2 µA) was nearly identical to the basal level of channel activity in the GIRQ1/CIR injected oocytes (~1.3 ± 0.15 µA). These data suggest that introduction of three amino acids into the pore region of GIRQ1 disrupted GIRQ1 channel function, creating a dominant negative GIRQ1 capable of inhibiting GIRQ1/CIR channels. The most likely mechanism of inhibition would be the association of GIRQ1-PAU5 with CIR producing a non-functional GIRQ1-PAU5/CIR or GIRQ1-PAU5/GIRQ1/CIR complex. Preliminary experiments indicate that GIRQ1-PAU5 is co-immunoprecipitated by CIR in oocytes. This is unlike the proposed mechanism for inhibitory effects of Kir3.4 (CIR) on Kir4.1 channels coexpressed in oocytes, i.e. the increased degradation of Kir4.1 channels associated with Kir3.4 (Tucker et al., 1996).

Immunofluorescence staining of GIRQ1-AU5 and CIR-AU1 in COS cells

Permeabilized CIR-AU1 transfected COS-7 cells displayed a diffuse membrane staining pattern consistent with the plasma membrane [Fig. 4(A)]. There was little detectable intracellular staining near the nucleus (indicative of endoplasmic reticulum) in most cells expressing CIR-AU1 as assessed by confocal microscopy. This was an unusual finding since, in transiently transfected cells where single cell expression levels are very high, integral membrane proteins are often present in the ER, but are consistent with efficient CIR targeting to the cell surface in COS-7 cells. The specificity of the AU1 antibody was supported by the low level of staining of surrounding, non-transfected COS-7 cells present in the field [Fig. 4(A,B)]. In contrast, GIRQ1-AU5-expressing COS-7 cells showed a strikingly different staining pattern (Fig. 4B). Intracellular staining was web-like throughout the cell and very intense around the nucleus. The web-like pattern of GIRQ1 staining was reminiscent of that seen for intermediate filaments, homopolymers of proteins, such as vimentin or keratins which begin at the nucleus and insert at plasma membrane plaques.
Fig. 4. (A) Permeabilized COS-7 cells transfected with CIR-AU1, stained with anti-AU1 antibody and FITC-conjugated goat-antimouse secondary antibody. (B) Permeabilized GIRK1-AU5 expressing COS-7 cells stained with anti-AU5 antibody and FITC-conjugated goat-antimouse secondary antibody. (C and D) FITC signal (green) from cells transfected with GIRK1-AU5 and stained with both anti-AU5 and anti-vimentin V9 monoclonal antibodies. (D) Anti-vimentin V9 antibodies were visualized with rhodamine-conjugated secondary antibodies (red). The cell nucleus was visualized by staining with 500 nM 4′,6-diamidino-2-phenylindole (DAPI) for 10 min before mounting. Cells were imaged using a 40× (A, B) or 100× (C, D) objective and photographed using Kodak Ektachrome 400 slide film. Slides were scanned using a Kodak slide scanner and processed to grayscale images or in color using Adobe Photoshop 3.0.

(Steinart and Roop, 1988). Double immunofluorescence labeling experiments using the AU5 antibody against GIRK1-AU5 and a monoclonal antibody specific for vimentin, suggest that GIRK1-AU5-staining colocalizes with vimentin-staining patterns [Fig. 4(C) and (D)]. Intermediate filament tracks follow a pathway similar to microtubules in some cell types. Thus, we cannot conclude whether GIRK1 is localized primarily to vimentin-containing intermediate filaments or to closely associated microtubules. No obvious plasma membrane staining, as seen consistently for CIR-AU1, was observed for GIRK1-AU5-expressing COS-7 cells. Wild type GIRK1 expressed in COS-7 cells demonstrated a similar staining pattern as GIRK1-AU5 when detected with a polyclonal antibody directed against a C-terminal GIRK1 fusion protein (Krapivinsky et al., 1995a) (data not shown). The intermediate filament localization pattern of GIRK1-AU5 in COS cells [Fig. 4(B), (D)] is consistent with the hypothesis that GIRK1-AU5 is not present at the plasma membrane. Since tagged GIRK1 and CIR subunits produced significantly different staining patterns and GIRK1/CIR channels represent the physiologically relevant G-protein activated inward rectifier in atria, double immunofluorescence labeling studies in GIRK1-
AU5/CIR-AU1 expressing COS cells were carried out. CIR-AU1 staining patterns were shifted significantly towards that of GIRK1-AU5 [Fig. 5(A)]. There was a shift in the distribution of GIRK1-AU5 from a primarily intermediate filament pattern to a pattern of both intermediate filaments and plasma membrane staining [Fig. 5(B)]. GIRK1-AU5 and CIR-AU1 staining overlaps almost completely as indicated by the yellow color in the overlay image [Fig. 5(C)]. These data support the interaction of GIRK1 and CIR and suggest that CIR may function to facilitate GIRK1 expression at the plasma membrane.

