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Kinase-Inactive G-Protein-Coupled Receptor Kinases Are Able to Attenuate Follicle-Stimulating Hormone-Induced Signaling

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Homologous desensitization of G-protein-coupled receptors (GPCR) is thought to occur in several steps: binding of G-protein-coupled receptor kinases (GRKs) to receptors, receptor phosphorylation, kinase dissociation, and finally binding of β-arrestin to phosphorylated receptors and functional uncoupling of the associated Gα protein. It has recently been reported that GRKs can inhibit Gαq-mediated signaling in the absence of phosphorylation of some GPCRs. Whether or not comparable phosphorylation-independent effects are also possible with Gαs-coupled receptors remains unclear. In the present study, using the tightly Gαs-coupled FSR receptor (FSH-R) as a model, we observed inhibition of the cAMP-dependent signaling pathway using kinase-inactive mutants of GRK2, 5, and 6. These negative effects occur upstream of adenylyl cyclase activation and are likely independent of GRK interaction with G protein α or βγ subunits. Moreover, we demonstrated that, when overexpressed in Cos 7 cells, mutated GRK2 associates with the FSH activated FSH-R. We hypothesize that phosphorylation-independent dampening of the FSH-R-associated signaling could be attributable to physical association between GRKs and the receptor, subsequently inhibiting G protein activation.

Key Words: **GRKs; FSH; FSH receptor; desensitization.**

G-protein-coupled receptor kinases (GRKs) specifically recognize and phosphorylate the agonist-occupied form of numerous G-protein-coupled receptors (GPCRs) (1, 2). GRK-mediated receptor phosphorylation facilitates binding of the β-arrestins (3). As a consequence, activated receptors are uncoupled from G proteins and subsequently endocytosed. The overall process, generally termed homologous desensitization, is thought to adapt cellular responsiveness to high agonist concentrations and to prevent overstimulation of the cells in the continued presence of agonist. It has long been considered that inhibition of G protein activation by receptors was a consequence of β-arrestin binding to the phosphorylated receptors. From that, it is generally assumed that GRK major function is to enable β-arrestin to bind to the receptor and to exert its inhibitory function (4–6).

The use of GRK2 K220R, a phosphorylation-inactive GRK2 mutant with an arginine substituted for a lysine at position 220 to disrupt kinase activity, has led to conflictual results. This mutant has been shown to decrease phosphorylation of different GPCRs: β2 adrenergic- (7), β1-adrenergic- (8), δ opioïd- (9), α1B-adrenergic- (10), type 1A angiotensin II- (11), endothelin- (12), parathyroid hormone- (13), follicle stimulating hormone (FSH)- (14) and calcitonin- (15) receptors. When signaling associated to different GPCRs in the presence of K220R was evaluated, in some instances the mutant was shown to potentiate cell response (9, 11, 15–17). In contrast, it attenuates signaling associated with the endothelin- (12), parathyroid hormone- (13) or α-trombin- (18) receptors and inhibits growth factor-dependent cell proliferation and Erk activation of osteoclasts, thus behaving like the wild-type kinase (19). Moreover, kinetic experiments suggest that the sole binding of rhodopsin kinase (GRK1) to rhodopsin is sufficient to deactivate it (20, 21). Taken together, these data suggest that, depending on the targetted GPCR, GRKs might inhibit receptor functions independently of phosphorylation and binding of β-arrestins, while in other cases, phosphorylation is required to achieve desensitization. This idea is nicely illustrated by two recent reports showing that

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in a same cell type, distinct GPCRs can be differentially modulated by overexpression of the GRK2 K220R mutant (22, 23). These data suggest that physical association between the GRK and the activated receptor is sufficient to disrupt receptor coupling to effector G proteins (11, 13). However, two recent reports have established that, besides phosphorylating GPCRs, GRK2 is able to selectively bind to Gαq via a RGS (Regulator of G protein Signaling) domain located in its N-terminal domain and to enhance Gαq GTPase activity and to attenuate signaling consequently (24, 25). Since the available examples of phosphorylation-independent effects of GRKs are restricted to Gαq-coupled responses, this concept may not be generalized to receptors coupled to other Gα subtypes.

In the present study, we took advantage of the FSR receptor (FSH-R), which is tightly coupled to the Gs/adenylyl cyclase/PKA pathway, to investigate the consequence of kinase-inactive GRK overexpression on FSH-induced responses (26). GRKs are involved in agonist-induced phosphorylation and homologous desensitization of this receptor and kinase-dead mutants of GRK2 and 6 attenuate its agonist-dependent phosphorylation (14, 27). However, the effects of mutated GRKs on FSH-R associated signaling are still unknown. The importance to investigate whether or not a Gαs-associated signaling pathway could be inhibited by kinase-inactive GRKs is reinforced by the fact that, to our current knowledge, Gαs subunits are not regulated by and do not interact with any RGS proteins (28).

