Expression of GIRK (Kir3.1/Kir3.4) channels in mouse fibroblast cells with and without β1 integrins

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Received 22 November 1999

Edited by Maurice Montal

Abstract G protein-activated K^+ channel (GIRK) subunits possess a conserved extracellular integrin-binding motif (RGD) and bind directly to β1 integrins. We expressed GIRK1/GIRK4 channels labeled with green fluorescent protein in fibroblast cell lines expressing or lacking β1 integrins. Neither plasma membrane localization nor agonist-evoked GIRK currents were affected by the absence of β1 integrins or by incubation with externally applied RGD-containing peptide. Mutation of the aspartate (D) of RGD impaired currents, GIRK glycosylation, and membrane localization, but the interaction with β1 integrins remained intact. Thus, β1 integrins are not essential for functional GIRK expression; and the GIRK-integrin interactions involve structural elements other than the RGD motif.

Key words: Voltage clamp; Inward rectifier K channel; Fibroblast; Green fluorescent protein

1. Introduction

Integrins are a large family of transmembrane adhesive receptors that mediate interactions both with the extracellular matrix and with neighboring cells. Integrin receptors are αβ heterodimers with a transmembrane segment, a short cytoplasmic domain, and a longer extracellular domain [1]. The extracellular ligand-binding domain in many cases recognizes a conserved RGD sequence on adhesion proteins [2,3], while the cytoplasmic domains interact with the cytoskeleton [1].

Besides acting as physical links between molecules in the extracellular matrix and the cytoskeleton, integrins function as transducers of various cellular signals [4]. The transduction pathways are initiated by interactions of integrins with extracellular matrix or cell surface ligands and include activation of tyrosine kinases [5], protein kinase C, MAP kinases [6–8], and modulation of membrane ion conductances [4]. Integrins are involved in the regulation of many cellular functions, including cell adhesion, proliferation, differentiation, migration, growth, and alteration of gene expression [1,9–11].

Integrin signaling pathways may participate in modulation of K^+ channel function. Integrin-ligand interactions enhance K^+ conductance, leading to membrane hyperpolarization in neuroblastoma and erythroleukemia cells after interaction with fibronectin [12,13]. The synthetic RGD-containing peptides that bind the αvβ3 integrins expressed by the vascular smooth muscle cause pronounced vasodilation in rat skeletal muscle arterioles [14]. Recently it was shown that an RGD-containing peptide, presumably working through α5β3, activates K^+ efflux from vascular smooth muscle cells, causing hyperpolarization and subsequent inactivation of voltage-gated Ca^{2+} channels and leading to a decrease in Ca^{2+} levels and to vasodilation [15].

G protein-activated K^+ channels (GIRKs) mediate the parasympathetic inhibition of heart rate and the inhibitory effects of many neurotransmitters in the brain [16]. In the heart, GIRK channels are activated by acetylcholine (ACh) via the muscarinic m2 receptor (m2R) and pertussis toxin-sensitive G proteins [17,18]. Cardiac GIRK is normally a heterotetramer of stoichiometry $GIRK1_2/GIRK4_2$. All known GIRK (Kir3.*x*) subunits, but no other known ion channels, have a conserved peptide sequence RGD on their extracellular surface between the putative first membrane spanning region (M1) and the P region. It has been shown recently that GIRK1 and GIRK4 channel subunits bind directly to β1 integrins and that mutation of the aspartate (D) of the integrin-binding RGD sequence of GIRK4 (and, to a lesser extent, of GIRK1) abolishes functional GIRK expression in *Xenopus* oocytes [19]. To extend this observation, we compared the expression and subcellular distribution of heteromultimeric GIRK1/4 channels in two mouse fibroblast cell lines: one expressing the β1A splice variant, and the other (GD10) lacking β1 integrins due to the disruption of the β1 integrin subunit gene [20]. Using chimeric constructs of GIRK with the fluorescent green protein (GFP), we observed similar plasma membrane localization of heteromultimeric GIRK1/4 channels in cell lines both with and without β1 integrins, and we observed no differences in the magnitude of GIRK currents in the two cell lines. The results indicate that GIRK1/4 inward rectifier channels do not require β1 integrins for expression and function.

2. Materials and methods

2.1. Materials

GIRK1 antibody was raised in rabbits against a peptide from the C-terminus of rat GIRK1 [21]. The integrin antibody was raised in rabbits against the C-terminus of chick β1 integrin (a gift of Dr. Richard Hynes) [22]. Restriction enzymes were from New England Biolabs. Other reagents were from Sigma, including the RGDS peptide (Cat. #A 9041).

Four cDNA constructs – GIRK1 WT (wild-type), GIRK4 WT, and their mutants in which the RGD sequences of the protein were

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PII: S0014-5793(99)01738-X

changed to RGE – were subcloned into pMXT [19]. RNA was transcribed in vitro using the mMessage Machine kit (Ambion).

