Selective Regulation of G Protein-Coupled Receptor-Mediated Signaling by G Protein-Coupled Receptor Kinase 2 in FRTL-5 Cells: Analysis of Thyrotropin, α₁B-Adrenergic, and A₁ Adenosine Receptor-Mediated Responses

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ABSTRACT

G protein-coupled receptor kinases (GRKs) play a key role in the process of receptor homologous desensitization. In the present study, we address the question of whether a variety of receptors coupled to different G protein subtypes and naturally expressed on the same cell are selectively regulated by GRK2. The signaling stimulated by thyrotropin (TSH), α₁B-adrenergic, and A₁ adenosine receptors was studied in FRTL-5 cells permanently transfected to overexpress GRK2 and GRK2-K220R, a kinase dead GRK dominant negative mutant. In FRTL-5 overexpressing GRK2, TSH-induced cyclic AMP response was attenuated, indicating that TSH receptor is desensitized by this kinase. Consistently, FRTL-5 cells overexpressing GRK2-K220R show increased TSH-induced cyclic AMP response, demonstrating that this receptor is under tonic control by GRK. Unlike TSH receptor, α₁B-adrenergic receptor response (activation of mitogen-activated protein kinases) was efficiently desensitized by GRK2 but was unaffected by GRK2-K220R overexpression. The present study documents that overexpression of GRK2 results in a selective regulation of different G protein-coupled receptors expressed on the same cell and that this kinase can regulate preferentially only one of the different pathways activated by the same receptor. The preferential regulation of the A₁ adenosine receptor-stimulated mitogen-activated protein kinases by GRK2 indicates that this kinase can have additional regulatory effects on Gᵢ₅-stimulated pathways, possibly through direct binding and regulation of the receptor-Gᵢ₅ complex.

When A₁ adenosine receptors were stimulated, Gᵢ₅-mediated cyclic AMP inhibition was totally unaffected by overexpression of either GRK2 or GRK2-K220R. By contrast, Gᵢ₇-mediated response (activation of mitogen-activated protein kinases) was efficiently desensitized by GRK2 but was unaffected by GRK2-K220R overexpression. The present study documents that overexpression of GRK2 results in a selective regulation of different G protein-coupled receptors expressed on the same cell and that this kinase can regulate preferentially only one of the different pathways activated by the same receptor. The preferential regulation of the A₁ adenosine receptor-stimulated mitogen-activated protein kinases by GRK2 indicates that this kinase can have additional regulatory effects on Gᵢ₅-stimulated pathways, possibly through direct binding and regulation of the receptor-Gᵢ₅ complex.

G protein-coupled receptors (GPCRs) mediate the signal transduction of a wide array of molecules, ranging from neurotransmitters, hormones, chemokines, and lipids to light and odors. On binding of the agonist to its GPCR, the heterotrimeric G protein dissociates into the Gₛ and Gₛₐ₇ subunits (Hamm, 1998). The dissociated subunits can either activate or inhibit a number of effector enzymes such as adenylyl cyclase, phospholipase C, ion channels, tyrosine kinases, and many others (Hamm, 1998). Each of these enzymes accounts for the intracellular transmission of the receptor signal that finally results in a variety of cellular functions. Some of the biological events evoked by GPCR stimulation, such as proliferation, differentiation, calcium, or ion homeostasis, are common to all cells; other functions depend on the cellular specialization, such as chemotaxis for neutrophils, iodide uptake for thyrocytes, visual stimuli for retina, aggregation for platelets, and so on.

GPCR signal transduction appears as a multistep process, whose regulation is fundamentally important to ensure a coherent organization of cellular, multicellular, and, hence, whole organism functions. One of the early regulatory mechanisms that is activated after GPCR stimulation is receptor homologous desensitization (Chuang et al., 1996b). This is an agonist-dependent adaptive process in biological systems that modulates responsiveness of the cell to repeated stimuli over time. Two classes of proteins play a major role in hormonal desensitization mechanism: G protein-coupled receptor kinases (GRKs), which phosphorylate agonist-occupied re-

ABBREVIATIONS: GPCR, G protein-coupled receptor; FSK, forskolin; A₁r, A₁ adenosine receptor(s); CaM, calmodulin; CPA, N⁶-cyclopentyladenosine; HEK, human embryonic kidney; cAMP, cyclic AMP; HBSS, Hanks’ balanced salt solution; ERK, extracellular signal-regulated kinase(s); MAPK, mitogen-activated protein kinase; TSH, thyrotropin; TSHr, thyrotropin receptor(s); NE, norepinephrine; IP, inositol phosphate(s); GRK, G protein-coupled receptor kinase; PC, phosphatidylcholine; ROS, rod outer segment(s); PiP₂, phosphatidyl inositol-4,5-bisphosphate.
ceptor and their functional cofactors arrestins (Chuang et al., 1996b; Freedman and Lefkowitz, 1996; Palczewski, 1997). The essential steps of homologous desensitization can be summarized as follows: on binding of the agonist to the receptor, the G protein is activated and then the receptor kinase is translocated to the membrane, where it gets in the vicinity to the receptor regions to be phosphorylated. Once membrane localized, the receptor kinase may interact with different GPCRs, but only those occupied by the agonist are in the suitable conformation that allows phosphorylation by the kinase. This process results in minimal desensitization, but it increases the affinity of the receptor for arrestin. Binding of arrestin to the phosphorylated receptor results in maximal homologous desensitization. A role of arrestin in the process of receptor internalization has been documented (Ferguson et al., 1996).

