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Evidence for a Functional Interaction between Integrins and G Protein-activated Inward Rectifier K^+ Channels*

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Heteromultimeric G protein-activated inward rectifier K^+ (GIRK) channels, abundant in heart and brain, help to determine the cellular membrane potential as well as the frequency and duration of electrical impulses. The sequence arginine-glycine-aspartate (RGD), located extracellularly between the first membrane-spanning region and the pore, is conserved among all identified GIRK subunits but is not found in the extracellular domain of any other cloned K^+ channels. Many integrins, which, like channels, are integral membrane proteins, recognize this RGD sequence on other proteins, usually in the extracellular matrix. We therefore asked whether GIRK activity might be regulated by direct interaction with integrin. Here, we present evidence that mutation of the RGD site to RGE, particularly on the GIRK4 subunit, decreases or abolishes GIRK current. Furthermore, wild-type channels can be co-immunoprecipitated with integrin. The total cellular amount of expressed mutant GIRK channel protein is the same as the wild-type protein; however, the amount of mutant channel protein that localizes to the plasma membrane is decreased relative to wild-type, most likely accounting for the diminished GIRK current detected. GIRK channels appear to bind directly to integrin and to require this interaction for proper GIRK channel membrane localization and function.

G protein-activated inward rectifier K^+ (GIRK)[1] channels are found in both the heart and the brain, where they are associated with slowing of the heart rate and suppression of neuronal firing (1). The channel found in the sinoatrial node and on atrial myocytes, K_{ACh}, is formed from two homologous channel subunits, GIRK1 (KGA or Kir3.1) and GIRK4 (CIR or Kir3.4) (2–4). This channel is activated by the release of acetylcholine from the vagus nerve acting on the m_2AChR, as well as by adenosine and other transmitters the receptors of which activate specific G proteins containing the $G\alpha_i$ subunit. The channel is opened by the direct binding of freed $G\beta\gamma$ subunits (5–9). GIRK channels are part of the larger family of inward rectifier K^+ channels the members of which have similar subunit topology, two or more membrane spanning domains connected by a pore-forming P loop that dips partially into the membrane. Interestingly, all known GIRK subunits and no other K^+ channels (including other inward rectifier K^+ channels) have a conserved peptide sequence, arginine-glycine-aspartate (RGD), on their proposed extracellular surface between the putative first membrane spanning region and the P region (Fig. 1) (2, 4, 10). These three amino acids form the recognition sequence for some of the ligands that bind to another class of membrane proteins, integrins (11). The presence of this amino acid sequence on GIRKs suggests that these channels may directly bind to and be modified by integrins.

Integrins are formed from two subunits, α and β, each with a single transmembrane domain. The extracellular portions of each subunit are involved in the recognition and binding to RGD sites on ligands, whereas intracellular portions associate with cytoskeletal proteins; integrins are thus ideally suited for roles in signal transduction, and complexes of intracellular signaling molecules and their activities are found associated with integrins. Their functions are diverse and include cell adhesion, migration, polarity, survival, growth, proliferation, differentiation, and alteration of gene expression (11–14). The cycling of integrin to and from the plasma membrane is important to its function in many cells and regulates the function of other molecules that bind to it (15–17). Integrin has also been specifically associated with nervous system functions (18), such as the level of hippocampal neuronal excitability (19), stretch-induced neurotransmitter release (20), short and long term memory (21), and neurite outgrowth (22).

Could some actions of GIRK channels in excitable tissues be mediated by interaction with integrin and *vice versa*? We have begun to address this question by asking whether the function of GIRK channels is affected by a direct binding interaction with integrin. The results reported here address this question.

EXPERIMENTAL PROCEDURES

Peptide and Antibodies—GRGDSP peptide was made at the Biopolymer Synthesis Facility of the Beckman Institute at the California Institute of Technology. The GIRK1 antibody was raised in rabbits against a peptide from the C terminus of rat GIRK1 (23). The GIRK4 antibodies were a rabbit antibody against a rat GIRK4 N-terminal peptide (Upstate Biotechnology; no longer available) and a guinea pig antibody against a mouse C-terminal peptide (a generous gift of Dr. Florian Lesage, Institut de Pharmacologie Moleculaire et Cellulaire (24)). The integrin antibodies included 8C8, a mouse monoclonal anti-*Xenopus* β1 integrin antibody (Developmental Studies Hybridoma Bank), and a rabbit antibody raised against the C terminus of chick β1 integrin (a generous gift of Dr. Richard Hynes, MIT (25)). The pan-Trk antibody was TrkC-14, raised in rabbits against the C terminus of human TrkA precursor (Santa Cruz Biotech).

Mutagenesis—The GIRK1 and GIRK4 cDNAs were both cloned into pMXT, a derivative of pBluescript containing β globin sequences known to stabilize transcripts in oocytes (26). Oligonucleotides containing a mismatch to these WT DNA templates were designed and used in PCR amplification to generate a mutant cDNA in which the RGD site of the protein was changed to RGE (codons CGG GGC GAA in GIRK1 and CGA GGT GAA in GIRK4). Full-length mutant clones were isolated and screened by dideoxy terminator sequencing. For CHO cell transfection, the WT GIRK4 construct was in pCDNA3; WT GIRK1 and mutant

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[1] The abbreviations used are: GIRK, G-protein-regulated, inwardly rectifying K^+ channel; Kir, inwardly rectifying K^+-selective channel; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; ACh, acetylcholine; m_2AChR, M2 muscarinic ACh receptor; CHO, Chinese hamster ovary; WT, wild-type.