**DISCUSSION**

We have developed a strategy for the biochemical detection of GIRK1 and CIR channels using the AU5 and AU1 epitopes. The advantage of this approach is that the antibody tagging is independent of the native GIRK1 and CIR amino acid sequences. Although antipeptide polyclonal antibodies to native GIRK1 and CIR are available (Krapivinsky et al., 1995a,b), the creation of chimeric channels and mutants of GIRK1 or CIR will, in many cases, result in the removal of regions of antigenicity. The epitopes, therefore, can be substituted into any chimeric
channel by mutagenesis. The AU5 and AU1 epitopes have proven to be specific and do not alter any of the measured intrinsic GIRQ/CIR channel properties, such as conductance, selectivity and GIRQ activation. The AU1 antibody efficiently co-immunoprecipitated GIRQ1-AU5 with CIR-AU1, thus demonstrating the usefulness of this system for the study of channel subunit assembly using a direct biochemical and functional approach as opposed to functional expression alone. Similar results were found in co-immunoprecipitation experiments using either the AU5 antibody to GIRQ1 or the AU1 antibody to CIR. GIRQ1 channels containing a 31-amino acid epitope tag, have been expressed previously in HEK-293 cells and βTC3 cells (Philipson et al., 1995). Functional currents were observed in βTC3 and not HEK-293 cells.

Epitopes were placed in several regions within the channel subunits. The placement of the AU5 epitope in the putative pore region was done to create an extracellular antigen for immunolabeling experiments. Unfortunately, the GIRQ1-PAU5 construct was non-functional and was not efficiently recognized by the AU5 antibody. However, GIRQ1-PAU5 was capable of efficiently inhibiting wild type GIRQ1/CIR currents by 71%, nearly to basal levels in Xenopus oocytes. GIRQ1-PAU5 may function to inhibit GIRQ1/CIR channels via several mechanisms, including competition with wild type GIRQ1 for interaction with CIR, or by sequestering wild type GIRQ1 from CIR. Preliminary experiments indicate that GIRQ1-PAU5 staining patterns in COS cells are identical to GIRQ1-AU5 and that GIRQ1-PAU5 is co-immunoprecipitated by CIR in oocytes. Regardless of the mechanism of GIRQ1-PAU5 inhibition of GIRQ1/CIR channels, this dominant negative subunit could prove useful in delineating the contribution of GIRQ1 containing inward rectifiers to the regulation of complex cellular processes such as secretion.

Immunofluorescence studies of GIRQ1-AU5 and CIR-AU1 subunits transiently expressed in COS-7 cells revealed an unusual staining-pattern for GIRQ1-AU5 in which the subunit appeared to colocalize with intermediate filaments. Double staining with AU5 and anti-vimentin monoclonal antibodies matched intermediate filament protein staining. Intermediate filaments form rope-like structures that tend to follow pathways similar to microtubules and provide structural rigidity to the cell (Geiger, 1987; Steinart and Roop, 1988). It is not known if the GIRQ1-AU5 staining pattern is atypical due to the high expression levels of GIRQ1-AU5 in COS cells. However, the pattern could reflect better detection of a normally occurring process. We would predict that GIRQ1-AU5, as an integral membrane protein, is present in vesicles localized to intermediate filaments but whether GIRQ1-AU5 channels are directly associated with intermediate filaments is under investigation. The lack of functional expression of GIRQ1 channels in mammalian cell lines devoid of CIR or CIR homologs could be due to inappropriate trafficking of GIRQ1. Double staining experiments with coexpressed GIRQ1-AU5 and CIR-AU1 revealed a dramatic shift in the staining pattern of CIR-AU1 from membrane localized to both membrane and intermediate filament localization. GIRQ1-AU5 again displayed predominant staining on intermediate filaments, but some plasma membrane staining was evident. It is possible that CIR functions in part to promote the localization of heteromultimeric GIRQ1/CIR channels at the cell surface. Alternatively, the lack of an undefined subunit, analogous to Kvβ2 subunit interaction with Kv1.2 (Shi et al., 1996; Xu et al., 1996; Yu et al., 1996), could prevent efficient cell surface localization of channel complexes. Currently, biochemical studies are under way to define the fraction of GIRQ1 channels present at the plasma membrane in intact cells under varying expression conditions.

Acknowledgements—We would like to thank Ryan Bortolon of the LADM facility (Mayo Foundation) for excellent technical assistance with laser confocal microscopy. Dr Grigorii Krapivinsky for bovine brain GIRQs, and Dr Bratislav Velimirovic for critical reading of the manuscript. This work was supported in part by an NRSA award to MEK and grants from the NIH to DEC.

REFERENCES