Both GRK and arrestins are members of multigene families. The GRK family consists of six cloned members, GRK1 to GRK6. Three of these kinases were previously known as rhodopsin kinase (GRK1), β-adrenergic receptor (AR) kinase 1 (βARK1) (GRK2), and βARK2 (GRK3), whereas GRK4, GRK5, and GRK6 were more recently cloned. The arrestin family consists so far of four members: retinal arrestin and cone arrestin, which are localized in the retina where they regulate phototransduction, and β-arrestin1 and β-arrestin2, which are widely distributed. For β-arrestin1, two splice variants, β-arrestin1A and β-arrestin1B (Parruti et al., 1993), are known.

The extraordinarily large number of GPCRs identified so far and the relatively small number of GRK and arrestin subtypes imply that more than one receptor has to be regulated by the same subset of GRK and β-arrestin. When levels and activity of GRK/arrestin regulatory proteins are increased, many GPCRs expressed on the cell surface could be turned off by GRK/β-arrestin-mediated regulatory mechanisms. Several studies documented that a variety of GPCRs are either phosphorylated and/or desensitized by the same GRK subtype (Chuang et al., 1996b). In the majority of such studies, desensitization of receptor-mediated intracellular response was induced by transient overexpression of GRK, using the coexpression of receptor and kinase in heterologous cell system as the experimental approach. It was suggested that the majority of GPCRs are substrates for GRK when the levels of these kinases is high enough, but the high levels of regulatory proteins usually reached in such experiments make it difficult to address the issue of selectivity.

The aim of the present study was to investigate whether a variety of GPCRs coupled to different G protein subtypes and naturally expressed on the same cell can be selectively regulated when GRK levels and activity are increased. We measured the receptor-mediated intracellular responses in cells permanently transfected to obtain moderate overexpression of GRK2. In parallel, we studied the same parameters in cell lines permanently transfected with GRK2 mutant (GRK2-K220R) in which the catalytic activity was disrupted (Kong et al., 1994). This mutant acts as a kinase dominant negative. We found that regulation of GPCR in FRTL-5 cells by GRK2 is highly selective and that thyrotropin receptors (TSHr), which in these cells mediate fundamental responses such as proliferation and differentiation, are strictly controlled by GRK-mediated regulatory mechanisms.

**Experimental Procedures**

**Cell Culture and Transfection.** FRTL-5 cells are a continuous line of Fisher rat thyroid cells that maintain in vitro the proliferation and differentiation properties of the rat thyroid. FRTL-5 cells were cultured as previously described (Iacovelli et al., 1996). Briefly, FRTL-5 were maintained in Coon’s modified F-12 medium supplemented with 5% calf serum, 20 mM glutamine, and a mixture of six hormones (TSH, insulin, transferrin, somatostatin, cortisol, and glycyrl-L-histidine-L-lysine acetate; 6H medium) and grown at 37°C in a humidified atmosphere of 5% CO₂.

Human GRK2 cDNA was previously cloned in our laboratory (Chuang et al., 1992). GRK2-K220R is a GRK2 mutant in which lysine in position 220 has been mutated to arginine to disrupt kinase activity (Kong et al., 1994). These cDNA constructs and pBJ1 vector were used for permanent transfection of FRTL-5 using the calcium-phosphate precipitation method. Colonies of resistant cells appeared after about 3 weeks and were picked for expansion 2 weeks later. Cells were always grown under antibiotic-selective pressure (400–800 μg/ml G418), and at least three different clones for each transfection were used in the experiments. Clones of FRTL-5 cells transfected with pBJ1 vector to be used as control were tested in parallel experiments with FRTL-5 to ensure that they still maintained the characteristics of wild-type strain. Transient transfection in COS7 cells was as previously reported (Iacovelli et al., 1996).

**Northern Blot Analysis.** Northern blot analysis was performed as previously described (Iacovelli et al., 1996). Total RNA (20 μg) isolated by the guanidium isothiocyanate-cesium chloride method was fractionated on 1% agarose-formaldehyde gel and transferred to a GeneScreen Plus membrane. Northern blot analysis of TSH receptor was performed using the random-primed cDNA fragments as probes. RNA blots were hybridized, washed, and subjected to autoradiography at –80°C.

**Western Blot Analysis.** Western blots were performed as described (Iacovelli et al., 1996). Monoclonal antibody C5/1, which recognizes an epitope common to GRK2 and GRK3, and monoclonal antibody A16-17, which recognizes GRK5 and GRK6 (Oppermann et al., 1996), were kindly provided by R. J. Lefkowitz. Proteins (40–100 μg) were electrophoresed on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes with a tank transfer system. Efficiency of transfer was verified by Coomassie blue staining of gels and Ponceau red staining of the blots after transfer. The blots were quantified by scanning four times with LKB Ultrascan XL laser densitometer (LKB Instruments, Rockville, MD) or by National Institutes of Health Image Version 1.59.