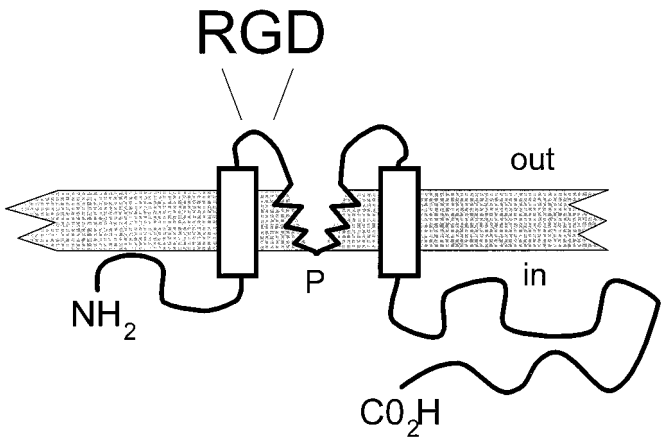

FIG. 1. **Schematic diagram of the GIRK subunits showing the location of the conserved RGD sequence.**

GIRK1 and GIRK4 cDNAs were subcloned into pCDNA3.1 (Invitrogen).

Oocytes and Mammalian Cells—Xenopus laevis oocytes were prepared and maintained essentially as described (27). RNA was transcribed *in vitro* (mMessage Machine, Ambion) by T3 or T7 RNA polymerase for the GIRK subunits in pMXT and the m_2AChR in pGEM, respectively. For most electrophysiological studies, oocytes were injected with 50 nl containing 1 ng of each of the WT GIRK1 and GIRK4 subunit cRNAs and the m_2AChR cRNA in H_2O. However, in electrophysiological experiments with the GIRK1*/GIRK4* channel, 10 ng of each GIRK subunit cRNA was injected. For experiments with the GRGDSP peptide, 100 μM peptide was included in the bathing medium just after injection of channel RNA into the oocytes and in all recording solutions. Electrophysiological recordings were performed 1–4 days after injection. For biochemical experiments, oocytes were injected with 5–10 ng of each GIRK subunit cRNA and assayed 2–4 days after injection. For metabolic labeling with [^{35}S]methionine, oocytes were injected with 50 nl containing the GIRK cRNAs (5–10 ng/oocyte) as well as 0.25 μCi [^{35}S]methionine (Amersham Pharmacia Biotech).

CHO cells were maintained in Ham's F-12 with 10% fetal bovine serum, 1 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin under an atmosphere of 5% CO_2 at 37 °C. For recording from transfected CHO cells, the LipofectAMINE reagent (Life Technologies, Inc.) was used to cotransfect a combination of WT or mutant GIRK1 and GIRK4 and m_2AChR (0.5 μg each/35-mm dish) and green fluorescent protein (pGreen Lantern, 0.125 μg/35-mm dish, Life Technologies, Inc.) DNA in serum-free medium, and recording was performed 24 h after transfection (28).

Whole Cell Recording—Two-electrode voltage clamp recording was performed on *X. laevis* oocytes using an Axoclamp 2A amplifier (Axon Instruments). Data acquisition, voltage command signals, and analysis were executed using pCLAMP software (Axon). The initial recording solution was a high Na^+ solution containing 98 mM NaCl, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.5. To record inward GIRK currents, the solution was changed to either a 98 K^+ or a 20 K^+ solution similar to the high Na^+ except that the NaCl was either fully replaced by KCl or partially replaced by 20 mM KCl. To activate the GIRK currents via the m_2AChR, 1 μM ACh was included. In order to block GIRK currents, 300 μM Ba^{2+} was also included. GIRK currents were recorded using a voltage ramp from −80 to +30 mV from a holding potential of −80 mV. Voltage jumps from the −80 mV holding potential to 0 mV and then to −100 mV were also performed in order to examine the activation kinetics of the GIRK channels.

Whole cell patch-clamp recording from CHO cells was performed using an Axopatch 1D amplifier (Axon Instruments). 3–5 MΩ electrodes were used to form high resistance seals on the cell membranes. The recording pipette solution contained 144 mM KCl, 2 mM $MgCl_2$, 5 mM HEPES, 5 mM EGTA, 4 mM ATP, and 0.2 mM GTP, pH 7.2. Initial recording was performed in an external solution of low K^+ containing 140 mM NaCl, 30 mM D-glucose, 5.4 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 10 mM HEPES, pH 7.4. The 98 K^+ solution, with or without 5 μM ACh, was applied from movable tubes near the cell and contained 120 mM NaCl, 30 mM D-glucose, 25 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, pH 7.4.

Coimmunoprecipitation—For coimmunoprecipitation from whole oocytes, 10–30 oocytes were homogenized in 10 μl/oocyte of lysis buffer (150 mM NaCl, 1 mM $MgCl_2$, 50 mM Tris, pH 7.6, 1% CHAPS, 2 μM aprotinin, 2 μM pepstatin, 2 μM leupeptin, 2 mM iodoacetamide, 1 mM *N*-ethylmaleimide, and 2 mM phenylmethylsulfonyl fluoride) for 10 min on ice. Insoluble material was removed by 1 min of centrifugation at 16,000 × *g*, 4 °C. The supernatant was precleared by incubation at 4 °C with protein G-Sepharose (Amersham Pharmacia Biotech) for 1 h. Sepharose was removed by 20 s of centrifugation in a Picofuge (Stratagene). Primary antibody (GIRK1, GIRK4, anti-chick integrin β1, or pan-Trk) and protein G-Sepharose were added to the precleared lysate and incubated for 3 h at 4 °C and pelleted. Immunoprecipitates were washed 4 times with 1 ml of lysis buffer, heated at 95 °C for 5 min in 2× nonreducing Laemmli sample buffer, and electrophoresed on SDS-polyacrylamide gels (7% polyacrylamide for integrin and 10% for GIRKs).