For the construction of the GIRK-GFP chimeras, the stop codon was removed and a *Not*I site was introduced at the 3′ end of the coding sequences of GIRK1 WT, GIRK4 WT, GIRK1 D111E, and GIRK4 D117E using the polymerase chain reaction (PCR). The resulting constructs were ligated into pcDNA3.1 containing the GFP coding sequence so that the latter was fused in-frame to the 3′ end of the GIRK subunits. To determine whether the addition of GFP to the C-terminus of the GIRKs has any effect on the channel function, two-electrode voltage-clamp experiments were performed to measure current in *Xenopus* oocytes expressing these construct. No differences in function were detected between WT channels and those containing the GFP sequences.

2.2. Metabolic labeling with [^{35}S]methionine and co-immunoprecipitation

This was performed as described [23]. Oocytes were injected with GIRK1/4 WT and GIRK1/4(RGD→RGE) mutant cRNAs (5 ng each subunit per oocyte) and incubated in ND96, containing 0.5 mCi/ml [^{35}S]methionine (Amersham Pharmacia Biotech) for 3 days at 22°C. Plasma membranes together with the vitelline membranes (extracellular collagen-like matrix) were removed manually with fine forceps after a 5–10 min incubation in a low osmolarity solution. The remainder of the cell, consisting of cytoplasm and intracellular organelles ('internal fraction') was processed separately. Thirty plasma membranes and 30 internal fractions were homogenized separately for 10 min on ice in 300 μl of lysis buffer (150 mM NaCl, 1 mM $MgCl_2$, 50 mM Tris, pH 7.6, 1% CHAPS, 1 mM phenylmethylsulfonylfluoride and 2 μg/ml each of leupeptin, pepstatin and aprotinin). Insoluble material was removed by centrifugation for 1 min 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 centrifugation in a Picofuge (Stratagene). Primary antibody (GIRK1 or anti-chick integrin β1) 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. Samples were boiled in SDS-gel loading buffer and electrophoresed on 10% polyacrylamide-SDS gel.

2.3. Oocyte expression and electrophysiology

These procedures have been described [19]. Injected oocytes were incubated for 3 days in ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.5) supplemented with 2.5 mM sodium pyruvate and 50 μg/ml gentamicin. 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 one containing 98 mM KCl in place of NaCl. To activate the GIRK currents via the m2R, 1 μM ACh was included. GIRK1/4 currents were recorded using a voltage ramp from −80 to +30 mV from a holding potential of −80 mV.

2.4. Cell culture and transfection

The β1-null mouse fibroblast cell line GD10, which lacks expression of β1 integrin due to disruption of the β1 integrin subunit gene, and a control GD10 line expressing the β1A splice variant of β1 integrin were employed [20]. GD10 cells were cultured in DME+10% fetal