MATERIALS AND METHODS

Materials. Leibovitz's (L 15) medium and Dulbecco's Minimum Essential Medium (DMEM) were purchased from Gibco-BRL Life Technologies (Gaithersburg, MD). Penicillin, streptomycin, trypsin, trypsin inhibitor (Soybean), deoxyribonuclease type-I, human Transferrin (Tf) and M1 anti-FLAG monoclonal antibody were obtained from Sigma Chemical Co (St. Louis, MO). Porcine (pFSH) and ovine FSH (oFSH) was purified in our laboratory (CY 1737 III: 41× NIH FSH P1 in homologous porcine radio receptor assay).

Plasmids. pCMVRenilla was from Promega (Madison, WI). pEGFP-N1 was purchased from Clontech (Palo Alto, CA, USA). The constructs for the different GRKs and the cAMP responsive reporter construct "pSOMLuc" were already described (27). pCMV5-K220R was a generous gift from Dr. R.J. Lefkowitz (Durham, NC).

Mutations in the cDNAs encoding human GRK5 and GRK6 were introduced by site-directed mutagenesis, according to the pSELECT-1 mutagenesis protocol from Promega. A dominant negative form of GRK6 in the expression vector pBC12BI was prepared by replacing Lys^{215} by an arginine (29). A dominant negative form of GRK5 was similarly obtained. Briefly, the coding sequence of GRK5 was excised from the plasmid pBC12BI by *Nsi*I and *Bam*HI and inserted into pSELECT-1 cleaved with *Pst*1 and *Bam*HI. The mutant primer 5′-CTCCAAGCGCCTGCAGGCATACAT-3′ (Arg codon is underlined) complementary of the coding strand was used to replace Lys^{215} with an arginine residue (GRK5 K215R). Clones with the mutation were identified by DNA sequencing. The mutated sequence was further digested with *Hin*dIII and *Bam*HI, blunted with the Klenow enzyme and inserted into the expression vector pCDM8 after digestion with *Xba*I, treatment with the Klenow enzyme and dephosphorylation.

pRK-FSHR/3 was a kind gift from Dr R. Sprengel (Heidelberg, Germany). The FLAG epitope was inserted at the N-terminus of the FSH-R as described (12).

Cell culture and transfection. The mouse Ltk cell line stably expressing the rat FSH receptor (Ltk 7/12 FSHR) was a kind gift from Dr E. Nieschlag (Münster, Germany) (30). These cells were cultured in MEM supplemented with 10% heat-inactivated fetal calf serum and Geneticin (0.4 mg/ml). Cos-7 cells were cultured in DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS), glutamine and antibiotics. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2.

Cells (2.5×10^5 per well in 12-well culture plates) (Corning) were transfected 24 h after seeding with various quantities of the appropriate plasmid mixtures. Calcium phosphate precipitation method was used as previously described (27). After transfection, cells were rinsed with MEM and were incubated without serum for 40 h. Cells were stimulated with either 100 ng/ml FSH or saline for 4 h and were then collected to determine luciferase activity. In all the experiments, the empty vector was added in order to equalize the transfected DNA quantities. The cAMP-sensitive pSOMLuc construct was used as a reporter system [i.e., the luciferase reporter gene driven by cAMP-responsive elements of the somatostatin gene promoter region]. This reporter gene system has already been proven suitable to measure the degree of FSH-R coupling to Gs/adenylyl cyclase/PKA pathway (27). Co-transfections of the pSOMLuc reporter gene with the empty vector were used as controls. Each transfection was repeated at least four times, with at least two different DNA preparations for each construct. Results were expressed as percentage of RLU values.

Isolation, culture, and transfection of rat Sertoli cells. Sertoli cells were prepared from testes of 11- to 12-day-old rats (Wistar Janvier), according to the method of Dorrington *et al.* (31). Collagenase digestion was replaced by one additional mechanical dispersion step performed after trypsin treatment. Contaminations of our current Sertoli cell preparations with other components of seminiferous tubules were less than 10% of germ cells and 2% of myoid cells as previously described (32).

