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Polarity Exchange at the Interface of Regulators of G Protein Signaling with G Protein α-Subunits*

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Thomas Wieland‡§, Nehat Bahtijari‡, Xiao-Bo Zhou‡, Christiane Kleuss¶, and Melvin I. Simon‖

From the ‡Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Universitäts-Krankenhaus Eppendorf, Martinistrasse 52, D-20246 Hamburg, the ¶Institut für Pharmakologie, Freie Universität Berlin, Thielallee 67-73, D-14195 Berlin, Germany, and the ‖Division of Biology, California Institute of Technology, Pasadena, California 91125

RGS proteins are GTPase-activating proteins (GAPs) for G protein α-subunits. This GAP activity is mediated by the interaction of conserved residues on regulator of G protein signaling (RGS) proteins and Gα-subunits. We mutated the important contact sites Glu-89, Asn-90, and Asn-130 in RGS16 to lysine, aspartate, and alanine, respectively. The interaction of RGS16 and its mutants with $G\alpha_t$ and $G\alpha_{i1}$ was studied. The GAP activities of RGS16N90D and RGS16N130A were strongly attenuated. RGS16E89K increased GTP hydrolysis of $G\alpha_{i1}$ by a similar extent, but with an about 100-fold reduced affinity compared with non-mutated RGS16. As Glu-89 in RGS16 is interacting with Lys-210 in $G\alpha_{i1}$, this lysine was changed to glutamate for compensation. $G\alpha_{i1}$K210E was insensitive to RGS16 but interacted with RGS16E89K. In rat uterine smooth muscle cells, wild type RGS16 abolished G_i-mediated α_2-adrenoreceptor signaling, whereas RGS16E89K was without effect. Both $G\alpha_{i1}$ and $G\alpha_{i1}$K210E mimicked the effect of α_2-adrenoreceptor stimulation. $G\alpha_{i1}$K210E was sensitive to RGS16E89K and 10-fold more potent than $G\alpha_{i1}$. Analogous mutants of $G\alpha_q$ ($G\alpha_q$K215E) and RGS4 (RGS4E87K) were created and studied in COS-7 cells. The activity of wild type $G\alpha_q$ was counteracted by wild type RGS4 but not by RGS4E87K. The activity of $G\alpha_q$K215E was inhibited by RGS4E87K, whereas non-mutated RGS4 was ineffective. We conclude that mutation of a conserved lysine residue to glutamate in $G\alpha_i$ and $G\alpha_q$ family members renders these proteins insensitive to wild type RGS proteins. Nevertheless, they are sensitive to glutamate to lysine mutants of RGS proteins. Such mutant pairs will be helpful tools in analyzing Gα-RGS specificities in living cells.

Many hormones, neurotransmitters, and sensory stimuli use surface receptors coupled to heterotrimeric ($G\alpha\beta\gamma$) guanine nucleotide-binding proteins (G proteins)[1] to transmit extracellular signals (1–3). To elicit an appropriate cellular response the strength and duration of intracellular signals must be tightly regulated. The duration of G protein activation itself is controlled by the intrinsic GTPase activity of Gα-subunits. GTP hydrolysis converts the active GTP-bound Gα-subunit to the inactive GDP-bound form, which then reassembles with the $G\beta\gamma$ dimer, and thus terminates signaling. Recently, a novel superfamily of GTPase-activating proteins (GAPs) for Gα-subunits, termed "regulators of G protein signaling" (RGS) proteins, with at least 21 different mammalian members, has been discovered (for review see Refs. 4–6). Besides their obvious role in regulation of signal strength and duration, RGS proteins have been shown to be involved in desensitization processes.

In *in vitro* reconstitution experiments, a variety of RGS proteins exerts GAP activity for members of the $G\alpha_i$ and $G\alpha_q$ subfamilies with a limited degree of specificity (for review see Ref. 6). On the molecular level, this lack of specificity is not very surprising. When the structure of the RGS4-$G\alpha_{i1}$ complex was solved by crystallization and x-ray diffraction (7), it became evident that interaction of these molecules involved the $G\alpha_{i1}$ amino acids Thr-182, Gln-204, Glu-207, Lys-210, and RGS4 amino acids Glu-87, Asn-88, and Asn-128. These important contact sites are highly conserved in many of the RGS proteins and all members of the $G\alpha_i$ and $G\alpha_q$ subfamilies. Nonetheless, there appears to be specificity with regard to G protein and receptor so that certain RGS proteins are part of particular signaling circuits (8). To explore such specificities in cells, it would be helpful to have mutants of RGS proteins available that do not interact with wild type Gα-subunits but are still able to interact with a specific mutant of the Gα-subunits.

We report herein that mutation of the RGS16 amino acids Asn-90 and Asn-130 to aspartate and alanine, respectively, causes a large reduction of GAP activity for the $G\alpha_i$ subfamily members $G\alpha_t$ and $G\alpha_{i1}$. Mutation of Glu-89 to lysine, reduces only the potency of RGS16 about 100-fold. In addition, mutation of Lys-210 in $G\alpha_{i1}$ to glutamate renders this Gα-subunit insensitive to wild type RGS16, whereas this mutant is fully responsive to the mutant RGS16E89K with regard to maximal GAP activity. By generating analogous mutations in $G\alpha_q$ and RGS4, we could demonstrate that functionally active mutant pairs can be generated from several members of RGS proteins and Gα-subunits. They can be studied in living cells, where they interact with each other but not with their wild type counterparts.

EXPERIMENTAL PROCEDURES

Animals—Female Wistar rats were obtained from a colony bred and maintained at the animal house of the University Hospital Eppendorf. Myometria were dissected from the rats after anesthesia with halothane followed by cervical dislocation. All experimental procedures

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§ To whom correspondence should be addressed: Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Universitäts-Krankenhaus Eppendorf, Martinistrasse 52, D-20246 Hamburg, Germany. Tel.: 49-40-42803-3177; Fax: 49-40-42803-4876; E-mail: wieland@uke.uni-hamburg.de.

[1] The abbreviations used are: G protein, heterotrimeric ($G\alpha\beta\gamma$) guanine nucleotide-binding protein; GTPγS, guanosine 5′-γ -thiotriphosphate; AC, adenylyl cyclase; BK_{Ca}, large conductance Ca^{2+}-activated potassium channel; RGS, regulator of G protein signaling; GAP, GTPase-activating protein; PKA, protein kinase A; PLC, phospholipase C; NTA, nitrilotriacetic acid; PAGE, polyacrylamide gel electrophoresis.

were carried out according to the animal welfare guidelines of the University Hospital Eppendorf.