**Extracellular Signal-Regulated Kinase (ERK)1/2 Activity Assay.** The activity assay for ERK1/2 was performed as previously described with minor modifications (Brondello et al., 1997; della Rocca et al., 1997).Briefly, cells were grown to confluence in 12-well plates and starved from hormones and serum for 48 h. Cells were stimulated in the same buffer as for cyclic AMP (cAMP) assay without 3-isobutyl-1-methylxanthine at 37°C for 30 min and then washed twice with ice-cold PBS and lysed in Triton X-100 lysis buffer for 15 min at 4°C. Samples were clarified by centrifugation at 12,000g for 10 min at 4°C. Equal amounts of proteins from supernatants (50 μg) were separated by SDS-polyacrylamide gel electrophoresis (12.5% acrylamide, 0.0625% bisacrylamide) plus 4 M urea. Proteins were transferred as specified in the section on Western blotting and probed using a commercial anti-phospho-specific antibody against phosphorylated ERK1/2 (Promega, Madison, WI).

**Binding of GRK2 and GRK2-K220R to G₆₅.** This binding was performed according to Pitcher et al. (1995), with minor modifications. Vesicles composed of 95% phosphatidylcholine (PC) and 5% phosphatidyl inositol-4,5-bisphosphate (PIP₂) (w/w) were incubated with or without G₆₅ (400 nM) for 1 h on ice. Vesicles were then incubated in polycarbonate tubes (Beckman) with cytosolic proteins (10 and 20 μg) from COS7 cells overexpressing comparable amounts of GRK2 and GRK2-K220R (as assessed by immunoblot), and the
reaction mixture was diluted to 50 μl with PBS. The final lipid concentration was 1.7 mg/ml in each sample. After incubation for 10 min at room temperature and 5 min at 4°C, samples were centrifuged at 100,000 rpm (TL-100 rotor) for 15 min at 4°C. The supernatants were saved, and the pellets were rinsed once with PBS. After the second centrifugation, the pellets were diluted in 50 μl of PBS, and SDS-loading buffer was added to either supernatants and pellets. Proteins were electrophoresed on 10% polyacrylamide gel and subjected to Western blot analysis using monoclonal C5/1 antibody.

**cAMP Assay.** The intracellular content of cAMP in transfected cells was determined by a method previously described (Iacovelli et al., 1996). Briefly, cells were seeded onto 96-well plates at the density of 5 × 10³ cells/well, grown to confluence, and then starved from hormones and serum for 48 h. Cells were washed twice with prewarmed Hank's balanced salt solution (HBSS) and added to HBSS containing 0.4% BSA, 10 mM HEPES, and 0.5 mM 3-isobutyl-1-methylxanthine. Incubations were continued at 37°C for 30 min with appropriate stimuli. The reaction was stopped by aspiration of the incubation medium and the addition of ice-cold ethanol. The intracellular cAMP content was measured using a commercial radioimmunoassay. Data are expressed as picomoles of cAMP per well. Experiments were performed in triplicate.

**Phosphoinositide Hydrolysis.** [3H]Inositol phosphate (IP) formation was performed as previously described (Molino et al., 1995). Briefly, cells were seeded onto 12-well plates at the density of approximately 2.5 × 10⁵ cells/well, grown for 3 days, and then incubated for 48 h with 3 μCi/ml myo-[3H]inositol in M199 serum-free medium. Cells were washed twice with prewarmed HBSS, incubated for 15 min at 37°C in HBSS buffer containing 10 mM HEPES and 10 mM LiCl, pH 7.3, and then stimulated with norepinephrine (NE) for 30 min at 37°C in the presence of 100 μM ascorbic acid. Total [3H]IP was extracted with perchloric acid, neutralized, and analyzed by ion exchange chromatography on Dowex columns. Experiments were performed in duplicate.

**A₁ Adenosine Receptor Binding.** Cells were harvested, pelleted, and then resuspended in binding buffer (PBS plus 10 mM MgCl₂ and 5 U/ml adenosine deaminase) to a final concentration of 0.5 mg protein/ml. Total binding was evaluated by using [3H]2-chloro-N⁶-cyclopentyladenosine (5 nM) as ligand and N⁶-cyclopentyladenosine (CPA; 100 μM) to define nonspecific binding in 0.5 ml of final volume. Incubations were carried on at 21°C for 1 h, followed by rapid filtration and liquid scintillation counting. Experiments were performed in duplicate.

**α₂b-Adrenergic Receptor Binding.** Cells were trypsinized, harvested, pelleted, and then resuspended in HBSS buffer containing 10 mM HEPES, pH 7.4, and then sonicated. Total binding was evaluated by using [3H]prazosin (0.6 nM) as ligand and phentolamine (10 μM) to define nonspecific binding in 0.5 ml of final volume. Incubations were carried on for 45 min at 37°C, followed by rapid filtration and liquid scintillation counting. Each experiment was performed in duplicate.

**Rod Outer Segment (ROS) Preparation and Phosphorylation.** ROS containing ~90% pure rhodopsin were isolated from bovine retina by stepwise sucrose gradient sedimentation, and endogenous rhodopsin kinase was denatured by treatment with 5 M urea. The ROS phosphorylation mixture contained 50 μM γ[32P]ATP, 20 mM Tris, 8 mM MgCl₂, 3 mM EDTA, 5 mM NaF, and 12 mM NaCl. The concentration of ROS was 0.6 μM, and 20 μg of cytosolic proteins prepared from L5pBJI, L5GRK2, and L5K220R were used as the source of GRK. The reaction was carried out for 30 min at 30°C in the presence of light, stopped with Laemmli’s buffer, and electrophoresed on 10% SDS-polyacrylamide gel electrophoresis, followed by autoradiography. Phosphorylation was quantified by Instant Imager (Packard).