Plasma Membrane Preparations—Plasma membranes were separated mechanically, with modifications from a previously described procedure (29). Defolliculated oocytes were incubated for 10 min in ice-cold hypotonic solution (5 mM NaCl, 5 mM Hepes, 0.07% SDS, containing protease inhibitors as described above). Plasma membranes together with vitelline membranes (extracellular collagen-like matrix) were removed manually with fine forceps. Six plasma membranes from each group were solubilized in SDS loading sample buffer, heated at 95 °C for 5 min, and analyzed by 10% SDS-polyacrylamide gel electrophoresis.

Western Blots and Autoradiography—Proteins were separated on 7 or 10% SDS-polyacrylamide gels to visualize the integrin and the GIRK channels, respectively (Protean System, Bio-Rad). The proteins were then transferred to nitrocellulose membranes and visualized with Western blots or autoradiography in the following manner. For Western blots, membranes were blocked with 5% nonfat dried milk in PBS with 0.1% Tween 20 for 1 h. After blocking, membranes were incubated with appropriate primary antibody for 3 h at room temperature, washed in blocking solution diluted 1:1 with PBS, and then incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies for 1 h. Primary and secondary antibodies were diluted in the blocking solution. Proteins were visualized using an ECL detection system (Amersham Pharmacia Biotech). Using antibodies from different species for the immunoprecipitation and immunoblot minimized their tendency to obscure the proteins of interest on Western blots of immunoprecipitated proteins. For the same reason, when visualization of immunoprecipitated GIRK proteins expressed in oocytes was desired, [^{35}S]methionine was coinjected to allow for radioactive labeling of the channel proteins during synthesis and autoradiographic visualization of the proteins on nitrocellulose blots. Integrins were not labeled detectably by [^{35}S]methionine because integrins are synthesized throughout oogenesis and before injection of the mRNA-[^{35}S]methionine mixture, in contrast to GIRK1 and GIRK4, which are fully labeled because synthesis occurs only during the 2–4 days after injection of the mRNA-[^{35}S]methionine mixture. Western blots with the 8C8 antibody used nonreducing conditions, because this antibody recognizes the nonreduced form of integrin.

RESULTS

Mutagenesis Shows That the RGD Site Is Required for Functional GIRK Activity—We used site-directed mutagenesis to alter the RGD site on both the GIRK1 and GIRK4 subunits. The mutations, converting the aspartate to glutamate, change only the size of the amino acid side chains. Thus, they are unlikely to change channel expression or assembly while disrupting the integrin binding site. An asterisk is used to indicate subunits containing the RGE mutation.

GIRK1/GIRK4 channel activity was assessed after co-injection of mRNA for the m_2AChR, GIRK1, and GIRK4 into *X. laevis* oocytes. GIRK current was elicited with 1 μM ACh, which stimulates the expressed m_2AChR, activating endogenous G_i to liberate $G\beta\gamma$, which in turn activates the expressed GIRK1/GIRK4 heteromultimeric channels. In Figs. 2 and 3*a*, the ACh-evoked GIRK currents from various combinations of mutant and WT GIRK subunits are compared. Similar quantities of RNA were coinjected into the oocytes, except for the GIRK1*/GIRK4* construct, which had 10-fold more RNA, in the following combinations: GIRK1/GIRK4, GIRK1*/GIRK4, GIRK1/GIRK4*, and GIRK1*/GIRK4*. Recordings were made 2 days after injection. The data show that the GIRK1*/GIRK4, GIRK1/GIRK4*, and double mutant GIRK1*/GIRK4* combinations produced <25%, <10%, and <2% the current of WT channels,