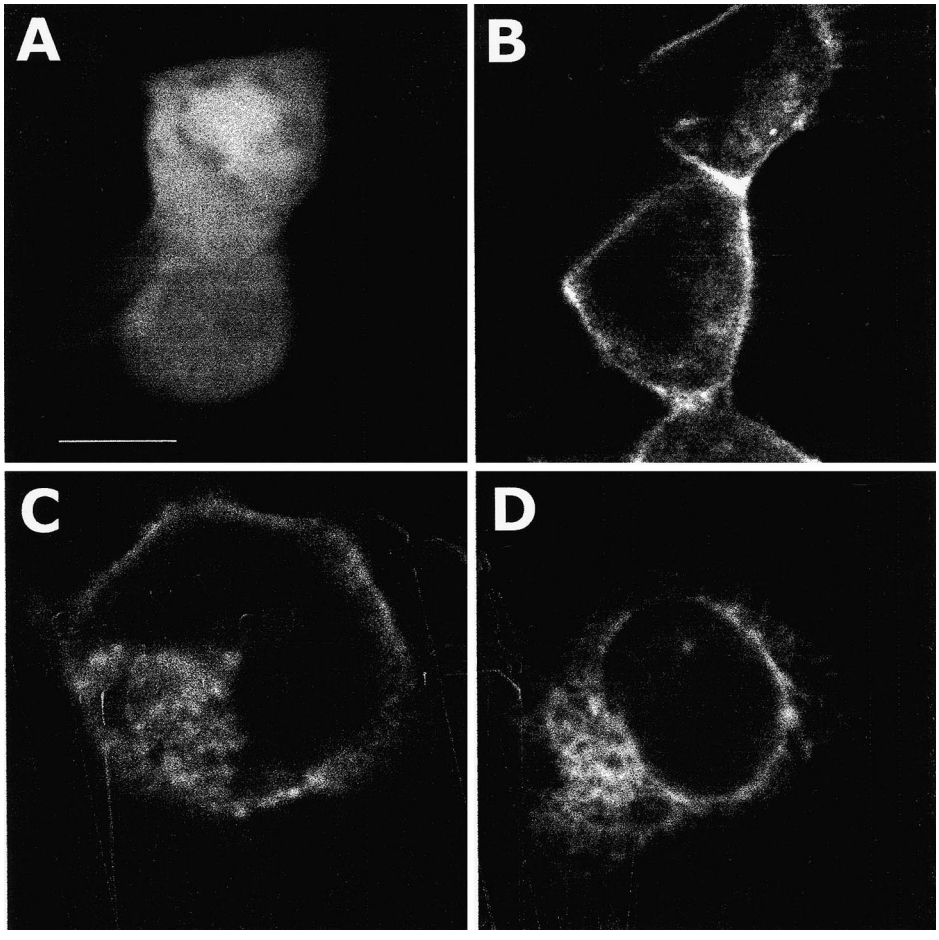

Fig. 1. Subcellular distribution of GFP-tagged GIRK1/4 subunits in control fibroblasts and in GD10 β1-null cells. A: Control fibroblasts with β1 integrin, transfected with GFP alone. Note the diffuse cytoplasmic fluorescence. B: Control fibroblasts transfected with WT GIRK1/4. Note the plasma membrane localization of fluorescence. C: GD10 β1-null fibroblasts transfected with WT GIRK1/4. Note the plasma membrane localization of fluorescence. D: Control fibroblasts after transfection with GIRK1/4(RGD→RGE) mutant. The GFP fluorescence does not reach the plasma membrane. Scale bar represents 25 μm in A, B and 15 μm in C, D.

bovine serum+L-glutamine (2 mM)+penicillin-streptomycin (non-selection medium). β1 integrin-expressing cell lines were continuously cultured in the same medium with puromycin (10 μg/ml) (selection medium). Cells were transiently transfected using Effectene (Qiagen) according to the manufacturer's instructions. For microscopy, 1 ng each of cDNA for GIRK1-GFP and GIRK4-GFP were transfected; for electrophysiology, 1 ng of cDNA for m2R was cotransfected in addition. All constructs were in pcDNA3.1. For experiments with the RGDS peptide, 0.4 mM peptide was included in the medium 2 h after transfection. Confocal microscopy and voltage-clamp recordings were performed 2 days after transfection.

2.5. Electrophysiology on fibroblasts

Recordings were begun in extracellular recording saline (mM): NaCl 140, KCl 5.4, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, D-glucose 30, pH 7.4. To elicit GIRK currents, the external solution was changed to one containing 25 mM KCl in place of 25 mM NaCl. Single-cell recording was performed at 21–24°C with pipettes pulled in four stages from 1.5 mm o.d. glass capillary tubes (WPI, Sarasota, FL, USA) with a P80/PC micropipette puller (Sutter Instruments, Novato, CA, USA). Patch pipettes were filled with a solution containing (mM): KCl 140, $MgCl_2$ 2, HEPES 5, EGTA 0.6, ATP 4, GTP 0.2, $CaCl_2$ 0.06, pH 7.4. Ionic currents were measured with a patch-clamp amplifier (Axopatch 200, Axon Instruments, Foster City, CA, USA), filtered at 2 kHz, digitized at 10 kHz, recorded on a computer (pCLAMP 7, Axon Instruments) and monitored on both a storage oscilloscope and a chart recorder. Pipette resistance ranged from 2 to 5 MΩ when filled with internal solution. Cells were voltage-clamped at a holding potential of −80 mV. Cells were chosen for study based on fluorescence of a transfected GFP reporter construct. Series resistance was monitored by measuring the instantaneous current in response to a 5 mV voltage step command. Results were discarded if the series resistance changed by more than 10% during the course of an experiment. The drugs were applied by a local perfusion system in which up to six solutions were connected to one port. The port fed a delivery tube (250 μM internal diameter) mounted ~500 μm from the recorded cell; flow rate was ~0.5 ml/min and changes among the solutions occurred within 2 s.