Mutation of cDNAs—Mutations of mouse RGS16 cDNA were performed by polymerase chain reaction using pET15B-His$_6$-RGS16 (9) as template and specific mutagenic primers as follows: RGS16N130A, primer 1, CCTAAAGAGGTGGCCATAGATCAC, primer 2, GTGATCTATGGCCACCTCTTTAGG; RGS16E89K, primer 1, GAATTCAGTGAGAAGAACCTGGAG, primer 2, CTCCAGGTTCTTCTCACTGAATTC; RGS16N90D, primer 1, TTCAGTGAGGAGGACCTGGAGTTC, primer 2, GAACTCCAGGTCCTCCTCACTGAA; RGS16E89K/N90D, primer 1, TTCAGTGAGAAGGACCTGGAGTTC, primer 2, GAACTCCAGGTCCTTCTCACTGAA; RGS16E89K/N90K, primer 1, AGTGAGAAGAAGCTGGAGTTCTGG, primer 2, CCAGAACTCCAGCTTCTTCTCACT. Mutants were subcloned into the *Nde*I and *Bam*HI sites of pET15B (Novagen).

Gα_{i1} mutant proteins were generated from the cDNA coding for internally His$_6$-tagged rat Gα_{i1} (10) as insert in pQE60 (*Nco*I-*Hin*dIII; Qiagen (11), with a site-directed mutagenesis kit (Gene Editor, Promega). The mutagenic oligonucleotides were GTGGGAGGCCAGCGATCGCAGCGGAAGAAGTG for Gα_{i1}E207Q, CAGAGCGGAAGGAGTGGATCCACTGCTTTGAAG for Gα_{i1}K210E, GTGGGAGGCCAGCGATCGCAGCGGAAGGAGTGGATCCACTGCTTTGAAG for the double mutant Gα_{i1}E207Q/K210E, and GTGGGAGGCCAGCGATCGGATCGGAAGGAGTGGATCCACTGC for the double mutant Gα_{i1}E207D/K210E.

Mutations of mouse Gα_qR183C and of human RGS4 were performed using QuikChange (Stratagene) with pCis-Gα_qR183C and pCR3-RGS4 as template, respectively, and specific mutagenic primers (Gα_qR183C/K215E, primer 1, TCAGAGAGAAGAGAATGGATACACTGC, primer 2, GCAGTGTATCCATTCTCTTCTCTCTGA; RGS4E87K, primer 1, GAATATAGTGAGAAGAATATTGACTTCTGG, primer 2, CCAGAAGTCAATATTCTTCTCACTATATTC). The sequences of all mutants were verified by automated DNA sequencing.

Purification of Proteins—His$_6$-RGS16, His$_6$-Gα_{i1}, and their mutants were expressed in the *Escherichia coli* strain BL21(DE3) and purified in a single step on a Ni^{2+}-NTA column (Qiagen) as described before (9). Gα_{i1} and RGS16 proteins were stored at −80 °C in 20 mM Hepes, 1 mM EDTA, 2 mM $MgCl_2$, 2 mM dithiothreitol, pH 8.0, and 50 mM Tris-HCl, 100 mM NaCl, 2 mM $MgCl_2$, 6 mM β-mercaptoethanol, 5% (v/v) glycerol, pH 8.0, respectively. Protein concentration was determined according to Bradford (12) with IgG as standard. The activity of Gα_{i1} was determined by binding of the metabolically stable GTP analog GTPγS (13).

Bleached bovine rod outer segment membranes were prepared from bovine retinae as described (14). Transducin (G_t) was eluted from the membranes by hypotonic elution in the presence of 100 μM GTP, and the subunits were separated by affinity chromatography on Blue Sepharose (Bio-Rad) (15). Urea-treated rod outer segment membranes were prepared as described (16).

GTPase Measurements—The multiple turnover GTPase and single turnover GTPase of G_t were determined as described before (9, 17).

Single turnover GTPase of Gα_{i1} or its mutants was performed essentially as described by Berman *et al.* (18). Briefly, Gα_i-subunits (250 nM) were loaded with [γ-^{32}P]GTP (1 μM, 0.2 μCi) in a reaction mixture (40 μl) containing 50 mM triethanolamine HCl, 5 mM EDTA, 2 mM dithiothreitol, and 0.2 mg/ml bovine serum albumin, pH 7.4, for 15 min at 30 °C and then cooled for 5 min at 0 °C. GTP hydrolysis was initiated by addition of the GTP-loaded Gα-subunits to 10 μl of a mixture containing $MgCl_2$ (15 mM final concentration), if indicated, RGS proteins, and an excess of unlabeled GTP (150 μM final concentration) to prevent reloading of Gα-subunits with [γ-^{32}P]GTP. If not otherwise indicated, the reaction was conducted for 1 min at 0 °C and then stopped by addition of 750 μl of ice-cold 5% (w/v) charcoal in 100 mM NaH_2PO_4, pH 2.0. Released $^{32}P_i$ was quantified as described before (17). The $^{32}P_i$ release observed in the absence of $MgCl_2$ was regarded as unspecific.

Interaction of Gα_{i1} and Gα_{i1}K210E with G$\beta\gamma_t$—As recombinant Gα_{i1} and its mutants contain an internal His$_6$ tag (10), their ability to form G protein heterotrimers was assayed by trapping purified transducin βγ dimers (G$\beta\gamma_t$) to a Ni^{2+}-NTA matrix. Briefly, 2.5 μM G$\beta\gamma_t$ was incubated without and with 2.5 μM Gα_{i1} or Gα_{i1}K210E in a reaction mixture (20 μl) containing 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mM $MgCl_2$, 6 mM β-mercaptoethanol, 5% (v/v) glycerol, and 10 μM GDP for 10 min at 30 °C. Thereafter, 50 μl of Ni^{2+}-NTA superflow beads were added and incubated for another 10 min at 30 °C. The beads were pelleted by centrifugation and washed three times with 1 ml of the above described buffer including 25 mM imidazole. Bound proteins were eluted from the beads by addition of 20 μl of sample buffer and boiling (5 min, 95 °C). Proteins were visualized by staining with Coomassie Brilliant Blue after SDS-PAGE (12% polyacrylamide in the resolving gel).

A

hRGS1	89	FLKSEFSE**EN**IE	100	133	AKQI**N**IDFR	141
hRGS2	98	FLKSEFCE**EN**IE	109	145	PKEI**N**IDFQ	153
hRGS3	408	FLRTEFSE**EN**LE	419	456	CKEV**N**LDSY	464
hRGS4	79	FLKSEYSE**EN**ID	90	124	TKEV**N**LDSC	132
mRGS16	81	FLKTEFSE**EN**LE	92	126	PKEV**N**IDHE	134
hGAIP	104	FLRTEYSE**EN**ML	115	152	PKEVSLDSR	160

B

bGα_t	199	GQRS**E**RK**K**W	207
rGα_{i1}	203	GQRS**E**RK**K**W	211
bGα_o	204	GQRS**E**RK**K**W	212
mGα_q	208	GQRS**E**RR**K**W	216
mGα_{11}	208	GQRS**E**RR**K**W	216

FIG. 1. **Sequence alignment of RGS proteins and Gα_i and Gα_q subfamily members.** The amino acid sequences of human RGS1 (*hRGS1*), RGS2 (*hRGS2*), RGS3 (*hRGS3*), RGS4 (*hRGS4*), GAIP (*hGAIP*), and mouse RGS16 (*mRGS16*) (*A*), as well as those of bovine Gα_t (*bGα_t*), rat Gα_{i1} (*rGα_{i1}*), bovine Gα_o (*bGα_o*), mouse Gα_q (*mGα_q*), and Gα_{11} (*mGα_{11}*) (*B*) are aligned in the regions where important interactions take place. Corresponding amino acids mutated herein are printed in *bold*.