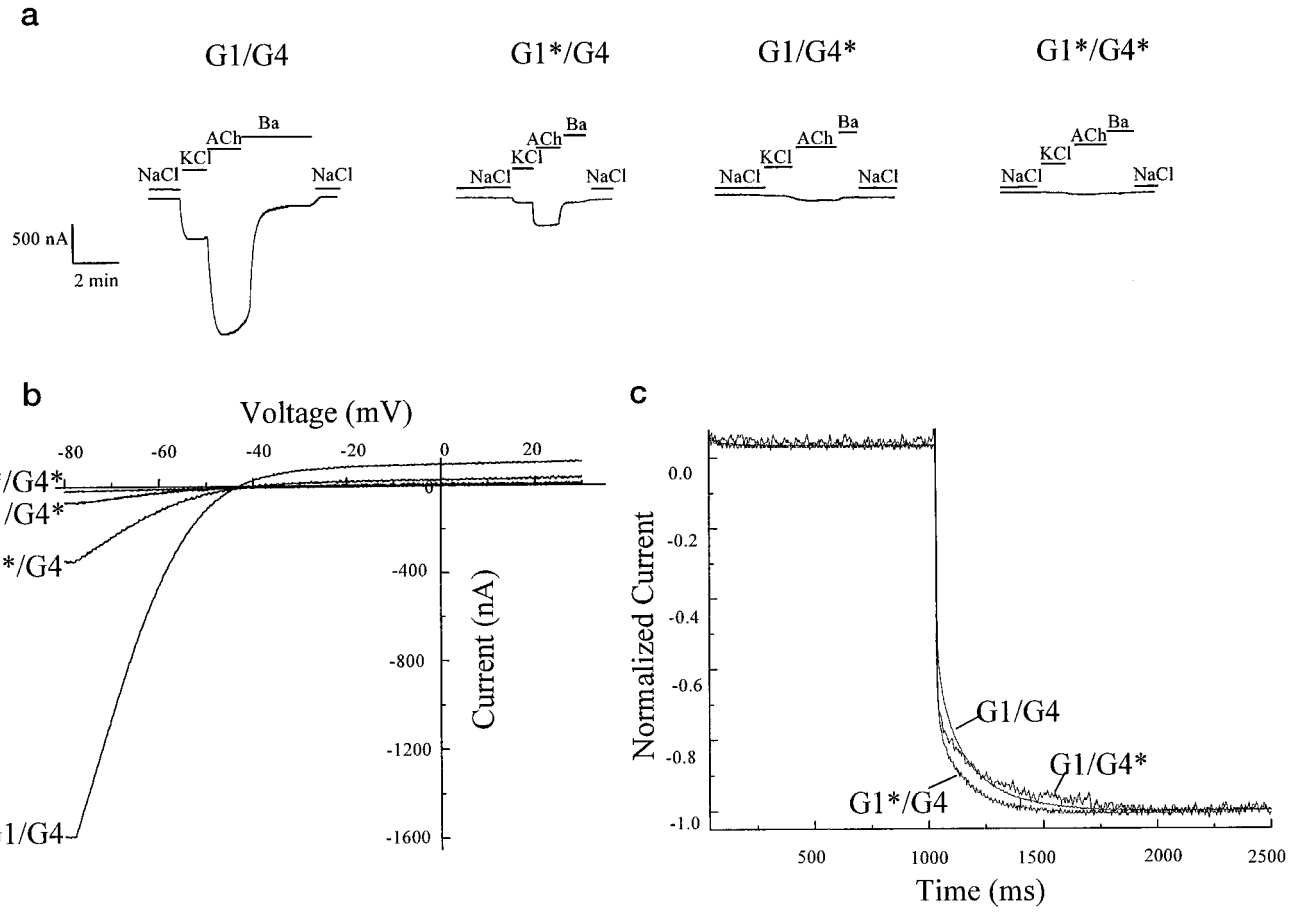

FIG. 2. **ACh-evoked currents from *X. laevis* oocytes expressing normal and RGD→RGE mutated GIRK channels 2 days after injection with RNA for various combinations of WT and mutant GIRK subunits and the m_2AChR**. The *asterisk* indicates subunits containing the RGE mutation. *a,* recordings after application of high Na^+ solution (*NaCl*), 20 K^+ solution (*KCl*), 20 K^+ solution with 1 μM ACh (*ACh*), and 20 K^+ solution with 1 μM ACh and 300 μM Ba^{2+} (*Ba*). *b,* ramps from −80 to +30 mV. *c,* voltage jump relaxations for a step from 0 to −100 mV. Waveforms have been scaled to superimpose. GIRK1*/GIRK4* currents are not included because they are too small for meaningful normalization.

respectively (currents at −80 mV are directly compared in Figs. 2*a* and 3*a*). The amount of GIRK1*/GIRK4 current continued to increase on days 3 and 4 after injection and reached levels comparable to those measured on day 1 or 2 for WT channels. In contrast, after waiting several more days for expression, we never observed large currents from the GIRK1/GIRK4* or the double mutant channels. GIRK channels display activation in the absence of agonists in heterologous expression systems; these basal currents were decreased to the same extent as the ACh-evoked currents (Fig. 2*a*). Even injecting larger quantities of RNA for mutant subunits (shown in Figs. 2 and 3*a* for the GIRK1*/GIRK4* mutant) or injecting Gβ1γ2 mRNA directly (2, 30) (data not shown) did not rescue channel function. No obvious difference in the extent of inward rectification or voltage-jump kinetics of the currents was observed for mutant subunits (Fig. 2, *b* and *c*). These data show that mutation of the RGD site on the channel to RGE disrupts GIRK currents in oocytes, consistent with the idea that the channel and endogenous oocyte integrin interact. The GIRK4 subunit, furthermore, seems to have a more important role in this interaction than the GIRK1 subunit. Interestingly, although integrin commonly interacts with ligands such as extracellular matrix proteins or with molecules found on other cells, the interaction of integrin with the GIRK channel seems to be occurring on the same cell (11).

WT and mutant channels were also expressed in CHO cells, which, unlike oocytes, express no endogenous GIRK subunits (31). Cells were transfected with DNA for the channel, m_2AChR, and green fluorescent protein, which provided a visual indicator of cotransfected cells. ACh-evoked currents were compared beginning 24 h after transfection (Fig. 3*b*). In the CHO cells, the GIRK1*/GIRK4 construct expressed as well as did the WT construct. Strikingly, any construct containing the GIRK4* subunit, either as GIRK1/GIRK4* or GIRK1*/GIRK4*, gave no detectable current. Thus, the relative importance of the GIRK4 subunit in the possible interaction of GIRK and integrin is revealed more dramatically by expression in the CHO cell system.

An Externally Applied RGD Peptide Does Not Detectably Alter GIRK Function—Externally applied RGD-containing peptides are often used to compete for an interaction of integrin with its ligand, disrupting integrin activity and thus implicating a role for integrin in various cellular phenomena (20, 22). We performed similar experiments with expressed GIRK channels. GIRK activity was again observed after co-injection of mRNA for the m_2AChR, GIRK1, and GIRK4 into *X. laevis* oocytes. In Fig. 4, ACh-evoked GIRK currents from oocytes incubated in the absence or presence of 100 μM GRGDSP were compared 1 day after RNA injection. Peptide was added to the oocyte bathing medium immediately following RNA injection and was included in all of the recording solutions. Desensitization was measured as the fractional ACh induced current remaining 5 min after the peak current. The peptide produced no dramatic change in the peak amplitude (Fig. 4*a*) or desensitization (Fig. 4*b*) of ACh-evoked currents.