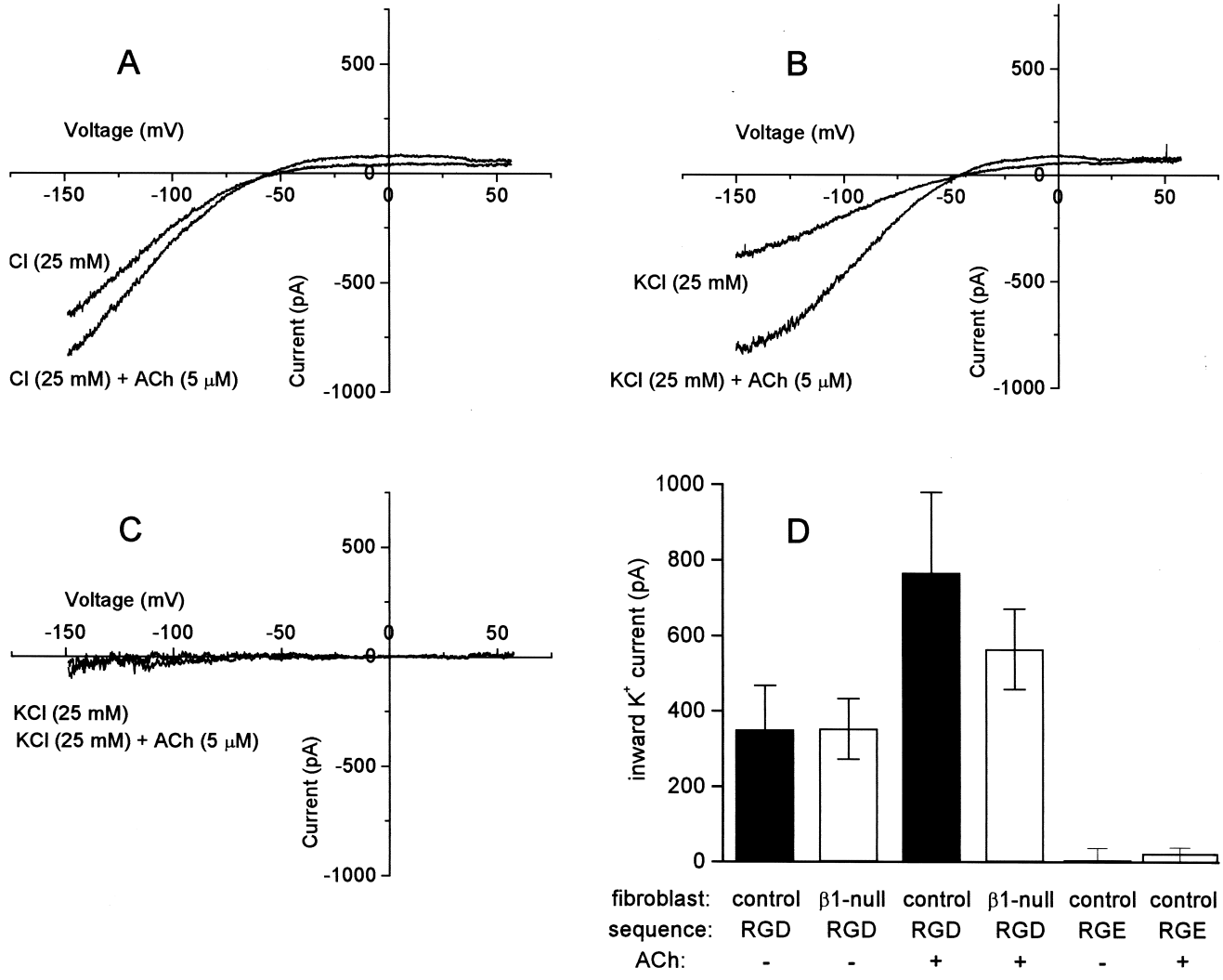

Fig. 2. GIRK currents in control and β1-null fibroblasts. Current-voltage relations were measured in the presence of KCl (25 mM) and in the presence of KCl+ACh (5 μM). Background current was blocked by Ba^{2+} (200 μM) and subtracted from traces in the absence of Ba^{2+}; resulting traces are plotted. A: Typical data for a control cell; B: β1-null fibroblast; C: control cell transfected with RGD→RGE mutant. D: Average currents induced by KCl (25 mM) and KCl+ACh (5 μM) for control and β1-null fibroblasts expressing WT GIRK1/4 and for control fibroblasts transfected with GIRK1/4(RGD→RGE). Currents were measured day 2 after cotransfection of the indicated fibroblast cell line with the GIRK1/GIRK4 heteromultimer channel and a GFP reporter plasmid. For measurement, cells were voltage-clamped at a holding potential of −80 mV and a voltage ramp (−150 mV to +60 mV) was used. Mean peak amplitudes of KCl and KCl+ACh-induced current were analyzed. Data represent mean ± S.E.M. of 10 control and 11 β1-null cells from three independent experiments.

3. Results

3.1. Distribution of GIRK1/4 WT-GFP and GIRK1/4(RGD→RGE)-GFP in GD10 cells

To study the role of the β1 integrins for expression and function of GIRK1/4 channels, we used the β1 integrin-deficient mouse fibroblast GD10 cell line. Because these cells do not express β1 integrins, we could directly determine whether heteromultimeric GIRK1/GIRK4 channel requires β1 integrin for proper function. We compared the expression level and localization of GIRK1/4 WT channel in two lines of fibroblasts – one expressing β1 integrins, and the other lacking expression of these proteins. We fused GFP in-frame to C-termini of GIRK1 and GIRK4 subunits and examined the localization of the labeled protein using a confocal microscope. In fibroblasts transfected with GFP-pcDNA3.1 alone, only a diffuse weak fluorescent background was seen (Fig. 1A). In fibroblasts expressing β1 integrins, the expressed GIRK1/4-GFP WT channels showed clear plasma membrane localization of fluorescence (Fig. 1B). In fibroblasts lacking β1 integrins, the strong plasma membrane signal was similarly obtained after transfection with GIRK1-GFP and GIRK4-GFP (Fig. 1C). This suggests that GIRK channels can reach the plasma membrane in cells lacking β1 integrins. In contrast to the distribution of the GIRK1/4-GFP WT channel, plasma membrane fluorescence of GFP fusions with RGD→RGE was less bright, and there was greater fluorescence in the endoplasmic reticulum and Golgi complex, indicating that the RGD→RGE mutant does not reach the plasma membrane but remains localized in the endoplasmic reticulum and Golgi complex (Fig. 1D).