Preparation of Uterine Smooth Muscle Cells and Electrophysiological Recording of Large Conductance Ca^{2+}-activated K^+ (BK_{Ca}) Channel Activity—Rat myometrial smooth muscle cells were isolated from the uterus as described before (19). BK_{Ca} activity was recorded using the whole-cell patch configuration. Recombinant proteins were dialyzed into the cells via the patch pipette. All experimental procedures were performed essentially as reported before (19).

Transfection of COS-7 Cells, Determination of Recombinant Protein Expression, and Phospholipase C Activity—COS-7 cells were seeded at a density of 10^5 cells per well in 12-well tissue culture plates 1 day before transfection. The amount of DNA used for transfection with pCis-Gα_qR183C and pCis-Gα_qR183C/K215E was 0.2 μg, for pCR3-RGS4 and pCR3-RGS4E87K 0.8 μg. A plasmid expressing the β-galactosidase gene under the control of the cytomegalovirus promoter (pCis-LacZ) was cotransfected to keep the total amount of DNA in each transfection constant at 1 μg; 0.5 ml of Opti-MEM (Life Technologies, Inc.) containing the DNA along with 5 μl of LipofectAMINE (Life Technologies, Inc.) was added to each well. Twelve hours later, the medium was replaced with 1 ml of Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Twenty-four hours post-transfection, cells were labeled with 10 μCi of *myo*-[2-^{3}H]inositol (NEN Life Science Products) per ml in 0.5 ml of inositol-free medium containing 10% dialyzed fetal bovine serum. Forty eight hours post-transfection, the levels of [^{3}H]inositol phosphate were determined as described (20, 21). For detection of recombinant protein expression, cells were kept for the same time in Dulbecco's modified Eagle's medium after replacing the transfection medium and then harvested in sample buffer (21). Proteins were separated by SDS-PAGE (15% polyacrylamide in the resolving gel) and electrophoretically transferred onto nitrocellulose membranes. G$\alpha_{q/11}$ proteins were visualized with an anti-G$\alpha_{q/11}$ antiserum raised against a C-terminal epitope (Calbiochem) and the ECL system (Amersham Pharmacia Biotech).

RESULTS

GAP Activity of RGS16 and Its Mutants for Gα_t—The crystal structure of the Gα_{i1}-RGS4 complex (7) indicated that side chains of several amino acids are required for the interaction of RGS proteins with Gα-subunits of the Gα_i and Gα_q subfamily members. We therefore mutated three amino acids corresponding to these important contact sites (Fig. 1), Glu-89, Asn-90, and Asn-130 in RGS16 to lysine, aspartate, and alanine, respectively. Wild type RGS16 and the mutants were expressed in *E. coli* and purified as described before (9). The ability of recombinant RGS16 mutants to act as GAP was studied in the multiple turnover GTPase assay for Gα_t that was originally used to characterize RGS16 GAP activity (9, 17). A maximal effective concentration of RGS16 (1 μM) increased GTP hydrolysis by Gα_t 7–8-fold (Fig. 2*A*). In contrast, the GAP activity of RGS16N130A (1 μM) was reduced by 90–95%. RGS16E89K and RGS16N90D did not exhibit any significant GAP activity. Single turnover GTPase of Gα_t, however, revealed a different

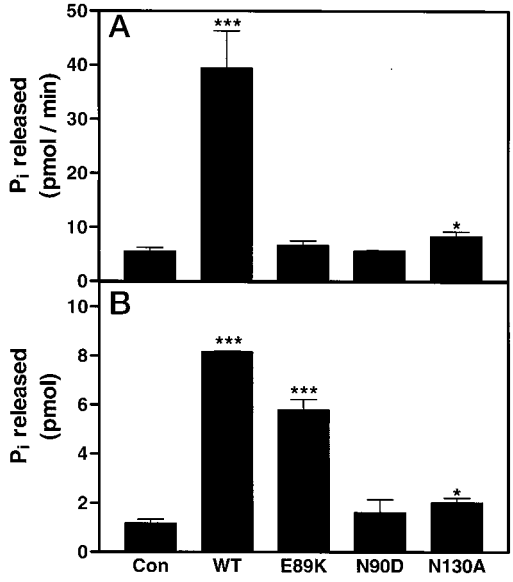

FIG. 2. **GAP activities of RGS16, RGS16E89K, RGS16N90D, and RGS16N130A for $G\alpha_t$.** Multiple turnover (*A*) and single turnover (*B*) GTPase of $G\alpha_t$ was measured for 10 min at 30 °C and 15 s at 0 °C, respectively, in the absence (*Con*) and presence of RGS16 (*WT*, 1 μM in *A*, 100 nM in *B*), RGS16N90D (N90D, 1 μM in *A*, 2 μM in *B*), RGS16E89K (E89K, 1 μM in *A*, 2 μM in *B*), and RGS16N130A (N130A, 1 μM in *A*, 2 μM in *B*). Data shown are means ± S.D. of assay triplicates. Statistical analysis was performed by Student's *t* test for unpaired observations. (***, $p < 0.001$; *, $p < 0.05$; *versus* basal activity (*Con*)).

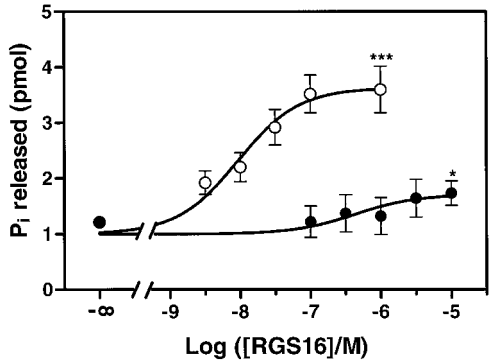

FIG. 3. **Reduced efficacy of RGS16N130A to act as GAP for $G\alpha_{i1}$.** Single turnover GTPase of recombinant $G\alpha_{i1}$ (250 nM) was measured in the absence and presence of increasing concentrations of RGS16 (○) or RGS16N130A (●) for 1 min at 0 °C. Means ± S.D. of assay triplicates are given. (***, $p < 0.001$; *, $p < 0.05$; *versus* basal activity).