**Statistical Analysis.** All the experiments are presented as the average of duplicate or triplicate determinations repeated at least three times. Statistical analysis was carried out either by Student’s t test or Dunnett’s ANOVA.

**Materials.** 5-Bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium were obtained from Life Technologies (Gaithersburg, MD). Forskolin (FSK) was purchased from Calbiochem (San Diego, CA). Glutamine, penicillin/streptomycin, and calf serum were obtained from Life Technologies (Grand Island, NY). Tris, SDS, bromophenol blue, and G418 were obtained from Fluka Chemical Corp. (Ronkonkoma, NY). Acrylamide, bisacrylamide, and anti-phospho ERK1/2 antibody were purchased from Promega. The cAMP assay kit, [3H]inositol, [3H]thymidine, [3H]prazosin, and [3H]CCPA were purchased from Amersham (Arlington Heights, IL). Alkaline phosphatase-conjugated goat anti-mouse IgG was obtained from Pierce Chemical (Rockford, IL). All other materials were from Sigma Chemical Co. (St. Louis, MO).

**Results**

**Expression of GRK2 in FRTL5 Cells.** Before studying the possible role of GRK2 in regulating TSHr and other receptors in thyroid cells, we wanted to further validate our previous findings showing that GRK2 is physiologically expressed in FRTL-5. The ability of FRTL-5 cytosolic protein preparation to phosphorylate rhodopsin, which represents GRK activity, was characterized to determine the subtype specificity. This approach is based on the different properties of GRK subfamilies: only the βARK subfamily members (GRK2 and GRK3) are activated by Gₛ, (Freedman et al., 1996), whereas only the GRK4 subfamily members (GRK4, GRK5, and GRK6) are substantially inhibited by nanomolar concentrations of calmodulin (CaM) in the presence of calcium (Chuang et al., 1996a; Iacovelli et al., 1999). Cytosolic preparations from FRTL-5 were able to phosphorylate light-activated ROS, indicating the presence of measurable GRK activity (Fig. 1A). ROS phosphorylation by GRK was not inhibited in the presence of calcium by 500 nM CaM (95 ± 6% of control, as quantified by phosphorimaging analysis, n = 3), which potently inhibits GRK4, GRK5, and GRK6 kinase activity. The addition of 100 nM Gₛ, increased kinase activity by 4.5 ± 0.9-fold (n = 3; Fig. 1A). Based on this approach, the cytosolic GRK activity in FRTL-5 could be ascribed to GRK2 and/or GRK3. It should be noted that with the use of our procedure to prepare cytosolic proteins, at least 50% of GRK5 is recovered in the cytosolic fraction (data not shown). The expression of GRK2, but not GRK3, was then confirmed by immunoblotting using the monoclonal antibody C5/1v, which specifically recognizes these two kinase subtypes (Fig. 1B). Western blotting with the monoclonal antibody A16-17, which recognizes GRK5 and GRK6, did not detect any specific immunoreactive band in FRTL-5 (Fig. 1C) even when the blot was developed for much longer (not shown). The expression of GRK2 was also confirmed by Northern blotting (Iacovelli et al., 1996; data not shown). Based on these different approaches, we are confident that GRK2 is expressed in FRTL-5 and that it is responsible for the majority of the cytosolic GRK activity.

**Overexpression of GRK2 and GRK2-K220R in FRTL-5 Cells.** We generated different cell lines by permanently transfecting FRTL-5 cells with GRK2 cDNA (named L5GRK2), with GRK2-K220R cDNA (L5K220R), and with the empty vector pBJI cDNA (L5pBJI) to be used as control (Fig. 2A). Clones were selected in which moderate (about 3- to 4-fold) and comparable overexpression of GRK2 and GRK2-K220R was observed, as measured by immunoblotting. The overexpression of GRK2 resulted in 2.3 ± 0.7-fold (n = 4)
increased ability of cytosolic preparations to phosphorylate ROS (as quantified by phosphorimaging analysis). The kinase activity was reduced by 61.3 ± 9% in cells overexpressing the GRK2-K220R clone, which acts as dominant negative for the endogenous GRKs (Fig. 2A).

We performed Western blot analysis to compare the GRK2 protein levels in transfected cells; in cells endogenously expressing this regulatory protein such as COS7 and human embryonic kidney (HEK) 293 cells, commonly used in transient transfection studies, and in a variety of other cell types (Fig. 2B). According to previous results (Ménard et al., 1997), GRK2 levels in COS7 cells were lower than those in HEK 293 cells and were comparable with those in L5pBJI and L5GRK2, respectively. Kinase levels in L5GRK2 cells were about 8- and 5-fold lower than those of endogenous GRK2 in HL60 and mononuclear leukocytes cells, respectively.