3.2. GIRK currents in control and β1-null fibroblasts

The two lines of fibroblasts coexpressing GIRK1/4 and the muscarinic m2 receptors were also studied in voltage-clamp experiments to characterize the functionality of the expressed channels by monitoring the K^+ current of these channels. To measure basal GIRK currents, the initial low K^+ perfusion solution was changed to a high K^+ (25 mM KCl solution); the coexpressed m2R was then activated by application of 5 μM ACh in high K^+ solution. Current-voltage relations were obtained using a voltage ramp (−150 to +60 mV). Representative current-voltage relations for ACh-activated GIRK1/4 current in wild-type and in β1-null fibroblasts are shown in Fig. 2A and B, respectively. The basal and ACh-activated K^+ currents typically showed inward rectification and were completely blocked by the application of external Ba^{2+} (0.2 mM, not shown). At −80 mV, currents induced by ACh were 766 ± 214 pÅ in cells expressing β1 integrins and 588 ± 99 pÅ in cells without β1 integrins (mean ± S.E.M., $n = 10$ or 11). There also was no significant difference in basal GIRK currents (Fig. 2). Fig. 2C confirms that the RGD→RGE mutant produces no detectable GIRK currents in fibroblasts, as previously reported for CHO cells. There was no significant difference in whole-cell capacitance between control and β1-null cells.

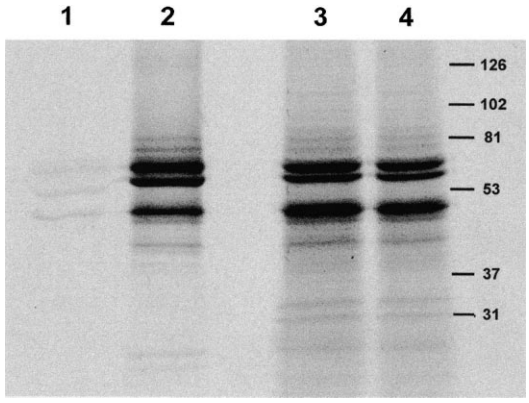

Fig. 4. Specificity of the antibody to integrin β1, examined with [^{35}S]methionine-labeled GIRK1/4 protein, translated in vitro in rabbit reticulocyte lysate. Lane 1, immunoprecipitation of GIRK1/4 with antibody to integrin β1. The antibody to integrin β1 does not recognize GIRK1/4 protein. Lane 2, immunoprecipitation ^{35}S-labeled GIRK1/4 channel expressed in lysate, using antibody to C-terminus of GIRK1. Lanes 3 and 4, GIRK1/4 translated in reticulocyte lysate. Each lane was loaded with 5 μl of lysate.

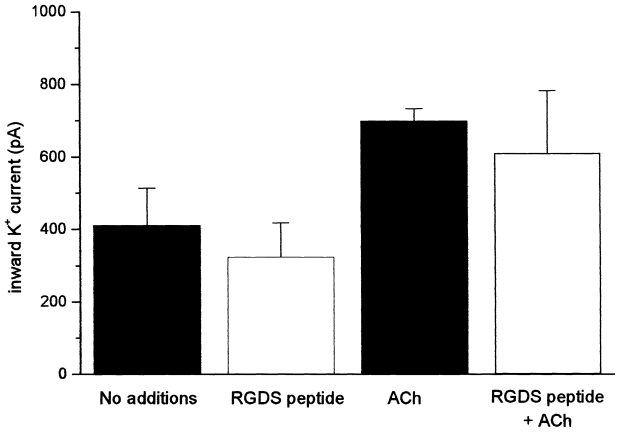

Fig. 3. KCl and ACh-induced GIRK current from β1-null fibroblasts in the presence and absence of extracellular RGD peptide (0.4 mM). Currents were measured at day 2 after cotransfection of CHO cells with the GIRK1/GIRK4 heteromultimer channel and a GFP reporter plasmid. Measurements were made as in Fig. 2. Data represent mean ± S.E.M. of six (with RGD peptide) and seven (control) cells from two independent experiments.

3.3. External application of RGD peptide

Because β1-null fibroblasts express αvβ3 and αvβ5 [24] which also recognize the RGD sequence, it was possible that these integrins could be involved in the interaction with GIRK1/4 channels. In order to examine this possibility, we used the RGDS peptide to disrupt this interaction. This RGDS-containing peptide is known to bind to integrins, competitively blocking the interaction with other proteins containing the RGD sequence [25]. For this purpose, β1-deficient fibroblasts were transfected with GIRK1/4-GFP WT constructs and incubated in the presence of 0.5 mM RGDS peptide for 2 days, and the distribution of GIRK1/4 channels in the fibroblasts was examined with the confocal microscope. In spite of the presence of RGDS peptide, GIRK1/4 channels were localized to the plasma membranes (data not shown). Whole-cell current measurements showed that the incubation with 0.4 mM RGDS peptide did not affect basal or ACh-induced GIRK currents (Fig. 3) (the peptide was added to all external solutions 1 h after the DNA transfection and remained in the solution throughout the experiment).