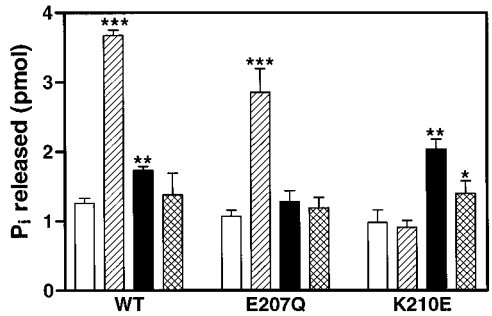

FIG. 4. **GAP activities of RGS16, RGS16E89K, and RGS16N90D for $G\alpha_{i1}$, $G\alpha_{i1}$E207Q, and $G\alpha_{i1}$K210E.** Single turnover GTPase of 250 nM recombinant $G\alpha_{i1}$ (*WT*), $G\alpha_{i1}$E207Q (E207Q), or $G\alpha_{i1}$K210E (K210E) was measured in the absence (*open bars*) and presence of 1 μM RGS16 (*hatched bars*), RGS16E89K (*filled bars*) or RGS16N90D (*cross-hatched bars*) for 1 min at 0 °C. Means ± S.D. of assay triplicates are given. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; *versus* basal activity.

pattern (Fig. 2*B*). When measured for 15 s at 0 °C, RGS16 (100 nM) stimulated GTP hydrolysis by about 7-fold. RGS16E89K (2 μM each) increased P_i release by about 5-fold, whereas RGS16N130A and RGS16N90D (2 μM each) displayed also in this assay a largely reduced (about 90%) GAP activity, which reached statistical significance in the case of RGS16N130A.

GAP Activity of RGS16 and Its Mutants for $G\alpha_{i1}$ and Its Mutants—To test whether the observed reduction in GAP activity of the RGS16 mutants is unique for the interaction with $G\alpha_t$ or can also be observed with other $G\alpha_i$ family members, the influence of RGS16 on the single turnover GTPase of recombinant $G\alpha_{i1}$ was studied. When measured for 1 min at 0 °C, basal P_i release of 250 nM $G\alpha_{i1}$ was about 1 pmol. RGS16 increased GTP hydrolysis about 3-fold (Fig. 3). The half-maximal and maximal increase in P_i release was observed at 20 and 100 nM RGS16, respectively. In contrast, RGS16N130A exhibited only marginal GAP activity for $G\alpha_{i1}$ (Fig. 3). The maximal increase (about 1.5-fold, $p < 0.05$) was observed at 3–10 μM RGS16N130A. Similarly, when compared at a concentration of 1 μM, the GAP activities of RGS16E89K and RGS16N90D were largely reduced or abolished (Fig. 4). The crystallographic data on the $G\alpha_{i1}$-RGS4 interaction surface (7) indicated that Glu-89 of RGS16 most likely interacts with Lys-210 of $G\alpha_{i1}$. Asn-90 interacts with Thr-182 in $G\alpha_{i1}$ and Thr-182 in this conformation with Gln-207 in $G\alpha_{i1}$. Thus, it could be possible to introduce compensating mutations into $G\alpha_{i1}$ that restore the interaction with Lys-89 and Asp-90 in these RGS16 mutants. Therefore, we expressed and purified the mutants $G\alpha_{i1}$E207Q and $G\alpha_{i1}$K210E. Both mutants exhibited a similar basal rate of single turnover GTPase (see Fig. 4) and binding of the GTP analog GTPγS (data not shown). The GAP activities of RGS16 and its mutants (1 μM) for $G\alpha_{i1}$E207Q were similar to those for unmutated $G\alpha_{i1}$ (Fig. 4). RGS16 increased P_i release from $G\alpha_{i1}$E207Q about 2.8-fold, whereas GAP activities of RGS16E89K and RGS16N90D were not evident on this $G\alpha_{i1}$ mutant. In contrast, $G\alpha_{i1}$K210E displayed sensitivity toward RGS16 mutants but not the wild type protein. RGS16E89K (1 μM) increased GTP hydrolysis by $G\alpha_{i1}$K210E about 2-fold. A weak GAP activity (1.4-fold increase in P_i release) was observed for RGS16N90D. The interaction of RGS16 and RGS16E89K with $G\alpha_{i1}$ and $G\alpha_{i1}$K210E was studied in more detail. In contrast to RGS16N130A (see Fig. 3), the efficacy of RGS16E89K toward $G\alpha_{i1}$ was not altered (Fig. 5*A*). RGS16E89K increased P_i release by $G\alpha_{i1}$ about 3-fold, however, with a largely reduced potency. Half-maximal GAP activity of RGS16E89K was observed at about 2 μM. Wild type RGS16 did not display any GAP activity for $G\alpha_{i1}$K210E even when used at a concentration of 10 μM (Fig. 5*B*). RGS16E89K showed similar GAP activity for $G\alpha_{i1}$K210E as for wild type $G\alpha_{i1}$; however, its potency was reduced compared with the RGS16-$G\alpha_{i1}$ interaction. Half-maximal increase was observed at about 1 μM. Similarly effective concentrations of RGS16 (0.1 μM) and RGS16E89K (10 μM) were used to determine the extent of GTP hydrolysis acceleration (Fig. 6). The calculated rate constants for the basal GTPase activity were $7.9 \pm 0.2 \times 10^{-3}$ s^{-1} and $4.6 \pm 0.3 \times 10^{-3}$ s^{-1} for $G\alpha_{i1}$ and $G\alpha_{i1}$K210E, respectively. In the presence of RGS16 and RGS16E89K, the rate constant for $G\alpha_{i1}$ increased to $94.5 \pm 1.1 \times 10^{-3}$ s^{-1} and $16.2 \pm 0.1 \times 10^{-3}$ s^{-1}, respectively. The rate constant for $G\alpha_{i1}$K210E increased to $42.7 \pm 0.4 \times 10^{-3}$ s^{-1} in the presence of RGS16E89K, indicating that under the experimental conditions used, maximal acceleration of GTP hydrolysis is similar (about 10-fold) for the interaction of $G\alpha_{i1}$ with RGS16 and of $G\alpha_{i1}$K210E with RGS16E89K, respectively.

In order to increase the apparent affinity of $G\alpha_{i1}$K210E for RGS16E89K, double mutants of $G\alpha_{i1}$ and RGS16 were studied. First, we generated the double mutant pair RGS16E89K/N90**D**

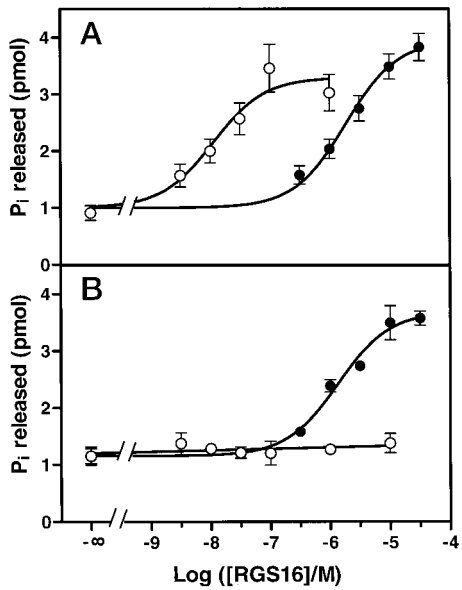

FIG. 5. **Concentration dependence of the GAP activity of RGS16 and RGS16E89K for $G\alpha_{i1}$ and $G\alpha_{i1}$K210E.** Single turnover GTPase of 250 nM recombinant $G\alpha_{i1}$ (*A*) or $G\alpha_{i1}$K210E (*B*) in the absence and presence of increasing concentrations of RGS16 (○) or RGS16E89K (●). Data shown are means ± S.D. of assay triplicates.