**Regulation of TSHr.** The functionality of TSHr in FRTL-5 was assessed by the ability of TSH to stimulate intracellular cAMP accumulation. Basal levels of cAMP were similar in L5pBJI (0.36 ± 0.007 pmol/well), L5GRK2 (0.38 ± 0.009 pmol/well), and L5K220R (0.38 ± 0.008 pmol/well). The levels of cAMP after direct stimulation of adenyl cyclase by FSK were similar in L5pBJI (10.7 ± 0.97 pmol/well), L5GRK2 (9.8 ± 0.63 pmol/well), and L5K220R (10.6 ± 0.59 pmol/well). In L5pBJI, exposure to TSH resulted in up to 25-fold increase in cAMP. This effect was reduced by 30 to 45% in FRTL-5 cells overexpressing GRK2, indicating that overexpression of this kinase induces TSHr desensitization (Fig. 3A). By contrast, TSH-stimulated cAMP accumulation was significantly enhanced in cells overexpressing the GRK2-K220R mutant, suggesting that in FRTL-5 the TSHr is tonically controlled by endogenous GRKs (Fig. 3A).

The TSHr mRNA levels were similar in L5pBJI, L5GRK2, and L5K220R, indicating that transfection did not modify the expression of receptors (Fig. 3B). As expected, Northern blot analysis of TSHr identified two mRNA species, 5.6 and 3.3 kb in size (Akamizu et al., 1990). The expression of TSHr could not be detected by Western blotting, as the affinity of the anti-TSHr antibodies presently available in our laboratory (kindly provided by L. Kohn) is not high enough to detect TSHr in FRTL-5 membranes (Ban et al., 1992).

**Regulation of TSHr by Different GRK Subtypes in COS7 Cells.** To define the possible role of different GRK subtypes in TSHr rapid homologous desensitization, we transfected the TSHr cDNA in COS7 cells and measured the effect of GRK subtype coexpression on TSH-stimulated cAMP accumulation. As previously shown, transient transfection of COS7 cells with TSHr cDNA results in the expression of functional receptors coupled to Gs, as indicated by up to 10- to 15-fold increase in cAMP levels after exposure to TSH. Overexpression of GRK2 induced a substantial blunting of cAMP accumulation after exposure to TSH for 15 min, whereas overexpression of GRK5 and GRK6 did not affect TSHr-

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**Fig. 1.** A, characterization of GRK activity in FRTL-5. Rhodopsin phosphorylation by cytosolic GRK activity obtained from FRTL-5 in the presence of the indicated concentrations of CaM (plus 1 mM Ca^{2+}) or in the presence of G_{b\gamma} (100 nM) purified from bovine brain. The arrow indicates the bands corresponding to phosphorylated rhodopsin as revealed by autoradiography after polyacrylamide gel electrophoresis. B, GRK immunoactivity in FRTL-5. Cytosolic proteins prepared from COS7 cells transiently transfected with GRK2 (40 \mu g) and from FRTL-5 (100 \mu g) were blotted, and immunoreactivity was probed using C5/1 monoclonal antibody, which recognizes GRK2 and GRK3. The arrow indicates the position of GRK3 that is not detectable. C, membrane proteins from COS7 cells transiently transfected with GRK6 (50 \mu g) and GRK5 (50 \mu g) and from FRTL-5 (100 \mu g) were blotted, and immunoreactivity was probed using A16-17 monoclonal antibody that recognizes GRK5 and GRK6. Data are representative of three independent experiments.

**Fig. 2.** A, characterization of a FRTL-5 cell line permanently overexpressing GRK2 and GRK2-K220R. Top row, 80 \mu g of cytosolic proteins from L5pBJI, L5GRK2, and L5K220R were blotted, and GRK immunoreactivity was detected by the C5/1 monoclonal antibody. The positions corresponding to GRK2 and GRK3 (which is not detectable) are indicated. Data are representative of two independent experiments. Bottom row, rhodopsin phosphorylation by cytosolic GRK obtained from L5pBJI, L5GRK2, and L5K220R. The incubation was at 30°C for 30 min. Data are representative of three independent experiments. B, comparative analysis between GRK2 and GRK2-K220R protein levels in permanently transfected FRTL-5 cells and endogenous GRK2 protein levels in different cells. GRK immunoreactivity was detected by the C5/1 monoclonal antibody using 80 \mu g of cytosolic proteins from each preparation. Arrow indicates the position corresponding to GRK2. 1, L5pBJI; 2, L5GRK2; 3, L5K220R; 4, COS7; 5, HEK 293; 6, HeLa; 7, HL60; and 8, MNL. Data are representative of two independent experiments.
mediated response. Basal cAMP levels were not affected by GRK transfection (3 to 4.5 pmol/well; data not shown). These results indicate that GRK2 is the subtype mainly involved in the rapid homologous desensitization of TSHr. This is in agreement with previous evidence from different laboratories that documented that overexpression of GRK2 in transfected COS7 cells (Iacovelli et al., 1996), but not GRK5 and GRK6, in FRTL-5 cells (Nagayama et al., 1996) was able to desensitize the TSHr after short-term (15- to 30-min) exposure to agonist. GRK5 and GRK6 were able to desensitize the TSHr only after long-term (2- to 24-h) exposure to TSH (Nagayama et al., 1996).