3.4. Co-immunoprecipitation of β1 integrins and GIRK1/4 WT and GIRK1/4(RGD→RGE) mutant channels

The interaction between β1 integrins and GIRK1/4 WT and double mutant GIRK1/4 channels was examined in *Xenopus* oocytes. First, to examine whether the antibody against β1 integrin recognizes GIRK1 or GIRK4 proteins, we expressed the GIRK1/4 channel in vitro in the rabbit reticulocyte lysate and immunoprecipitated the solubilized channel with antibodies for GIRK1 and for β1 integrin separately. As shown in Fig. 4 (lane 1), the β1 integrin antibody did not immunoprecipitate the GIRK1/4 channel, whereas the GIRK1 antibody did.

Oocytes were injected with cRNAs and metabolically labeled by incubation for 3 days in medium containing [^{35}S]methionine. The channels were isolated separately from plasma membranes and from the rest of the cell (internal fraction) by detergent solubilization, followed by immunoprecipitation, and subjected to SDS–PAGE and autoradiography. To study the interaction of GIRK1/4 WT and GIRK1/4(RGD→RGE) mutant channels with the *Xenopus* oocyte endogenous β1 integrins, we carried out co-immunoprecipitation using the antibody to the C-terminus of β1 integrin. In the same experiments, using the antibody against the C-terminus of GIRK1, we immunoprecipitated the GIRK1/4 channels to examine the levels of expression of WT and RGD→RGE mutant channels. Autoradiograms of typical gels are shown in Fig. 5A and B. Data presented in these figures were obtained in the same batch of oocytes. As shown previously, the immunoprecipitated GIRK1 protein migrated as a doublet of 58–56 kDa, and GIRK4 ran as a band at 45 kDa. The WT GIRK1 also has a diffuse band of approximately 80 kDa, which represents a heavily glycosylated form of the protein [26]. The level of expression of GIRK1/4 WT and GIRK1/4(RGD→RGE) mutant in the internal fraction was equal. In the plasma membrane fraction, the RGD→RGE mutants were found in much smaller amounts than the WT proteins, and the GIRK1(RGD→RGE) mutant also lacked the glycosylated form (Fig. 5A, lane 5). Band intensities were measured in three experiments using a PhosphorImager. In all experiments, the major difference between WT and RGD→RGE mutant channels was the absence of a glycosylated form in the GIRK1(RGD→RGE) mutant expressed in the plasma membrane. The amount of GIRK1 subunit mutants in the plasma membrane was 3 times less than that of the 58–56 kDa unglycosylated form of the WT protein.

Fig. 5B shows the results of co-immunoprecipitation of GIRK WT and mutant, using the antibody against β1 integrin. We observed co-immunoprecipitation of both GIRK1/4 WT and GIRK1/4 mutant. Again there was less GIRK1/4 intensity in the plasma membrane fraction from the oocytes expressing the RGD→RGE mutant, and the glycosylated form was essentially absent in the plasma membrane fraction. These results show that the RGD sequences are not essential for the interaction between GIRK1/4 and β1 integrins.

Two-electrode voltage-clamp recordings were made 3 days after RNA injection (m2R was coexpressed in all cases). One μM ACh, which stimulated large current via the WT GIRK1/4 channels, produced no detectable current in oocytes expressing the GIRK1/4(RGD→RGE) mutant (data not shown), in confirmation of previous data [19].