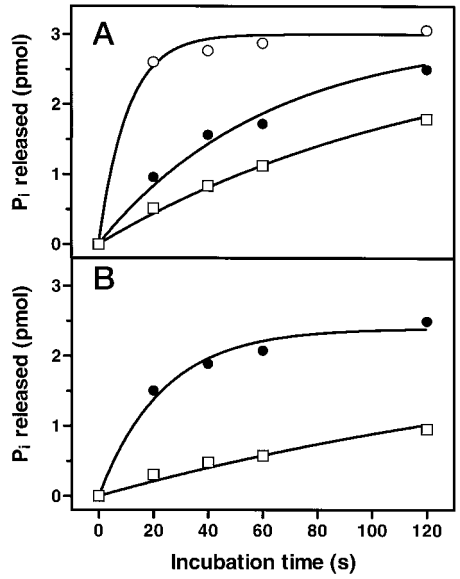

FIG. 6. **Acceleration of P_i release from $G\alpha_{i1}$ and $G\alpha_{i1}$K210E by RGS16 and RGS16E89K.** P_i release from 250 nM recombinant $G\alpha_{i1}$ (*A*) or $G\alpha_{i1}$K210E (*B*) was determined in the absence (□) and presence of RGS16 (○, 100 nM) or RGS16E89K (●, 10 μM) at the indicated periods. The data shown are the average of two independent experiments.

and $G\alpha_{i1}$E207**Q**/K210E. Second, as $G\alpha_{i1}$K210E apparently has a rather acidic surface consisting of Glu-207 and Glu-210, a more basic counterpart was created with the double mutant RGS16E89K/N90**K**. Finally, as in this mutant the side chain of Lys-90 has two more carbon atoms than that of the original asparagine, and the side chain of Glu-207 was shortened in the double mutant $G\alpha_{i1}$E207**D**/K210E for compensation. All the $G\alpha_{i1}$ double mutants exhibited an unaltered rate of basal GTP hydrolysis and GTPγS binding (data not shown). They failed, however, to be stimulated in their rate of GTP hydrolysis by RGS16 or any of its mutants. Also the double mutants of RGS16 did not increase the GTPase activity of $G\alpha_{i1}$, $G\alpha_{i1}$E207Q, or $G\alpha_{i1}$K210E (Table I).

Influence of RGS16, RGS16E89K, $G\alpha_{i1}$, and $G\alpha_{i1}$K210E on BK_{Ca} Channel Activity in Rat Uterine Smooth Muscle Cells—If RGS16E89K and $G\alpha_{i1}$K210E bare the potential to serve as tools in analyzing Gα-RGS protein interaction, the differences between the mutants and their wild type counterparts observed in the *in vitro* GAP assay have to cause distinguishable differences in the modulation of signaling pathways by the mutants and wild type proteins on the background of a living cell. To test this hypothesis we chose the model of the BK_{Ca} channel regulation via the adenylyl cyclase (AC)-protein kinase A (PKA) pathway in uterine smooth muscles cells. As shown before (19), under the experimental conditions chosen, the BK_{Ca} channel activity is a very sensitive probe to follow AC-PKA activation and even more important, defined concentrations of purified proteins can be dialyzed into the cell via the patch pipette. Therefore, this method allows us to study the influence of these proteins on regulatory components of the AC activity in detail. As shown in Fig. 7, stimulation of the G_s-coupled β-adrenoreceptors on these cells by isoproterenol (10 μM) caused a pronounced decrease of the outward current through BK_{Ca} channels (I_{out}) by about 50%. Co-stimulation of the G_i-coupled α_2-adrenoreceptors by the selective agonist clonidine (10 μM) antagonized the effect of isoproterenol by partially restoring I_{out} to about 80% of the drug-free control. After dialysis of 1 μM RGS16 into the cells, the G_s-mediated effect of isoproterenol was unaffected. The G_i-mediated effect of clonidine, however, was totally abolished. In contrast, RGS16E89K, even when used in a concentration of 10 μM, was without effect on the isoproterenol- and clonidine-evoked signals.

As the inhibitory effect of α_2-adrenoreceptors on AC activity is mediated by $G\alpha_i$-subunits (19), this inhibition should be mimicked by dialyzing recombinant $G\alpha_{i1}$ into the cells. As shown in Fig. 8, 1 μM $G\alpha_{i1}$ completely antagonized the inhibition of I_{out} caused by isoproterenol, whereas the currents in the presence of 0.1 μM $G\alpha_{i1}$ were virtually unaltered. Interestingly, $G\alpha_{i1}$K210E was about 10-fold more potent than wild type $G\alpha_{i1}$. At 0.1 μM $G\alpha_{i1}$K210E the inhibitory effect of isoproterenol was completely blunted. In contrast to its inability to regulate endogenous G_i proteins (see Fig. 8), RGS16E89K (10 μM) completely reversed the effect of 0.1 μM $G\alpha_{i1}$K210E when both proteins were applied simultaneously to the cells.

Interaction of $G\alpha_{i1}$ and $G\alpha_{i1}$K210E with Gβγ—The difference in potency of $G\alpha_{i1}$ and $G\alpha_{i1}$K210E to inhibit AC activity in the uterine smooth muscle cells might be explained by an impaired ability of the mutant to form heterotrimers with Gβγ. To exclude this possibility we tested heterotrimer formation of GDP-liganded $G\alpha_{i1}$ and $G\alpha_{i1}$K210E (2.5 μM) with an equimolar concentration of $G\beta\gamma_t$. As shown in Fig. 9, $G\alpha_{i1}$ and $G\alpha_{i1}$K210E similarly trapped $G\beta\gamma_t$ in an apparent 1:1 proportion to Ni^{2+}-NTA beads via their internal His_6 tag. $G\beta\gamma_t$ alone did not bind to the matrix. Thus, the ability of heterotrimer formation is apparently not impaired by the Lys to Glu mutation.

Interaction of RGS4 and RGS4E87K with $G\alpha_q$R183C and $G\alpha_q$R183C/K215E, Respectively—To study whether the concept of creating an interacting mutant pair can be extended to other RGS proteins and members of the $G\alpha_q$ subfamily, we introduced mutations analogous to RGS16E89K and $G\alpha_{i1}$K210E in RGS4 (RGS4E87K) and $G\alpha_q$ ($G\alpha_q$ K215E, see Fig. 1). As template for the $G\alpha_q$ mutation we used the cDNA of the $G\alpha_q$R183C mutant. This mutant has been shown to be GTPase-deficient by itself, whereas it regains GTPase activity in the presence of RGS4 (22). In addition, it can be used in transfection assays to stimulate directly phospholipase C (PLC) activity in COS-7 cells (23).