GIRK Channels Bind Directly to Integrins—That the GIRK

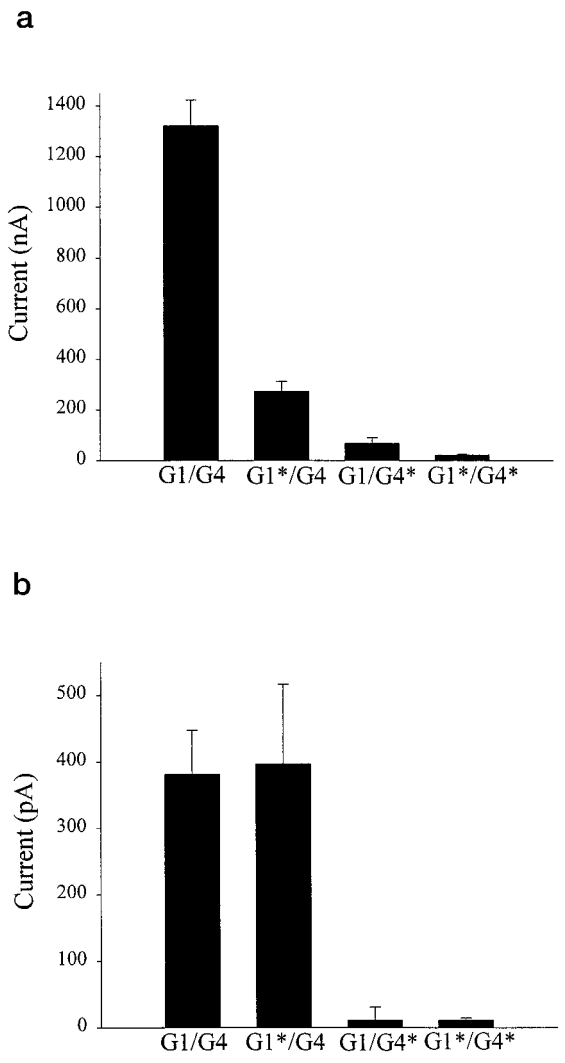

FIG. 3. **Direct comparison of ACh-evoked mean currents ± S.E. for WT and RGD→RGE mutated GIRK channels.** *a,* currents from oocytes measured at −80 mV; each *column* represents measurements from 4 or 5 oocytes as described in Fig. 2. *b,* currents from CHO cells measured at −120 mV 24 h after transfection for the m_2AChR and for various combinations of WT and mutant GIRK subunits (0.5 μg of DNA from each construct/dish). Each *column* represents measurements from 3–5 cells.

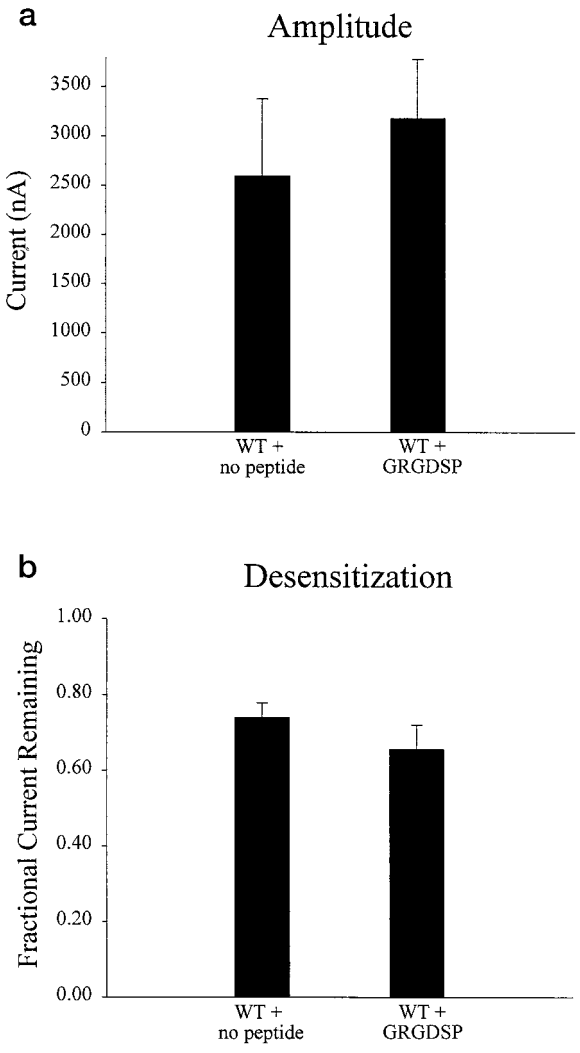

FIG. 4. **ACh-evoked currents measured in 98 K^+ solution 1 day after GIRK channel and m_2AChr RNA injection into *X. laevis* oocytes in the absence and presence of extracellular 100 μM GRGDSP peptide.** *a,* mean amplitude ± S.E. (four oocytes each) of peak ACh-induced current at −80 mV. *b,* mean desensitization ± S.E. (four oocytes each). Desensitization was measured as the fractional ACh-induced current remaining 5 min after the peak current. The data are representative of experiments on three oocyte batches and from two separate syntheses of peptide.