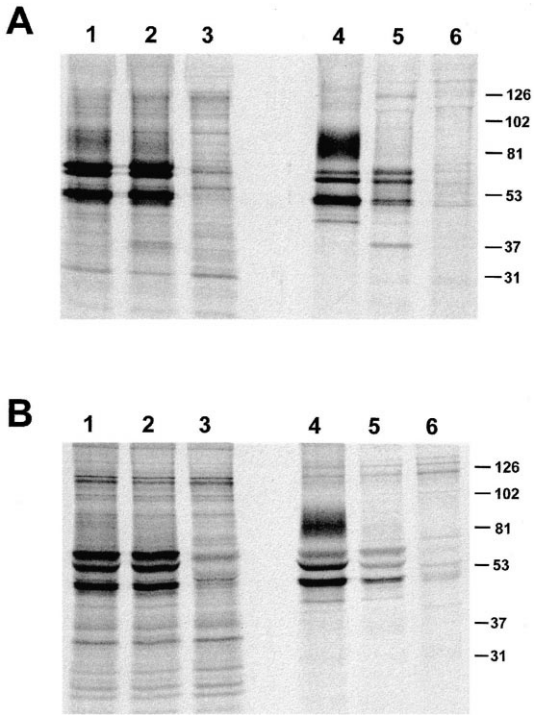

Fig. 5. SDS–PAGE analysis of immunoprecipitated GIRK1 and GIRK4 polypeptides expressed in cRNA-injected oocytes metabolically labeled with [^{35}S]methionine. A: Co-immunoprecipitation of GIRK1 and GIRK4 protein with antibody to GIRK1. B: Co-immunoprecipitation of endogenous β1 integrin and GIRK1 and GIRK4 with antibody to β1 integrin. In each lane, immunoprecipitates from 30 plasma membranes and 30 internal oocyte fractions were loaded. For both panels, lanes 1 and 4 contained immunoprecipitates from oocytes injected with GIRK1/4 cRNA; lanes 2 and 5 contained immunoprecipitates from oocytes injected with GIRK1/4(RGD→RGE) mutant cRNA; lanes 3 and 6 contained immunoprecipitates from uninjected oocytes.

4. Discussion

In the present study we provide evidence that, despite the interaction between inward rectifier GIRK1/4 channels and β1 integrins, these channels do not require β1 integrins for their expression and function. Moreover, we have shown that the RGD sequences of GIRK1 and GIRK4 channels are not essential for the interaction with β1 integrins. The RGD→RGE mutations result in loss of the GIRK1/4 function, apparently because proper channel processing and/or plasma targeting is disrupted. The mechanism of this crucial dependence on the conserved aspartate residue is unknown.

Confocal immunofluorescence microscopy analysis revealed no alterations in the distribution of GIRK1/4 protein in β1-null fibroblasts compared with cells expressing the β1A splice variant. The magnitude of the current in the two cell lines was not significantly different. These cells express α5β1, α6β1, and perhaps other αβ1A combinations. The possibility that other integrins (αvβ3 and αvβ5) present in the β1-deficient cells replaced the β1 integrin was addressed in part by experiments with an externally applied RGDS-containing peptide, which is expected to compete with GIRK channels for the extracellular interaction with integrins. The peptide affected neither the cellular distribution of the GIRK proteins nor the amplitude of basal and agonist-evoked GIRK currents. Thus, expression

and function of GIRK1/4 channels is not dependent on any RGD-mediated interaction that could be disrupted by externally applied peptide, with β1 or any other integrins present in GD10 cells. In control fibroblasts, β1-null fibroblasts, and oocytes, the GIRK(RGD→RGE) mutant was not expressed in the plasma membrane.

Results obtained in co-immunoprecipitation experiments in *Xenopus* oocytes showed that the endogenous β1 integrin interacts with GIRK1/4 WT, in agreement with earlier observations [19]. However, in contrast to the above work, we also observed interaction between β1 integrins and GIRK mutant RGD→RGE in the internal fraction and to some extent in the plasma membranes. This discrepancy may reflect differences in the level of expression of the GIRK1/4 WT and GIRK1/4(RGD→RGE) mutant present in the previously described co-immunoprecipitation experiments [19]. Our results demonstrate that the RGD sequences in GIRK1/4 are not essential for the interaction with β1 integrins.

It should be noted that, in *Xenopus* oocytes, we found no difference in the expression between WT and mutant RGD→RGE of GIRK in the internal fraction. However, the amount of the WT channels in plasma membranes was 3-fold greater than for the mutant channels. As mentioned above, the GFP-tagged GIRK1/4(RGD→RGE) mutant did not reach the plasma membrane in fibroblasts expressing β1 integrins. McPhee et al. [19] also observed that the RGD→RGE mutation decreased the quantities of the GIRK protein reaching the plasma membrane in *Xenopus* oocytes. McPhee et al. suggested that GIRK channels require β1 integrins for plasma membrane expression, and that mutation disrupts this interaction. However, the present results indicate that it is more likely that the RGD→RGE mutations cause inappropriate processing and/or trafficking of the GIRK protein via another, unknown mechanism. Interestingly, the RGD→RGE mutant did not have a glycosylated form in the plasma membrane. However, the absence of glycosylation alone cannot explain the loss of function, since the removal of the glycosylated site in GIRK1 does not affect GIRK function [27].

Our results are in agreement with a recently published work [28] showing that precursor convertase 1 containing a conserved RGD sequence interacts with α5β1 integrin in an RGD-independent manner. The authors suggest that the RGD motif of preprotein convertase 1 is critical for its cellular trafficking but not for its intracellular binding to integrin α5β1.

It should be noted that, although the RGD sequences are present only in GIRK but not in other K^+ channels, similar motifs are found in other inward rectifiers at the same position. The motif is HGD and other sequences; however, all inwardly rectifying K^+ channels have a conserved aspartate residue in this region. It was shown [29] that replacing this conserved aspartate for histidine in an HKD motif (which is not a recognition site for integrins) in Kir1.1 resulted in a complete loss of channel function. Perhaps only the aspartate residue, but not the entire RGD motif, is important for proper channel function.