First we checked the expression of $G\alpha_{q/11}$ proteins in transfected COS-7 cells by immunoblotting. Transfection with the

TABLE I

Maximal GAP activities of RGS16 and its mutants for $G\alpha_{i1}$ and its mutants

Single turnover GTPase of $G\alpha_{i1}$, $G\alpha_{i1}$E207Q, $G\alpha_{i1}$K210E, $G\alpha_{i1}$E207Q/K210E, and $G\alpha_{i1}$E207D/K210E (250 nM each) was measured for 1 min at 0 °C in the absence and presence of RGS16 (1 μM), RGS16N90D, RGS16E89K, RGS16E89K/N90D, and RGS16E89K/N90K (10 μM each). The relative increase (*x*-fold) in P_i release is given. The data are means ± S.D. of at least three independent determinations performed in triplicate. Basal GTP hydrolysis for all $G\alpha_{i1}$ proteins was about 1 pmol. Statistical analysis was performed by two-way analysis of variance.

	$G\alpha_{i1}$	$G\alpha_{i1}$E207Q	$G\alpha_{i1}$K210E	$G\alpha_{i1}$E207Q/K210E	$G\alpha_{i1}$E207D/K210E
RGS16	3.17 ± 0.34[a]	2.20 ± 0.55[b]	1.10 ± 0.09	0.97 ± 0.10	1.14 ± 0.20
RGS16E89K	2.86 ± 0.88[a]	1.10 ± 0.10	3.04 ± 0.23[a]	0.78 ± 0.13	1.00 ± 0.20
RGS16N90D	1.05 ± 0.05	1.07 ± 0.06	1.43 ± 0.20	0.86 ± 0.04	1.21 ± 0.21
RGS16E89K/N90D	0.96 ± 0.13	1.08 ± 0.20	1.00 ± 0.40	0.85 ± 0.09	1.09 ± 0.19
RGS16E89K/N90K	1.1 ± 0.09	1.32 ± 0.32	1.13 ± 0.21	1.09 ± 0.20	1.05 ± 0.04

[a] $p < 0.001$ *versus* basal activity.
[b] $p < 0.05$ *versus* basal activity.

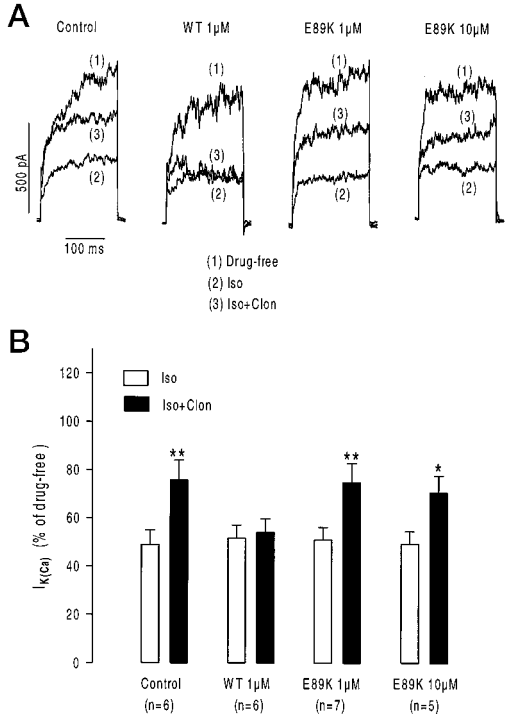

FIG. 7. **Influence of RGS16 and RGS16E89K on α_2-adrenoreceptor-mediated regulation of BK_{Ca} channel activity in rat uterine smooth muscle cells.** Currents were elicited in rat uterine myocytes every 10 s by 200-ms depolarizing pulses from −20 to +80mV in the presence of 0.3 μM intracellular Ca^{2+}. Cells were first dialyzed via the patch pipette for 20 min with intracellular pipette solution alone (*Control*) or the indicated concentrations of RGS16 (*WT*) and RGS16E89K (*E89K*), and thereafter superfused with 10 μM isoproterenol ((*2*), *open bars*), followed by application of 10 μM clonidine ((*3*), *closed bars*). *A*, a representative original recording for each condition is presented. *B*, average results from 5 to 7 cells obtained from different rats are shown. Current densities were used to calculate the percentage values. Data are expressed as means ± S.E. Statistical significance was determined by Student's *t* test for paired observations. **, $p < 0.01$; *, $p < 0.05$; *versus* isoproterenol.

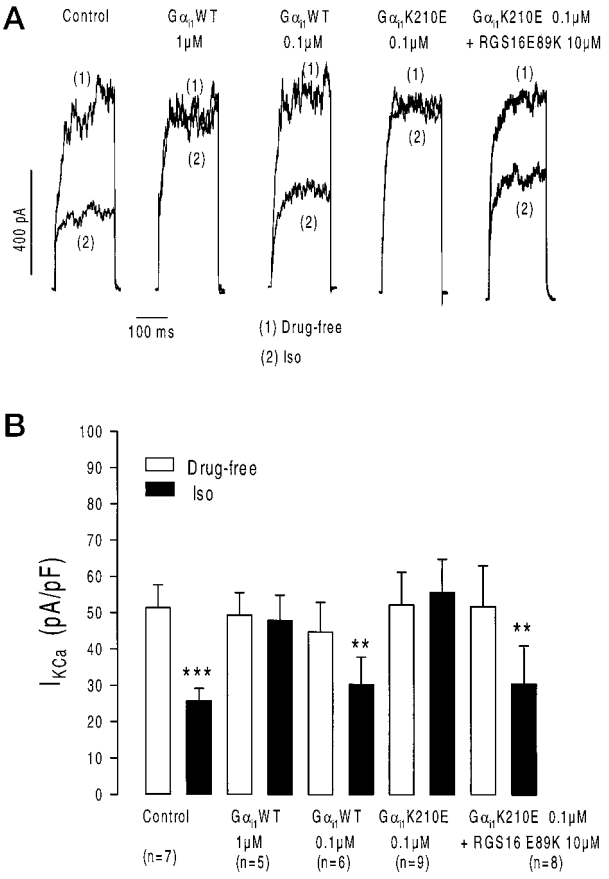

FIG. 8. **Influence of $G\alpha_{i1}$ and $G\alpha_{i1}$K210E on BK_{Ca} channel activity in rat uterine smooth muscle cells.** Currents were elicited in rat uterine myocytes every 10 s by 200-ms depolarizing pulses from −20 to +80mV in the presence of 0.3 μM intracellular Ca^{2+}. Cells were first dialyzed via the patch pipette for 20 min with intracellular pipette solution alone (*Control*) or the indicated concentrations of $G\alpha_{i1}$WT, $G\alpha_{i1}$K210E, and RGS16E89K. Thereafter, cells were superfused without ((*1*), *open bars*) and with 10 μM isoproterenol ((*2*), *closed bars*). *A*, a representative original recording for each condition is presented. *B*, average results from 5 to 9 cells obtained from different rats are shown. Current densities are given as means ± S.E. Statistical significance was determined by Student's *t* test for paired observations. **, $p < 0.01$; ***, $p < 0.001$; *versus* drug-free.