Sertoli cells (10^6 cells per well in 24-well culture plates, Falcon-Becton–Dickinson SA) were cultured in DMEM supplemented with 100 U/ml penicillin, 2.5 μg/ml amphotericin B, 100 μg/ml steptomycin, 2 μg/ml insulin, 5 μg/ml human transferrin, 2 mM glutamine, 200 ng/ml vitamin E (α-tocopherol) and 50 ng/ml vitamin A (retinol) at 34°C in a humidified atmosphere of 5% CO_2.

After 24 h of culture, Sertoli cells were co-transfected by the calcium phosphate precipitation method as previously described (33). Briefly, 500 ng/well of pSOMLuc-driven *firefly* luciferase reporter plasmid was used as a cAMP sensor and 25 ng of *renilla* luciferase plasmid was added to normalize transfection efficiency. Forty-eight hours after transfection, cells were added with fresh medium before stimulation with FSH (100 ng/ml) for 6 h, then rinsed in phosphate-buffered saline and lysed according to the manufacturer's instructions (Dual luciferase assay, Promega). Both *firefly* and *renilla* luciferase activities were quantified on a luminometer (Lumat LB 9507-EG & G Berthold, Turku, Finland).

Sorting and analysis of transfected cells. Ltk 7/12 cells (10^6 cells per 75 cm^2 culture flask, Falcon-Becton–Dickinson SA) were transiently co-transfected with both an expression vector for *Green Fluorescent Protein* and one of the mutated GRK construct described above. Transfast liposomes (Promega) were used in a 1/1 *ratio;* 2.5 μg of GFP expression vector and 40 μg of either control plasmid or mutated GRK construct were used for each 75 cm^2 culture flask. After 48 h, GFP-positive living cells were selected using a FACStar Plus cell sorter (Beckton-Dickinson). To avoid contaminations with untransfected cells, only 15–20% of cells presenting the more intense GFP signal (about 50% of the cells were GFP-positive) were sorted

for subsequent analysis. GFP-positive and negative cells were recovered for further analysis. About 3.10^5 cells recovered for each condition were replated in 96-well dishes (5×10^4 cells/well) in serum free medium and cultured for 24 h. Positive and negative cells were then stained with DAPI reagent and observed by fluorescence microscopy in order to monitor sorting efficiency.

cAMP assay. For cAMP assay, sorted cells were replated for 24 h and were stimulated by FSH for 15 min. Cells were then washed twice with cold PBS, frozen in ethanol and the intracellular cAMP content was determined using an ELISA after acetylation (Amersham Pharmacia Biotech, Buckinghamshire, England).

Binding assay. For binding assays, all solutions were dissolved in 0.05 M Tris HCl pH 7.4 containing 0.1% BSA, 6 mM $CaCl_2$. Crude membrane preparations from transfected or untransfected Cos-7 cells were incubated with increasing doses of unlabeled pFSH in the presence of [^{125}I]-oFSH for 16 h at 4°C. The reaction was stopped by the addition of ice-cold buffer followed by centrifugation at 2500*g* for 20 min at 4°C. The membrane pellets were then washed and the amount of labeled pFSH was counted.

Immunoprecipitation and Western blotting. To immunoprecipitate the FLAG-tagged FSH receptors in intact cells, Cos-7 cells were transiently cotransfected with mutated GRK2 construct in 75 cm^2 flasks using Transfast liposomes (Promega). After 48 h, cells were washed twice with DMEM and incubated for 15 min at 37°C in DMEM with or without pFSH (100 nM). This medium was replaced by 8 ml of DMEM with 2.5 mM dithiobis(succinimidyl propionate) (Pierce) with or without FSH and incubated for 30 min at 25°C. Then, cells were solubilized in 1 ml of RIPA buffer at 4°C for 1 h, and immunoprecipitation of the FLAG-tagged FSH receptor was done with 10 μg of M1 antibody for 16 h at 4°C (Sigma). Separation of immune complexes and cleavage of the crosslinker was done for 90 min at 37°C in Laemmli buffer. Immunoprecipitated proteins were resolved by SDS/PAGE and transferred to poly (vinylidene difluoride) membranes. GRK2 immunoreactivity on the membrane was detected with an anti-GRK2/3 monoclonal antibody (Upstate Biotech., Lake Placid, NY, USA) and revealed by a chemiluminescence detection system (NEN).

Statistics. Statistical analysis of the data was performed using a single mean Student's t-test (Statview, Abacus Concepts, CA).

RESULTS

First, we selected Ltk 7/12 cells which stably overexpress the rat FSH-R to compare the effects of different GRKs to their kinase-inactive mutant counterparts on FSH-induced signaling. Expression vectors for the wild type and mutated GRKs were transiently transfected in Ltk 7/12 cells. The functional consequences of GRK cotransfection with the pSOMLuc reporter on FSH receptor-mediated production of luciferase was measured.