channel binds to integrin is consistent with the mutagenesis studies described above (Figs. 2 and 3), but the lack of RGD peptide effects produced no further evidence for a direct interaction. The lack of effect with the RGD-containing peptide does not rule out an interaction between GIRKs and integrins, because the peptide may not be able to access the GIRK-integrin binding site or may not form a structure that competes effectively for a GIRK-integrin interaction. We initiated a series of studies to co-immunoprecipitate the channel and endogenous integrin from detergent-solubilized whole oocytes, with detection by Western blots and by autoradiography for the GIRK channels labeled radioactively during expression in oocytes. Fig. 5, *a* and *b*, shows that the GIRK channel and integrin were co-immunoprecipitated from oocytes with antibodies specific for either protein. An anti-chick integrin antibody, against a highly conserved portion of the integrin β1 subunit, immunoprecipitated the radiolabeled WT GIRK1 and GIRK4 subunits, as did the anti-GIRK1 antibody (Fig. 5*a*, autoradiogram). Data from cells expressing either the GIRK1 or GIRK4 subunit (immunoprecipitated with the anti-GIRK1 and GIRK4 antibodies, respectively) confirm that the GIRK1 protein runs as a doublet of about 56–58 kDa and that the GIRK4 protein runs as a major band of about 45 kDa. Western blots performed with an anti-*Xenopus* β1 integrin antibody revealed that the anti-GIRK1 antibody immunoprecipitated the endogenous oocyte integrin from cells expressing both WT GIRK subunits, as did the anti-chick integrin antibody (Fig. 5*b*, immunoblot). Integrin β1 runs as a doublet due to differentially glycosylated forms (32). A control antibody that binds to neither integrin nor the channel, pan-Trk, immunoprecipitated neither the channel nor integrin, ruling out the possibility that these proteins bound nonspecifically to the Sepharose used during immunoprecipitation. These studies show directly that GIRK and integrin do bind to each other when the channel is expressed in heterologous cells.

We performed similar co-immunoprecipitation experiments with the mutant GIRK subunits expressed in oocytes. If these mutations disrupt the binding of integrin to the GIRK channel, we would expect that the mutant channels and integrin would not co-immunoprecipitate. As shown in Fig. 5*a*, although the anti-chick integrin antibody immunoprecipitated both coexpressed WT GIRK channel subunits, it did not precipitate

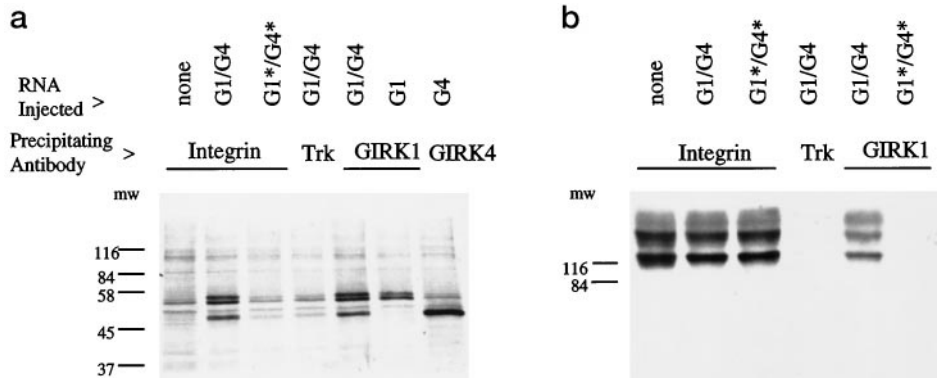

FIG. 5. **Co-immunoprecipitation of GIRK channels and endogenous integrin from GIRK1/GIRK4 RNA-injected oocytes.** *a,* GIRK1 and GIRK4 subunit protein visualized autoradiographically from uninjected oocytes or oocytes injected with GIRK1/GIRK4, GIRK1*/GIRK4*, GIRK1, or GIRK4 (blot representative of six experiments). All cells were also injected with [^{35}S]methionine. Protein in lanes labeled *Integrin* was immunoprecipitated with the integrin anti-chick antibody from cells injected with no channel RNA (*none*), GIRK1/GIRK4 channel RNA (*G1/G4*), or GIRK1*/GIRK4* channel RNA (*G1*/G4**). Protein in the lane labeled *Trk* was eluted after pan-Trk antibody immunoprecipitation from cells injected with GIRK1/GIRK4 RNA. Protein in the lanes labeled *GIRK1* was immunoprecipitated with anti-GIRK1 antibody from cells injected with GIRK1/GIRK4 channel RNA (*G1/G4*) or GIRK1 RNA only (*G1*), showing that the GIRK1 protein runs as a clearly identifiable doublet at about 56–58 kDa. Protein in the lane labeled *GIRK4* was eluted after anti-GIRK4 antibody immunoprecipitation from cells injected with GIRK4 RNA only, showing that the GIRK4 protein runs as a major band at about 45 kDa. Protein was from 30 oocytes each for lanes *Integrin* and *Trk* and from 10 oocytes each for *GIRK1* and *GIRK4*. *b,* endogenous integrin protein visualized by Western blotting with the 8C8 antibody from uninjected oocytes or oocytes injected with GIRK1/GIRK4 or GIRK1*/GIRK4* RNA (blot representative of two experiments). Protein in lanes labeled *Integrin* was integrin immunoprecipitated with the integrin anti-chick antibody from cells injected with no RNA (*none*), GIRK1/GIRK4 RNA (*G1/G4*), or GIRK1*/GIRK4* RNA (*G1*/G4**). *Trk,* absence of integrin in eluate after pan-Trk antibody immunoprecipitation. Protein in lanes labeled *GIRK1* was integrin immunoprecipitated with the anti-GIRK1 antibody from oocytes injected with GIRK1/GIRK4 RNA (*G1/G4*) or GIRK1*/GIRK4* (*G1*/G4**). Protein was from 10 oocytes for *Integrin* and *Trk* and from 20 oocytes for *GIRK1*.

either subunit of the GIRK1*/GIRK4* channel. Correspondingly, Fig. 5*b* demonstrates that although the anti-GIRK1 antibody immunoprecipitated the integrin from cells expressing WT channels, it did not immunoprecipitate integrin from cells expressing the double mutant channels. As expected, the binding of the mutant channel to integrin appears to be disrupted, and this disruption apparently eliminates its ACh-activated current.