cDNA encoding RGS4 proteins did not alter the amount of endogenously expressed $G\alpha_{q/11}$ proteins (Fig. 10, *upper panel*). The cDNA coding for $G\alpha_q$R183C or $G\alpha_q$R183C/K215E clearly increased the levels of immunodetectable $G\alpha_{q/11}$ proteins under all conditions tested. Cotransfection with the plasmid for RGS4 slightly decreased the expression levels of $G\alpha_q$R183C and $G\alpha_q$R183C/K215E, whereas the plasmid for RGS4E87K was without inhibitory effect. As shown in Fig. 10 (*lower panel*), PLC activity in COS-7 cells increased about 2-fold when transfected with the plasmid encoding $G\alpha_q$R183C or $G\alpha_q$R183C/K215E. PLC activity decreased by 57% after transfection of an RGS4 encoding plasmid alone. RGS4E87K encoding DNA had little if any effect. Coexpression of RGS4 with $G\alpha_q$R183C totally abolished $G\alpha_q$R183C-induced PLC activity, whereas coexpression of RGS4E87K had no effect. In contrast, $G\alpha_q$R183C/K215E-induced PLC activity was sensitive to coexpression of RGS4E87K but not of RGS4. The moderate differences in the expression levels of $G\alpha_q$R183C and $G\alpha_q$R183C/K215E are much smaller than the alterations in PLC activity. Therefore, the large reduction in PLC activity after cotransfection of RGS4 + $G\alpha_q$R183C and RGS4E87K + $G\alpha_q$R183C/K215E indicates a negative regulation of $G\alpha_q$R183C and $G\alpha_q$R183C/K215E by RGS4 and RGS4E87K, respectively.

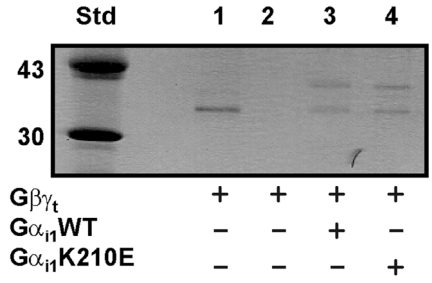

FIG. 9. **Interaction of $G\alpha_{i1}$ and $G\alpha_{i1}$K210E with $G\beta\gamma_t$.** $G\beta\gamma_t$ (2.5 μM) was incubated without (*lane 2*) and with an equimolar concentration of $G\alpha_{i1}$ (*lane 3*) or $G\alpha_{i1}$K210E (*lane 4*) in the presence of 10 μM GDP. Thereafter, His_6-tagged $G\alpha_{i1}$ proteins were trapped to a Ni^{2+}-NTA matrix, and beads were extensively washed as described under "Experimental Procedures." Bound proteins were eluted from the beads, separated by SDS-PAGE, and stained with Coomassie Brilliant Blue. *Lane 1*, $G\beta\gamma_t$ was directly applied to the gel as a loading control.

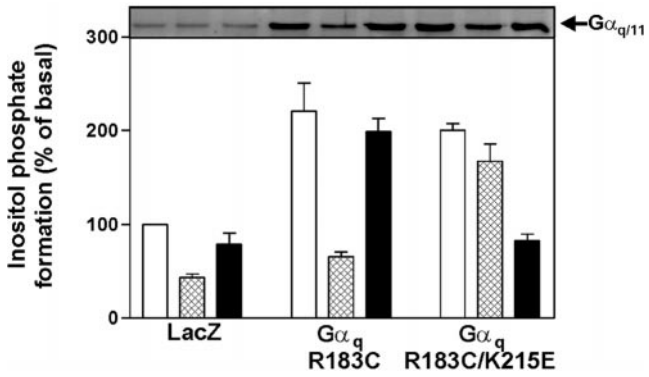

FIG. 10. **Interaction of RGS4 and RGS4E87K with $G\alpha_q$R183C and $G\alpha_q$R183C/K215E, respectively.** COS-7 cells were transiently transfected with the mammalian expression vector pCis encoding β-galactosidase (*LacZ*), $G\alpha_q$R183C, or $G\alpha_q$R183C/K215E (*open bars*) or were cotransfected with these vectors plus RGS4- (*cross-hatched bars*) or RGS4E87K-encoding constructs (*filled bars*). Forty-eight hours post-transfection, inositol phosphate accumulation (*lower panel*) and expression of $G\alpha_{q/11}$ proteins (*upper panel*) were analyzed as described under "Experimental Procedures." PLC activity in cells transfected only with pCis-LacZ was set to 100%. Data in the *lower panel* are means ± S.D. of one representative experiment performed in triplicate.

DISCUSSION

The majority of the so far identified RGS proteins are GAPs for members of the $G\alpha_i$ and $G\alpha_q$ subfamilies with a very limited degree of specificity (6). From a structural point of view, this is not very surprising. When the complex of RGS4 with $G\alpha_{i1}$ was crystallized (7), it became evident that RGS4 interacts with the important switch regions, switch I, II, and III in $G\alpha_{i1}$. The conformations of these switch regions are most sensitive to the binding of GDP or GTP to the Gα-subunit. Residues in switch I and II are directly involved in binding and hydrolysis of GTP. In the same region, residues in switch I (Thr-182) and switch II (Gln-204, Glu-207, and Lys-210) of $G\alpha_{i1}$ form hydrogen bonds with residues (Tyr-84, Glu-87, Asn-88, and Asn-128) of RGS4. All members of the $G\alpha_i$ and $G\alpha_q$ subfamilies on the one hand and a majority of RGS proteins on the other hand are highly conserved at these important contact sites (see Fig. 1). Tesmer *et al.* (7) also suggested that the direct interaction of Asn-128 in RGS4 with Gln-204 in $G\alpha_{i1}$ is of major importance to orient Gln-204 of $G\alpha_{i1}$ in an optimal conformation to the attacking water molecule and the γ-phosphate of GTP during GTP hydrolysis. The observation that GTPase-deficient Q204L mutants of $G\alpha_i$ family members are able to bind to RGS proteins (24), but do not regain GTPase function in the presence of RGS4 (18), further support this hypothesis. Our data obtained by mutation of the analogous Asn-130 in RGS16 (see Fig. 1) to alanine indicate that this residue is indeed critical for their GAP function. Although the GAP activity of RGS16N130A for $G\alpha_t$ and $G\alpha_{i1}$ was not completely abolished, its efficacy was reduced by 90–95%. Mutation of Asn-128 in RGS4 to alanine was reported to abolish its GAP activity (25). In addition, RGS proteins, like GAIP, with a serine at the position corresponding to Asn-128 in RGS4, are less potent than RGS4 but still exhibit GAP activity for several Gα-subunits (26–28). Recent work by others (29, 30) indicates that this residue is very important for binding of RGS proteins to Gα-subunits and variably modulates the maximal rates of GTP hydrolysis. Thus, the extent of loss in GAP activity by alteration of this important asparagine differs between certain pairs of RGS proteins and Gα-subunits.