Figure 1a shows that cotransfection of pSOMLuc with GRK2 or GRK3 constructs resulted in an inhibition of FSH-stimulated luciferase production. These data are compatible with the model that GRK-mediated receptor phosphorylation triggers inhibition of receptor function. However, the same inhibitory effect (60% inhibition) was observed with GRK2-K220R (Fig. 1a). In accordance with our previous results, the basal levels of luciferase activity were also inhibited by wild type or mutated GRK overexpression (27). GRK2 and 3 have been previously shown to bind with high affinity to G protein $\beta\&\gamma$; subunits via their C-terminal domain (34–36). To exclude phosphorylation-independent effects of GRKs that might be caused by binding of the overexpressed K220R mutant to G protein $\beta\gamma$ subunits, kinase-inactive mutants were constructed for GRK5 and 6 which do not directly interact with G protein subunits (2, 6). Figures 1b and 1c show that both wild type and mutated GRK5 and 6 significantly attenuated luciferase production when cotransfected in Ltk 7/12 cells (80 and 60% inhibition for mutated GRK5 and 6 respectively). Once again, basal and stimulated cells were affected by GRK transfection. From these data, the possibility that signaling inhibition mediated by mutated GRK might be due to direct binding to G protein $\beta\gamma$ subunits can be ruled out.

The Ltk 7/12 cell line presents an enforced expression of FSH receptors which could modify the stoechiometry within the signaling complexes and ultimately modify cell response to FSH. In this respect, we have tested the effects of mutated GRKs in natural FSH-R bearing cells. In primary rat Sertoli cells, wild-type GRK2, GRK5 and GRK6 are recruited by FSH and inhibit FSH-dependent signaling when overexpressed (our unpublished results). Primary Sertoli cells were cotransfected with pSOMLuc and with kinase-inactive GRK2, GRK5 and GRK6 (Fig. 2). We found that the three mutants significantly inhibited the basal and FSH-stimulated luciferase activities (75 to 90% inhibition).

To determine whether the mutated GRK-mediated loss of responsiveness was due to events occuring upstream of adenylyl cyclase, Ltk 7/12 and primary Sertoli cells cotransfected with pSOMLuc and with one of the GRK mutants, were stimulated with the adenylyl cyclase-specific activator forskolin. GRK-transfected cells remained fully responsive to forskolin (Fig. 3).

To investigate whether or not mutated GRK could also dampen short-term cAMP accumulation, Ltk 7/12 cells were transiently co-transfected with both an expression vector for Green Fluorescent Protein (GFP) and one of the kinase-inactive GRK constructs. Transfected cells were selected by flow cytometry, using the GFP fluorescence as a marker (Fig. 4a). Short-term (15 min) cAMP accumulation in response to FSH was measured both in GFP-negative (i.e.: untransfected) and GFP-positive (i.e.: cotransfected) cells. As displayed in Fig. 4b, transient overexpression of mutated GRK2, 5 or 6 significantly desensitized the FSH-promoted cAMP accumulation. When compared with their corresponding GFP-negative controls, the degree of inhibition achieved by the mutated GRK can be estimated respectively to 55% for GRK2 K220R, and more than 90% for GRK5 K215R and GRK6 K215R.

Such an inhibition presumably would be mediated by direct binding of mutated GRKs to the FSH-R. To prove such direct binding, we attempted to crosslink mutated GRK2 to a FLAG-tagged FSH-R in intact