Mutant Channel Expression Is Not Altered, but Plasma Membrane Localization Is Altered—Fig. 6, *a* and *b,* shows a comparison of the oocyte expression levels of GIRK channels composed of mutant *versus* WT subunits. After the oocyte was lysed, channel subunits were detected by Western analysis with antibodies specific to GIRK1 and GIRK4 (Fig. 6, *a* and *b*, respectively). When WT or mutant subunits were expressed in oocytes, the total amount of each channel subunit protein was similar.

Although roughly equal total amounts of protein were expressed for each channel combination, we found that less mutant channel protein reached the plasma membrane. The immunoblots in Fig. 6, *c* and *d,* compare the GIRK1 and GIRK4 channel subunit protein contained in manually isolated (29) plasma membranes of oocytes expressing WT and various mutant GIRK combinations. When either WT GIRK subunit was replaced by a mutated subunit, less GIRK1 (Fig. 6*c*) or GIRK4 (Fig. 6*d*) subunit reached the plasma membrane, even though the total cellular expression levels of the mutant proteins are the same as WT. The diminution in plasma membrane expression was greatest when both GIRK1 and GIRK4 subunits were replaced by the mutated subunit and was particularly striking for the more slowly migrating band corresponding to the glycosylated GIRK protein. In other experiments not shown, we injected oocytes with [^{35}S]methionine as they expressed normal and double mutant GIRK channels, manually isolated the membranes, and used the GIRK1 antibody to immunoprecipitate the GIRK channel protein. We measured >3-fold and >10-fold less radioactivity in the unglycosylated and glycosylated bands, respectively, for the double mutant GIRK channels. Interestingly, when we purified a plasma membrane fraction by isolating various pools of oocyte membranes from a total membrane preparation on sucrose gradients (33, 34), we observed no difference in the amount of mutant and WT channel protein, possibly because the mutant GIRK proteins may reach a vesicle pool that cofractionates with the plasma membrane.

DISCUSSION

Direct Binding of GIRK to Integrin Is Required for GIRK Channel Function—The data presented here support the idea that the GIRK channel binds to integrin directly, that mutation of the integrin recognition site on the channel eliminates this binding, and that without this binding, the GIRK current decreases or is eliminated. Evidence for this view is provided by the observations that GIRK RGD → RGE mutants have disrupted function and that WT but not RGD → RGE mutants co-immunoprecipitate from total oocyte lysate. Expression of the mutant channel protein is comparable to that of WT, but decreased quantities of mutant GIRK protein reach the plasma membrane (Fig. 6). Most likely, the decreased quantity of mutant channel protein in the plasma membrane explains most if not all of the decreased current for mutant-containing constructs.

The RGD → RGE mutation preserves the charge of the native amino acid and is thus unlikely to cause a major disruption of protein folding or membrane insertion. Recent atomic-scale crystallographic data on a *Streptomyces lividans* K^+ channel suggest that this region of the channel is part of an extracellular "turret" on the channel (35), conceivably poised to interact with the extracellular domain of an integrin molecule. Although such a mutation would be expected to destroy function if the aspartate residue interacts directly with permeant K^+ ions, the structural data also argue against this possibility. Interestingly, although the RGD sequence is preserved in GIRK channels, it diverges slightly in other inward rectifiers, often forming an HGD motif, but the aspartate residue of the motif is well conserved throughout the family of inward rectifiers. The residue is clearly important in Kir1.1 (ROMK1), for instance, because an aspartate-to-histidine mutation produces a nonfunctional channel in one allele of Bartter syndrome (36). This conservation is consistent with the idea that other inward