As shown herein, mutation of Asn-90 to Asp abolished GAP activity of RGS16 for $G\alpha_{i1}$ and $G\alpha_t$. Similarly, mutation of the analogous Asn-88 to Ser in RGS4 abolished interaction with $G\alpha_{i1}$ (31), indicating that this conserved residue is also essential for the interaction of RGS proteins with $G\alpha_i$ family members. In contrast, mutation of Glu-89 to Lys did not abolish interaction of RGS16 with $G\alpha_{i1}$ or $G\alpha_t$. It has been shown before that the EC_{50} of the GAP activity is a good estimate for the affinity of RGS proteins to Gα-subunits (17). Therefore, the high EC_{50} of RGS16E89K represents a markedly (about 100-fold) reduced affinity for $G\alpha_i$ family members. In addition, Glu-89 of RGS16 is most likely to interact with Lys-210 in $G\alpha_{i1}$. When this residue in $G\alpha_{i1}$ was changed to glutamate, this mutant was insensitive to RGS16. The GTP hydrolysis by $G\alpha_{i1}$K210E could, however, still be stimulated by RGS16E89K to a similar extent as observed for the RGS16-$G\alpha_{i1}$ interaction. Taken together, these data indicate that the interaction of this highly conserved glutamate in RGS proteins with the equally well conserved lysine in $G\alpha_i$ and $G\alpha_q$ family members is important for high affinity binding but is apparently not directly involved in the acceleration of GTP hydrolysis.

For many RGS proteins it is not known if they gain specificity for the different members of the $G\alpha_i$ and $G\alpha_q$ subfamilies. Thus, the insensitivity of $G\alpha_{i1}$K210E to RGS16 and its efficient interaction with RGS16E89K is of particular interest. To establish the significance between wild type and mutant proteins observed in the *in vitro* GAP assay, we tested all proteins *in vivo* for differential regulation of endogenous signaling pathways on a cellular background. In rat uterine smooth muscle cells, we monitored the α_2-adrenoreceptor $G\alpha_i$-mediated inhibition of AC by the PKA-mediated regulation of BK_{Ca} channel activity (19). Indeed, we obtained evidence that the RGS16E89K mutant poorly interacted with endogenous $G\alpha_i$. Interestingly, the differences in protein affinities were more pronounced in the *in vivo* model than in the test tube. At a concentration of 1 μM, RGS16 completely abolished α_2-adrenoreceptor signaling. In contrast, RGS16E89K, even when used in a 10-fold higher concentration, did not significantly alter the α_2-adrenoreceptor effect. Both, recombinant $G\alpha_{i1}$ and $G\alpha_{i1}$K210E mimicked the effect of the endogenous $G\alpha_i$ and inhibited AC activity to a similar extent. Their potency, however, was significantly (about 10-fold) different. The maximal inhibition by $G\alpha_{i1}$ and $G\alpha_{i1}$K210E was observed at 1 and 0.1 μM, respectively. As both proteins exhibited a similar basal GTPase rate (see Figs. 4 and 5), this difference could be best explained by their different susceptibility to the endogenous RGS proteins, of which we detected several in the uterine smooth muscle cells by RNase protection assay and immunoblot (data not shown). Whereas the half-life of $G\alpha_{i1}$-GTP was shortened by RGS proteins, that of $G\alpha_{i1}$K210E-GTP should not. Therefore, maximally inhibitory concentrations of of GTP-liganded α-subunit were obtained at a lower concentration of the mutant protein compared with wild type $G\alpha_{i1}$. This interpretation was further corroborated by the ability of RGS16E89K to reverse

$G\alpha_{i1}$K210E effects in the uterine smooth muscle cell (see Fig. 8). Together with the unimpaired ability of $G\alpha_{i1}$K210E to form a heterotrimer with $G\beta\gamma_t$ (see Fig. 9), these data argue against other possible explanations for the increased potency of $G\alpha_{i1}$K210E like impaired heterotrimer formation or increased affinity of the mutant for AC isozymes. In summary, our data suggest that in a living cell the action of $G\alpha_{i1}$K210E is independent of endogenously expressed RGS proteins, and RGS16E89K is a extremely poor negative regulator of endogenous Gα-subunits. In addition, corresponding mutants of RGS4 (Glu-87 to Lys) and $G\alpha_q$ (Lys-215 to Glu, see Fig. 1) exhibited similar properties when the stimulation of PLC activity by a GTPase-deficient $G\alpha_q$ mutant ($G\alpha_q$R183C) in transfected COS-7 cells was studied. The activity of overexpressed $G\alpha_q$R183C was negatively regulated by RGS4 but not by RGS4E87K, whereas $G\alpha_q$R183C/K215E was sensitive to coexpression of RGS4E87K but not to RGS4. In contrast to RGS4E87K, expression of RGS4 alone inhibited PLC activity by 57%. This inhibition is most likely due to the interaction of RGS4 with endogenously expressed $G\alpha_q$ family members and thus is in line with the other data. As outlined before, the inhibition of PLC activity should reflect the interaction and thus inactivation of $G\alpha_q$ and $G\alpha_q$K215E by RGS4 and RGS4E87K, respectively. Together with data obtained with RGS16E89K and $G\alpha_{i1}$K210E *in vitro* and *in vivo*, they therefore implicate that by mutation of the highly conserved glutamate to lysine in RGS proteins and lysine to glutamate in Gα-subunits a pair of interacting mutants can be created.

Two recent studies (32, 33) indicate that certain RGS proteins contain motifs in the N terminus that contribute to specificity in regulation of distinct signaling pathways. In rat pancreatic acinar cells, the Ca^{2+} release evoked by $G\alpha_q$-coupled receptors was differentially regulated by RGS4. The m3 muscarinic receptor-induced Ca^{2+} release was about 3-fold and 10-fold more sensitive to RGS4 than that induced by bombesin and cholecystokinin receptors, respectively. Moreover, the discrimination between m3 and cholecystokinin receptor-evoked signals differed between RGS family members. Whereas RGS2 was equally potent in inhibiting both signals, RGS1 was about 1000-fold more potent in inhibiting m3 receptor signaling (33). Experiments performed with knock-out mice lacking different members of the $G\alpha_{q/11}$ subfamily indicate that RGS proteins interact equally well with all members of this subfamily. These data question that this specific subset of RGS proteins does discriminate between members of the $G\alpha_i$ and $G\alpha_q$ subfamilies. Nevertheless, it is frequently encountered that $G\alpha_q$-coupled receptors coactivate $G\alpha_i$ subfamily members (34). This has also been demonstrated for the m3 receptor (35). It is not clear to date whether RGS proteins are involved in post-receptor signal sorting. We have shown herein for $G\alpha_i$ and $G\alpha_q$ that a lysine to glutamate mutation renders the Gα-subunit insensitive to wild type (*in vitro*) and endogenously (*in vivo*) expressed RGS proteins. On the other hand, glutamate to lysine mutants of interacting RGS proteins were able to restore the original signaling. Our data implicate that the introduced mutations in RGS proteins and Gα-subunits do not affect other domains, *e.g.* the interaction sites with receptor, effectors, or other proteins. Thus, the mutated proteins are selectively uncoupled from endogenous signal transduction at the level of RGS-Gα-subunit interaction but are otherwise functionally intact. Expression of a single mutant (RGS protein or Gα-subunit) might therefore create a phenotype that can be rescued by the corresponding mutant of a specific RGS-Gα couple. Therefore, the interacting mutant pairs are supposed to be a helpful tool to analyze RGS-Gα-subunit interaction in living cells or even transgenic animals.

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