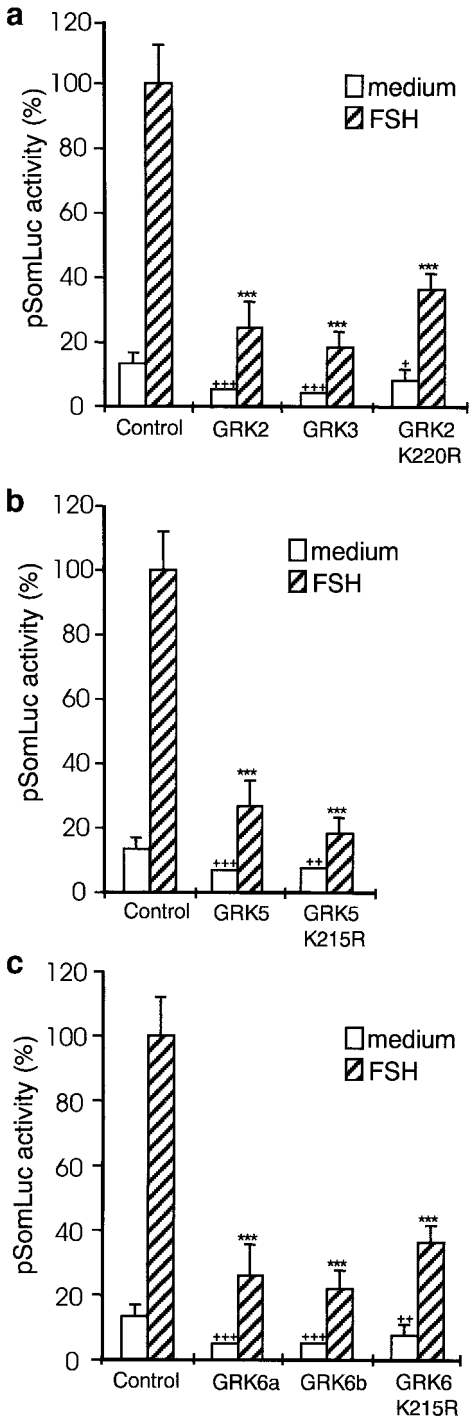

FIG. 1. FSH-R uncoupling in Ltk 7/12 cells by transiently overexpressed GRK phosphorylation-deficient mutants. Ltk 7/12 cells were transiently cotransfected with the cAMP-sensitive reporter gene pSOMLuc (1 μg/well) and an expression vector for a GRK or the corresponding kinase-dead mutant (3 μg/well). Twenty-four hours after transfection, cells were stimulated or not with FSH (100 ng/ml) for 4 h. (a) The ability to dampen the FSH-induced response was compared between control plasmid, GRK2, GRK3, or the K220R mutant of GRK2. (b) Control plasmid, GRK5, or the K215R mutant of GRK5. (c) Control plasmid, GRK6a, GRK6b, or the K215R mutant of GRK6. Luciferase activity was measured in the cell lysates. Values were expressed as percentage of the stimulated control activity taken as 100%. These data are representative of three independent experiments, each with four replicates. +, $P < 0.05$; **/++, $P < 0.01$; ***/+++, $P < 0.001$, significant statistical differences from unstimulated (+) or stimulated (*) control cells.

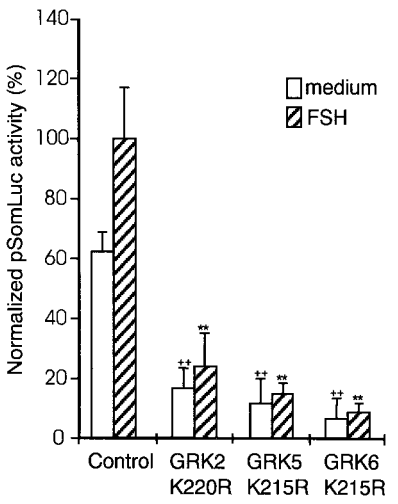

FIG. 2. FSH-R uncoupling in primary Sertoli cells by transiently overexpressed kinase-inactive mutants of GRKs. Primary Sertoli cells were transiently cotransfected with the cAMP-sensitive reporter gene pSOMLuc (0.5 μg/well) and with an expression vector for a phosphorylation-deficient GRK2, 5, or 6 (0.75 μg/well). *Renilla* luciferase plasmid (25 ng/well) was added to allow normalization of transfection efficiency. Twenty-four hours after transfection, cells were left unstimulated or were stimulated with FSH (100 ng/ml, as indicated). After 6 h FSH stimulation, luciferase activities (*Firefly* and *Renilla*) were measured in the cell lysates. Values were expressed as percentage of the stimulated control activity taken as 100%. These data are representative of three independent experiments, each with four replicates. **/++, $P < 0.01$, significant statistical differences from unstimulated or stimulated control cells.

cells, and then to immunoprecipitate the complex with an anti-FLAG antibody. The tagged rat FSH-R expressed in Cos-7 cells displayed the same binding ability (Fig. 5a) and cAMP response to FSH stimulation (Fig. 5b) than its *wt* counterpart expressed in the same conditions. Figure 5c shows that indeed mutated GRK2 was coimmunoprecipitated from the cells with the tagged FSH-R. The addition of pFSH increased the amounts of coprecipitated mutant GRK2.