**Regulation of α1B-ARs.** α1B-ARs are expressed on FRTL-5, where their activation results in phospholipase C stimulation, via Gq (Kanasaki et al., 1994). The functional response of these receptors was assessed by measuring IP production after NE stimulation. Basal IP production was 472 ± 45.7, 510 ± 31, and 536 ± 45 cpm/well in L5pBJI, L5GRK2, and L5K220R, respectively. Cotransfection of GRK2 and GRK2-K220R did not significantly alter the effect of NE (Fig. 4A). For example, 1 μM NE increased IP production by 2.3 ± 0.3-, 2.0 ± 0.2-, and 2.1 ± 0.3-fold, in L5pBJI, L5GRK2, and L5K220R, respectively. α1B-AR density, as assessed by [3H]prazosin binding, was also similar in L5pBJI, L5GRK2, and L5K220R (Fig. 4B).

**Regulation of A1 Adenosine Receptors (A1r).** Adenosine receptors that are coupled to Gi are expressed on FRTL-5. Stimulation of Gi-coupled receptors induces the dissociation of Gi heterotrimer and the release of free Ga and Gbg subunits that lead to the activation of two distinct intracellular pathways. Ga-mediated pathway induces inhibition of adenylyl cyclase activity and reduction in intracellular cAMP levels, whereas free Gbg interacts with a variety of effectors and activates mitogen-activated protein kinase (MAPK) cascade (Faure et al., 1994; Moolenaar et al., 1997). Therefore, Ga-coupled receptors, such as adenosine receptors, represent a useful model in which to investigate the regulatory role of GRK on different pathways activated by the same receptor.

In L5pBJI, exposure to adenosine reduced FSK-stimulated intracellular cAMP accumulation, and this effect appeared to be mediated by the A1 subtype of adenosine receptors (A1r), as the inhibitory effect of 10 μM adenosine on FSK-stimulated cAMP was fully reproduced by the A1-selective agonist CPA (1 μM), whereas the A2-selective agonist N6-iodobenzyl-5’-N-methylcarboxamidoadenosine (IBMECA) (10 μM) was almost completely ineffective (data not shown).

**Inhibition of FSK-stimulated cAMP by adenosine was dose dependent, and the IC50 value was 7.6 μM (Fig. 5A).** The ability of adenosine to reduce FSK-stimulated cAMP levels was not affected by overexpression of either GRK2 and GRK2-K220R (Fig. 5A). Similar results were obtained using the A1-selective agonist CPA (Fig. 5B). The density of A1r, as assessed by [3H]CCPA binding, was similar in L5pBJI (39.7 ± 3.5 fmol/mg of protein), L5GRK2 (33.7 ± 1.2 fmol/mg), and L5K220R (32 ± 5.6 fmol/mg) (Fig. 5C).

Activation of A1r by CPA (1 μM) induced 3- to 4-fold activation of ERK1/2, and this phosphorylation was reduced by 80% (as quantified by densitometric analysis) after 1 nM pertussis toxin pretreatment (Fig. 6A), confirming that this effect is mediated by Gi, presumably through a Gbg-depen-

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**Fig. 3.** Regulation of TSHr by GRK2 and GRK2-K220R. A, L5pBJI (○), L5GRK2 (●), and L5K220R (■) were stimulated for 30 min with TSH (0.01–100 nM), and cAMP accumulation was measured. Data are mean ± S.E.M. of three to six independent experiments, each assayed in triplicate. **Significantly different from L5pBJI at p < .01. B, Expression of TSHr mRNA in transfected cells. Blots of total mRNA (20 μg) were probed with labeled TSHr cDNA. The experiment shown is representative of two independent experiments.

**Fig. 4.** Regulation of α1B-AR by GRK2 and GRK2-K220R. A, L5pBJI (○), L5GRK2 (●), and L5K220R (■) were stimulated for 30 min with NE (0.1–10 μM), and the IP formation was measured. Data are mean ± S.E.M. of three to five independent experiments each assayed in duplicate. B, a1B-AR density as measured by [3H]prazosin-specific binding in membranes from transfected cells. Data are mean ± S.E.M. of five independent experiments, each assayed in duplicate.
dent pathway. The effect of CPA on ERK1/2 phosphorylation was markedly desensitized in L5GRK2 (Fig. 6B). Exposure to 0.1 and 1 µM CPA resulted in a 2.20 ± 0.13- and 3.53 ± 0.46-fold, respectively, increase in phosphorylation of ERK1/2 in L5pBJI (n = 6). This effect was substantially reduced in L5GRK2 in which CPA at 0.1 and 1 µM stimulated ERK1/2 phosphorylation by only 1.35 ± 0.03-fold (p < .05 versus L5pBJI) and 1.90 ± 0.19-fold (p < .01 versus L5pBJI), respectively (n = 6). In L5K220R, CPA stimulated ERK1/2 phosphorylation similar to control cells (Fig. 6B). Exposure to 0.1 and 1 µM CPA resulted in a 2.50 ± 0.40- and 3.06 ± 0.26-fold, respectively, increase in phosphorylation of ERK1/2 in L5K220R (n = 3).

We measured the binding of GRK2 and GRK2-K220R to Gβγ to test whether their different effects on MAPK could be due to an altered ability of the mutant to bind and to sequester Gβγ. Cytosolic proteins from COS7 cells expressing similar levels of GRK2 and GRK2-K220R were incubated with Gβγ reconstituted into vesicle composed of 95% PC and 5% PIP2. GRK2 and GRK2-K220R bound to Gβγ were separated by centrifugation and analyzed by Western blotting. The binding of GRK2 and GRK2-K220R to Gβγ was comparable, as shown in Fig. 7.