Based on the above results, we conclude that (1) inward rectifier heterotetrameric GIRK1/GIRK4 channels do not require β1 integrins for expression and function; (2) the RGD motif, and possibly mainly the conserved aspartate residue, is important for proper GIRK processing; and (3) intact RGD motifs are not essential for interaction of GIRK1 and GIRK4 channels with β1 integrins. The region responsible for this interaction has not been identified, and the functional consequences of GIRK1/4 binding to β1 integrins remain uncertain.

Acknowledgements: We thank Nathan Dascal for helpful discussion, Steve Potter for help with confocal microscopy, and Hai-Rong Li for help with the oocytes. This work was supported by grants from the National Institutes of Health (GM-29836, HL21644), the US-Israel Binational Science Foundation, and the Deutsche Forschungsgemeinschaft.

References

[1] Hynes, R.O. (1992) Cell 69, 11–25.
[2] Yamada, K.M. and Kennedy, D.W. (1984) J. Cell Biol. 99, 29–36.
[3] Ruoslahti, E. and Pierschbacher, M.D. (1987) Science 238, 491–497.
[4] Clark, E.A. and Brugge, J.S. (1995) Science 268, 233–239.
[5] Kornberg, L.J., Earp, H.S., Turner, C.E., Prockop, C. and Juliano, R.L. (1991) Proc. Natl. Acad. Sci. USA 88, 8392–8396.
[6] Vuori, K. and Ruoslahti, E. (1993) J. Biol. Chem. 268, 21459–21462.
[7] Chen, Q., Kinch, M.S., Lin, T.H., Burridge, K. and Juliano, R.L. (1994) J. Biol. Chem. 269, 26602–26605.
[8] Schlaepfer, D.D., Hanks, S.K., Hunter, T. and van der Geer, P. (1994) Nature 372, 786–791.
[9] Haas, T.A. and Plow, E.F. (1994) Curr. Opin. Cell Biol. 6, 656–662.
[10] Dedhar, S. and Hannigan, G.E. (1996) Curr. Opin. Cell Biol. 8, 657–669.
[11] Parsons, J.T. (1996) Curr. Opin. Cell Biol. 8, 146–152.
[12] Arcangeli, A., Becchetti, A., Mannini, A., Mugnai, G., De Filippi, P., Tarone, G., Del Bene, M.R., Barletta, E., Wanke, E. and Olivotto, M. (1993) J. Cell Biol. 122, 1131–1143.
[13] Beekhuizen, H. and van Furth, R. (1993) J. Leukoc. Biol. 54, 363–378.
[14] Mogford, J.E., Davis, G.E., Platts, S.H. and Meininger, G.A. (1996) Circ. Res. 79, 821–826.
[15] Platts, S.H., Mogford, J.E., Davis, M.J. and Meininger, G.A. (1998) Am. J. Physiol. 275, H1449–1454.
[16] Dascal, N. (1997) Cell Signal. 9, 551–573.
[17] Wickman, K.D. and Clapham, D.E. (1995) Curr. Opin. Neurobiol. 5, 278–285.
[18] Yamada, M., Inanobe, A. and Kurachi, Y. (1998) Pharmacol. Rev. 50, 723–760.
[19] McPhee, J.C., Dang, Y.L., Davidson, N. and Lester, H.A. (1998) J. Biol. Chem. 253, 34696–34702.
[20] Sakai, T., Peyruchaud, O., Fassler, R. and Mosher, D.F. (1998) J. Biol. Chem. 273, 19378–19382.
[21] Dascal, N., Doupnik, C.A., Ivanina, T., Bausch, S., Wang, W., Lin, C., Garvey, J., Chavkin, C., Lester, H.A. and Davidson, N. (1995) Proc. Natl. Acad. Sci. USA 92, 6758–6762.
[22] Marcantonio, E.E. and Hynes, R.O. (1988) J. Cell Biol. 106, 1765–1772.
[23] Ivanina, T., Perets, T., Thornhill, W.B., Levin, G., Dascal, N. and Lotan, I. (1994) Biochemistry 33, 8786–8792.
[24] Wennerberg, K., Lohikangas, L., Gullberg, D., Pfaff, M., Johansson, S. and Fassler, R. (1996) J. Cell Biol. 132, 227–238.
[25] Grooms, S.Y. and Jones, L.S. (1997) Neurosci. Lett. 231, 139–142.
[26] Krapivinsky, G., Gordon, E.A., Wickman, K., Velimirovic, B., Krapivinsky, L. and Clapham, D.E. (1995) Nature 374, 135–141.
[27] Kennedy, M.E., Nemec, J., Corey, S., Wickman, K. and Clapham, D.E. (1999) J. Biol. Chem. 274, 2571–2582.
[28] Rovere, C., Luis, J., Lissitzky, J.C., Basak, A., Marvaldi, J., Chretien, M. and Seidah, N.G. (1999) J. Biol. Chem. 274, 12461–12467.
[29] Derst, C., Konrad, M., Kockerling, A., Karolyi, L., Deschenes, G., Daut, J., Karschin, A. and Seyberth, H.W. (1997) Biochem. Biophys. Res. Commun. 230, 641–645.