DISCUSSION

The FSH-R signaling was already known to be profoundly dampened by different GRKs. In the present study, we confirmed this view in an established cell line and in primary Sertoli cells. Interestingly, we also observed inhibition using the GRK2 kinase-inactive K220R mutant. This kinase-inactive mutant is well documented to act as a dominant-negative mutant for phosphorylation of a wide array of GPCRs (7–16) but the functional consequence of its overexpression can be

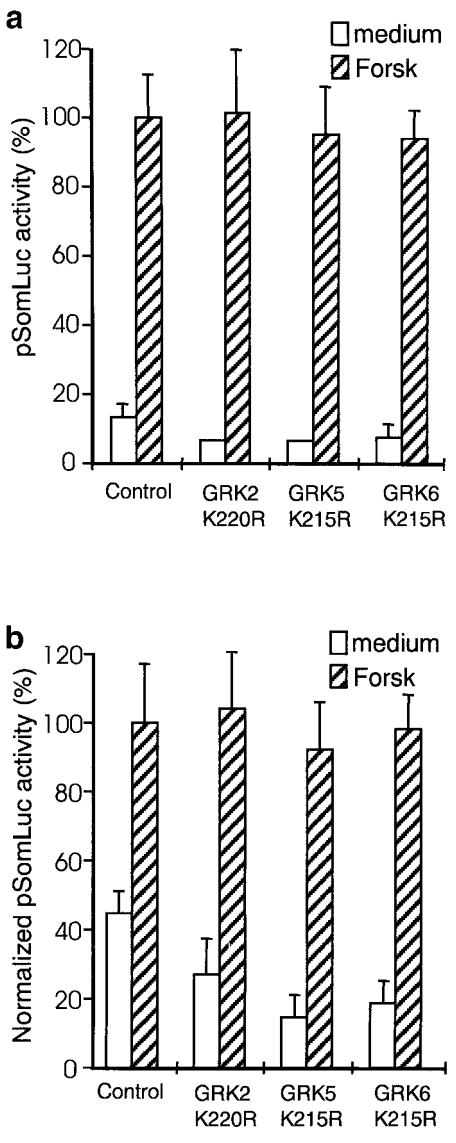

FIG. 3. Cells overexpressing kinase-inactive GRKs display unaltered response to forskolin. Cells were transiently cotransfected with pSOMLuc and with an expression vector for phosphorylation-deficient mutants of GRK 2, 5, or 6. Twenty-four hours after transfection, cells were left unstimulated or were stimulated with forskolin (10 μM). (a) Control plasmid, GRK2, GRK5, or GRK6 mutants were compared for their ability to modify the forskolin-induced response of Ltk 7/12 cells. (b) The same experiment was performed in primary Sertoli cells with *Renilla* luciferase plasmid to normalize transfection efficiencies. Transfection conditions were as described in the legend to Fig. 1. Values were expressed as percentage of the stimulated control activity taken as 100%. These data are mean ± SE of two independent experiments, each with four replicates.

either positive or negative, depending on the targetted GPCR. In particular, K220R dampens inositol phosphate generation in the cases of thyrotropin, serotonin 5HT, endothelin and parathyroid hormone receptors (12, 13, 24). Whether these latter effects were due to K220R direct association with the receptor (12, 13) or instead were attributable to a selective regulation of Gαq by the RGS domain present in the N-terminus of this GRK (24–25), remained unclear. Here, we show inhibitory effects of the GRK2-K220R mutant on cAMP accumulation mediated by the Gs-coupled FSH-R. We also demonstrate that kinase-inactive mutants of GRK5 and GRK6 behave similarly to GRK2-K220R since they also attenuate FSH-dependent cAMP accumulation both in Ltk 7/12 and in primary Sertoli cells. In addition, the observation that mutated GRKs do not affect forskolin responsiveness of the transfected cells strongly suggests that their site of action must be