These results show that the two pathways activated by A1r are differently regulated in L5GRK2. Although the ability of adenosine to inhibit intracellular cAMP was unaffected in L5GRK2, the A1r-mediated stimulation of MAPK cascade was substantially blunted. This effect is not due to binding, and sequestration of Gβγ by overexpressed GRK2, as GRK2-K220R expressed to similar levels, did not affect MAPK activation.

Another possibility is that the different effects of GRK2 on the two different pathways may reflect different sensitivity of the assays used to measure agonist-stimulated Giα- and Gβγ-mediated responses when measuring desensitization. Alternatively, it may be due to a selective effect of GRK2 on the different pathways. To address this point, we studied the two A1r-mediated pathways in two FRTL-5-derived cell lines permanently transfected to overexpress the two β-arrestin1 isoforms, β-arrestin1A and β-arrestin1B (named L5βarr and L5βarrB), described by Iacovelli et al. (1996). The density of A1r in L5βarr and L5βarrB was similar to that in controls (110% and 92% of L5pBJI, respectively).

The ability of adenosine to reduce FSK-stimulated cAMP levels was similar in L5pBJI and L5GRK2 cells, whereas this effect was substantially blunted in cells overexpressing β-arrestin1A and β-arrestin1B (Fig. 8A). For example, 10 µM adenosine, which was able to decrease cAMP by 54 ± 14 and 66 ± 5% in L5pBJI and L5GRK2, respectively, did not affect FSK-stimulated cAMP accumulation in L5βarr and L5βarrB (104 ± 1 and 100 ± 7% of FSK, respectively). In L5βarr and L5βarrB, the dose-response curve of adenosine was shifted to the right (IC50 = 116 and 82 µM, respectively), and the

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Fig. 5. Regulation of A1r by GRK2 and GRK2-K220R. L5pBJI (○), L5GRK2 (■), and L5K220R (■) were stimulated for 30 min with 10 µM FSK plus the indicated concentrations of adenosine (A) or CPA (B), and cAMP accumulation was measured. Data are mean ± S.E.M. of three to five independent experiments, each assayed in triplicate. C, A1r density as measured by [3H]CCPA-specific binding in L5pBJI, L5GRK2, and L5K220R. Data are the mean ± S.E.M. of four independent experiments performed in duplicate.

Fig. 6. Regulation of A1r-stimulated ERK1/2 phosphorylation by GRK2 and GRK2-K220R. A, immunoblot representing phospho-ERK1/2 in L5pBJI untreated or treated with 1 µM CPA and 1 nM pertussis toxin (overnight pretreatment). B, immunoblots representing phospho-ERK1/2 in L5pBJI, L5GRK2, and L5K220R after exposure to CPA. Data are representative of three independent experiments.
Discussion

The present study documents that the regulation of different GPCR expressed on the same cell by GRK2 is highly selective. Using the rat thyroid cell line FRTL-5, we found that TSHr functionality is strictly controlled by GRK, whereas under similar conditions, the α1B-AR appears to be insensitive to this regulatory mechanism. A moderate overexpression of GRK2 resulted in significant desensitization of TSHr, whereas the α1B-AR-mediated signaling was unaffected. Consistently, overexpression of the GRK dominant negative negative increased TSHr-stimulated cAMP response, indicating that in FRTL-5, the TSH receptor is tonically controlled by endogenous GRK. When coexpressed with GRK2 in heterologous systems (COS7 cells), both the TSHr and the α1B-AR appeared to be phosphorylated and/or desensitized by this kinase (Diviani et al., 1996; Iacovelli et al., 1996). This implies that these receptors are potentially regulated by similar mechanisms, but at least in FRTL-5 cells and under the experimental conditions used, the TSHr is more strictly “controlled” by GRK2. This is further supported by our previous data showing that in FRTL-5, overexpression of β-arrestin1 also induced TSHr desensitization (Iacovelli et al., 1996). We can speculate that in thyroid cells, in which TSHr mediates fundamental functions such as growth and differentiation, GRK/β-arrestin machinery must be active to ensure, along with other regulatory mechanisms located downstream, efficient signal transduction.

The analysis of A1r-mediated signaling revealed an unexpected level of complexity in the regulatory mechanism. Adenosine stimulation of this receptor, which is G1 coupled, activates two distinct pathways: the one mediated by Gi inhibits adenylyl cyclase, and the other mediated by Gαs induces, among other effects, MAPK activation (Faure et al., 1994). We observed that overexpression of GRK2 substantially blunted Gαs-mediated MAPK activation, whereas agonist-induced adenylyl cyclase inhibition was unaffected, indicating that different pathways stimulated by the same receptor can be differently regulated by GRK. This selective

maximal effect was reduced by ~30% compared with L5pBJI and L5GRK2 (Fig. 8A). Similar results were obtained using the A1-selective agonist CPA. In L5pBJI, 0.1 and 1 μM CPA inhibited FSK stimulated cAMP (17 and 83% of inhibition, respectively), whereas this effect was substantially blunted in L5βarrB (7 and 15% of inhibition, respectively).