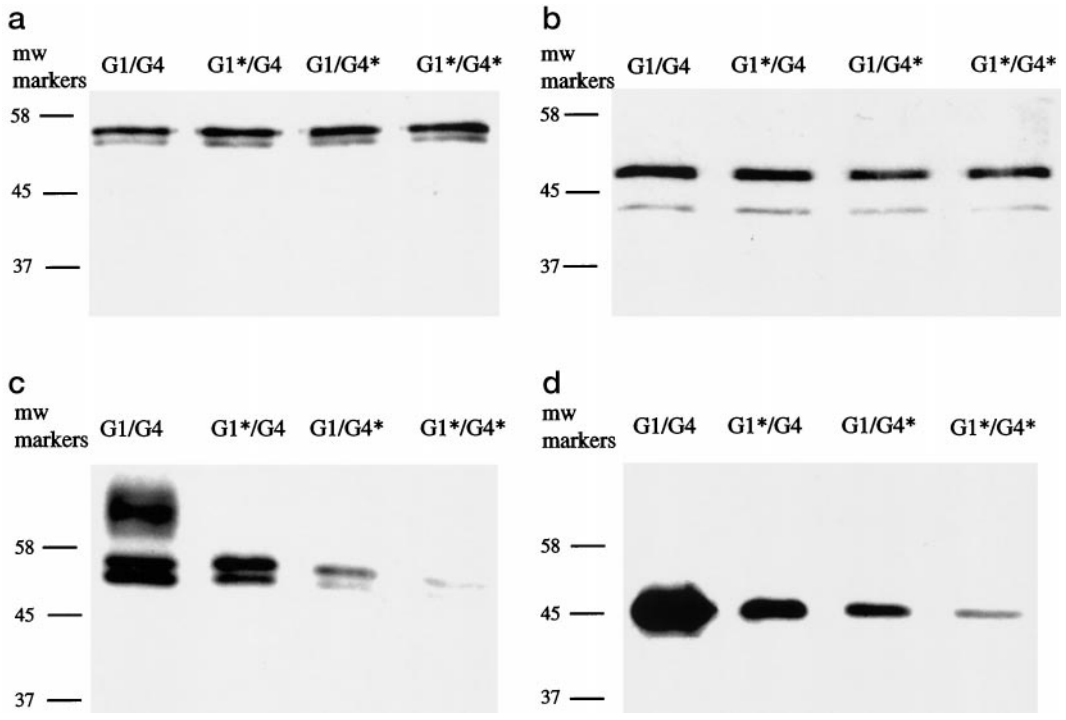

FIG. 6. *a* and *b,* total cellular GIRK subunit protein from various combinations of WT and mutant GIRK1 and GIRK4 RNA-injected oocytes. Cells were solubilized with 1× Laemmli sample buffer. Oocytes were injected with 5 ng of each channel subunit RNA. Protein was from ⅕ oocyte/lane. *a,* GIRK1 subunit protein visualized with Western blots using the anti-GIRK1 antibody. *b,* GIRK4 subunit protein visualized with Western blots using the rabbit anti-GIRK4 antibody. *c* and *d,* GIRK subunit protein found in plasma membranes manually stripped from oocytes injected with various combinations of WT and mutant GIRK1 and GIRK4 RNA. Oocytes were injected with 10 ng of each channel subunit RNA. Each lane contains protein from the plasma membranes of six oocytes. *c,* GIRK1 subunit protein visualized with anti-GIRK1 antibody. *d,* GIRK4 subunit protein visualized with the guinea pig anti-GIRK4 antibody.

rectifiers may also interact with integrin, a notion that we will explore in the future.

Mechanism of GIRK Regulation by GIRK-Integrin Binding—We suggest that integrin plays a role in the insertion, degradation, or stability of GIRK proteins in the plasma membrane. Perhaps the plasma membrane expression of the RGD → RGE GIRK mutant subunits decreased because the mutant subunits cannot bind to integrin in internal membrane compartments. If the GIRK-integrin interaction is in an intracellular compartment, this explains how WT channel function is not altered by incubation with externally applied, RGD-containing peptides.

Integrins themselves cycle in and out of the plasma membranes of neutrophils, and this system is stimulated by chemical signals in a Ca^{2+}-dependent manner, allowing changes in the migration behavior of these cells (15, 37, 38). Integrin receptors in the plasma membrane of fibroblasts are also regulated; their stability in the membrane requires the transmission of adhesion-dependent signals (39). In addition, evidence exists that integrin regulates the localization of other proteins. For example, integrin mediates the internalization of adenovirus by binding the virus, which is then endocytosed along with integrin (17). The degradation of vitronectin is also regulated by integrin in a similar manner, requiring the prior activation of protein kinase C (16, 40).

Integrins, particularly those including the integrin $\beta 1$ subunit that binds with GIRK in our experiments, are associated with the cytoskeleton via direct interactions with talin, paxillin, tensin, and possibly other cytoskeletal proteins (12). Therefore, the integrin-GIRK interaction may link the channel to the cytoskeleton. Several other channels have links to the cytoskeleton (41–47), and previous studies show that the actin cytoskeleton helps to stabilize some inward rectifiers (48). Mechanical stress, presumably transmitted via cytoskeletal elements, affects both the integrin-mediated transduction pathway (49) and GIRK activation (50).

It is also possible that integrin may mediate intracellular signals that regulate GIRK plasma membrane localization. Integrin intracellular signaling has been associated with tyrosine kinases, including src and focal adhesion kinase, and small GTPases, especially rho. Many of these signaling molecules are associated with integrin in signaling complexes, which are in turn ultimately linked to the cytoskeleton. Focal adhesion kinase also interacts with phosphatidylinositol 3-kinase, of interest considering that phosphoinositides may be required for GIRK activation (51, 52). Integrin-mediated signaling has also been associated with gene activation and the mitogen-activated protein kinase pathway (12). It is conceivable that one of these transduction pathways also modifies GIRK localization by either modifying its exocytosis, stability in the plasma membrane, or endocytosis.

Possible Sequelae Involving Integrin Signaling—It is not yet known whether the possible direct interaction between GIRK channels and integrin affects signaling by integrin itself via one the signal transduction pathways associated with the C-terminal cytoplasmic regions of the integrin molecule. Neurite formation in neuroblastoma cell lines has been associated with integrin, with the activation of an inward rectifier K^+ channel, and with tyrosine phosphorylation (22, 53). Mechanosensitive processes, including stretch-induced release of neurotransmitter (20), have also been associated with integrin function. GIRK channels are mechanosensitive (50), raising the possibility that GIRK and integrin may simultaneously be involved in mechanosensitive processes. Further studies may reveal details of the mechanism and physiological relevance of the GIRK-integrin interaction suggested by our data.

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