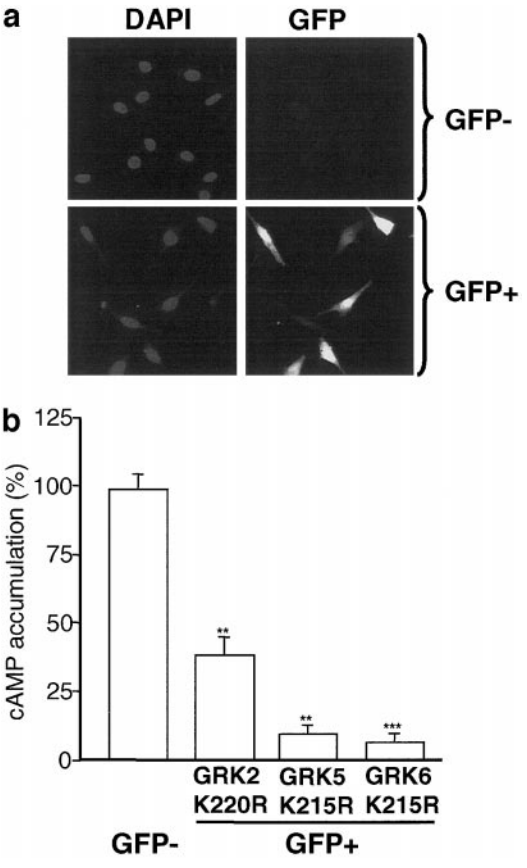

FIG. 4. Kinase-inactive GRKs inhibit the acute FSH-promoted cAMP accumulation in Ltk 7/12 cells. Ltk 7/12 cells were transiently co-transfected with both an expression vector for green fluorescent protein (GFP) and a kinase-inactive GRK contruct or pCMV5 as control (not shown). Twenty-four hours after transfection, cells were trypsinized; GFP-positive (GFP+) and -negative (GFP−) cells were sorted by flow cytometry and were then replated for 24 h. (a) Fluorescence microscopy visualization of GFP vs DAPI labeling in positive and negative populations after cell sorting. (b) Cells cotransfected with GRK2 K220R mutant, GRK5 K215R mutant, or GRK6 K215R mutant. Positive and negative cells were stimulated or not with 100 ng/ml of FSH for 15 min. Intracellular cAMP content was measured as described in both basal and stimulated conditions. Data were expressed as percent of cAMP accumulation, different pools of GFP negatives cells were taken as controls. The actual basal and FSH-stimulated cAMP values were comprised between 0.54 and 3.55 fmol/10,000 cells. These data represent the mean ± SE of two independent experiments, each with three replicates. $^{**}P < 0.01$, $^{***}P < 0.001$, significant statistical difference from GFP negative cells.

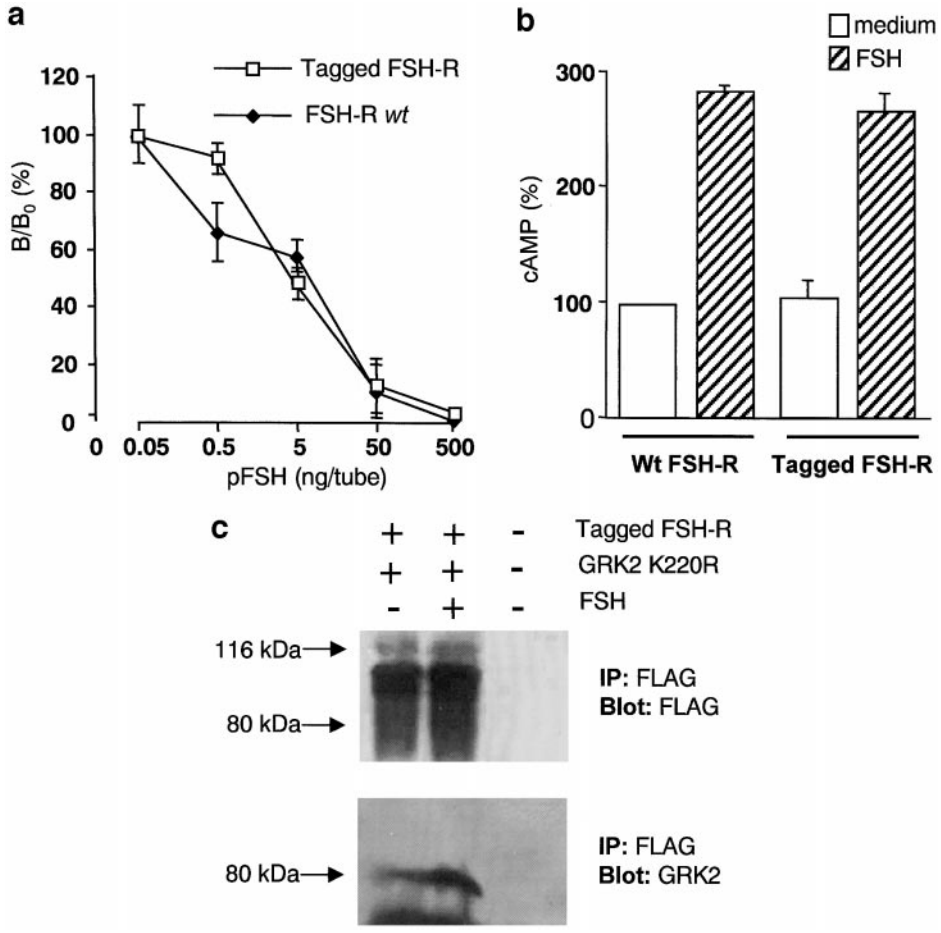

FIG. 5. Crosslinking of mutated GRK2 to the FSH-R. A FLAG-tagged rat FSH-R or its wild-type counterpart was transfected alone or together with mutated GRK2 into Cos-7 cells. (a) Competition curves of pFSH for the FLAG-tagged and *wild-type* rat FSH-R expressed in Cos-7 cells. [^{125}I]-oFSH (30,000 cpm) was incubated 16 h at 4°C with Cos-7 membranes in the presence of increasing amounts of unlabeled hormones (0–500 ng/tube). (b) FLAG-tagged and *wild-type* FSH-R-transfected cells were stimulated or not with 100 ng/ml of FSH for 15 min. Intracellular cAMP content was measured as described in both basal and stimulated conditions. Data were expressed as percent of cAMP accumulation. These data represent the mean ± SE of two independent experiments, each with three replicates. (c) Cos-7 cells were transfected with a FLAG-tagged rat FSH-R together with mutated GRK2. Control cells were transfected with equivalent amounts of an empty vector. Cells were incubated for 15 min with or without 100 ng/ml of pFSH and crosslinking with 2.5 mM dithiobis(succinimidyl propionate) was done as described under Materials and Methods. The receptors were solubilized and immunoprecipitated with anti-FLAG antibodies. Immunoprecipitates were analysed by Western blot with anti-FLAG and anti-GRK2/3 antibodies.

located upstream of the adenylyl cyclase. Since GRK5 and 6 interact with the plasma membrane via a polybasic region or a palmitoyl group respectively and not via a pleckstrin homology domain capable of interacting with G protein $\beta\gamma$ subunits (2, 6), the blockade of G$\beta\gamma$ by K220R, as previously reported for other GPCRs (34–36), can be ruled out. Moreover, to date, interaction between a RGS protein and Gαs subunit has never been reported (28) and no functionality has been attributed to the atypical RGS domains present in GRK5 and 6 as they do not bind to Gαs (25). These results point to the receptor as the most likely site of action because the inhibitory effects observed in the absence of phosphorylation are exerted neither at the G protein $\beta\gamma$ subunit level nor downstream from adenylyl cyclase. In the present study, we demonstrate that mutated GRK2 can be cross-linked to and co-immunoprecipitated with ligand activated FSH-R in living cells. As previouly proposed by Dicker *et al.* for the parathyroid hormone (13), the inhibition reported here might thus be caused by the formation of a receptor-GRK complex that prevents the receptor from coupling to G proteins. GRKs are thought to bind to and to phosphorylate agonist-occupied GPCRs via multiple contact point interactions (37). That GRK binding to GPCRs disturbs receptor/G protein interactions can be anticipated because GRKs are large proteins (between 66 and 80 kDa) when compared to the core of G protein-coupled receptors (<35 kDa). The existence of similar GRK/GPCR complexes has also been demonstrated *in vivo* for other GPCRs (12, 13, 16). Moreover, inhibitory effects of K220R GRK2 were reported at

mutant protein levels similar to those naturally expressed in brain (13) and Aragay *et al.* have achieved co-immunoprecipitation of endogenous GRK2 with the chemokine receptor CCR2B in Mono Mac 1 cells (16). These latter data suggest but not definitively establish that such stable complex formation and phosphorylation-independent inhibition of receptor signaling may represent a physiologically relevant mechanism for some particular GPCR/GRK combinations.

Our results are also informative concerning the consequences of GRK autophosphorylation. Autophosphorylation has been reported for GRK5 and 6 (2, 19), with functional implications unknown for GRK6 and unclear in the case of GRK5. When GRK5 is autophosphorylated on Ser 484 and Thr 485 in a lipid-dependent manner, its ability to phosphorylate GPCR is increased while autophosphorylation at distinct sites upon calmodulin binding inhibits GRK5 kinase activity (2). In this context, it is interesting to note that, in our experiments, GRK5 and 6 kinase-inactive mutants, which are by essence unable to autophosphorylate, might still be able to interact with the FSH-R.

In conclusion, our current study demonstrates that GRKs are able to exert phosphorylation-independent dampening of the FSH-mediated adenylyl cyclase/PKA activation. These effects are due to events located upstream of adenylyl cyclase activation and are likely independent of GRK interaction with G protein subunits. These effects could be attributable to physical association between GRKs and FSH-R, since we show here that mutated GRK2 associates in cell to liganted FSH-R.

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