The A1r-dependent activation of ERK1/2 was reduced in L5βarrB, in which CPA at 0.1 and 1 μM stimulated ERK1/2 phosphorylation by 1.40 ± 0.20-fold (p < .05 versus L5pBJI) and 2.61 ± 0.45-fold, respectively (n = 3) (Fig. 8B). Quantitative analysis of ERK1/2 experiments is summarized in Fig. 9.

Fig. 7. Binding of GRK2 and GRK2-K220R (K220R) to Gβγ. Cytosolic proteins (10 and 20 μg) from transfected COS7 cells expressing similar amounts of GRK2 and GRK2-K220R were incubated with 400 nM Gβγ reconstituted into vesicle composed of 95% PC and 5% PIP2 (+βγ) or vesicles without Gβγ (−βγ) and then centrifuged. Western blotting was used to detect GRK2 and GRK2-K220R bound to vesicles (in the pellet, P) and unbound proteins (in the supernatant, S). Data are representative of two independent experiments.

Fig. 8. Regulation of A1r by β-arrestin1 and GRK2. A, L5pBJI (□), L5βarr (■), L5βarrB (■), and L5GRK2 (○) were stimulated for 30 min with 10 μM FSK plus the indicated concentrations of adenosine, and cAMP accumulation was measured. Data are mean ± S.E.M. of three to five independent experiments, each assayed in triplicate. Differences versus L5pBJI were statistically significant at 10 μM for L5βarrB (p < .01) and for L5βarr (p < .05) and at 100 μM and 1 nM for L5βarr and L5GRK2 (p < .01), respectively. B, immunoblots representing phosphorylated ERK1/2 in L5pBJI, L5βarrB, and L5GRK2 after exposure to CPA. Data are representative of three independent experiments.

Fig. 9. Quantitative analysis A1r-stimulated ERK1/2 phosphorylation, as assessed in experiments similar to that in Figs. 6B and 8B. Experiments (n = 6) were performed in which L5pBJI, L5GRK2, and L5K220R (three experiments) or L5pBJI, L5GRK2, and L5βarrB (three experiments) were analyzed in parallel. The results are cumulated here and represent data (mean ± S.E.M.) obtained in L5pBJI (n = 6), L5GRK2 (n = 6), L5K220R (n = 3), and L5βarrB (n = 3). Data are expressed as fold of basal of ERK1/2 phosphorylation in unstimulated cells as defined 1. Statistically significant difference versus L5pBJI was observed at 0.1 μM CPA in L5GRK2 and L5βarrB (p < .05) and at 1 μM CPA in L5GRK2 (p < .01; Dunnett’s test). Open bars, basal; hatched bars, CPA 0.1 μM; stippled bars, CPA 1 μM.
regulation by GRK of one pathway in respect to the other is not due to intrinsic "refractoriness" of the $G_{i\alpha}$-dependent pathway to be desensitized in FRTL-5. We could demonstrate that overexpression of $\beta$-arrestin1 in FRTL-5 induced a robust blunting of adenosine-mediated adenyl cyclase inhibition, whereas MAPK activation was slightly affected. Another possibility is that GRK2 selectively desensitizes the $G_{i\beta\gamma}$-induced MAPK activation by its ability to bind to $G_{i\beta\gamma}$, thus inhibiting this effector through physical interaction. However, this is not the case because overexpression of GRK2-K220R mutant, which lacks kinase activity while having intact $G_{i\beta\gamma}$ binding site, did not affect agonist-induced MAPK activation. We have also documented a similar binding to $G_{i\beta\gamma}$ by GRK2 and GRK2-K220R. A recent study by Wu et al. (1998) could provide a possible explanation for these findings. Using the M2 and M3 muscarinic receptors, the authors documented that after the agonist-dependent G protein dissociation, $G_{i\alpha}$ may be "trapped" by the receptor third intracellular loop, thus forming a transduction complex that can be important for $G_{i\beta\gamma}$ signaling, such as activation of mitogenic pathway. GRK2 can bind to this complex and can phosphorylate this receptor loop. By analogy with the muscarinic receptor, the present observation that GRK2, but not the dominant negative GRK2-K220R, can desensitize the agonist-stimulated $G_{i\beta\gamma}$-dependent activation of MAPK may reflect the ability of this kinase to phosphorylate and regulate the domain of the $A_2$ receptor that binds and activates $G_{i\beta\gamma}$.

In the present work, we decided to use the approach of permanent transfection to obtain levels of overexpressed proteins lower than those after transient transfection. In particular, as shown in Fig. 2B, the GRK2 levels in L5GRK2 are comparable with the endogenous levels detected in HBEK 293 and HeLa cells and are far lower than the endogenous levels in HL60 and MNL cells. In addition, a 3- to 4-fold increase in GRK2 levels was shown to occur under physiological conditions. In fact, several studies reported that the level of expression of GRK and arrestins can be actively regulated. In different cell types, including rat thyroid cell line FRTL-5, $\beta$-arrestin1 levels were raised (up to 3- to 4-fold) by receptor agonists such as isoproterenol or TSH or by agents able to increase intracellular cAMP (Parruti et al., 1993; Iacovelli et al., 1996). Increased levels of GRK2 (up to 3- to 4-fold) and GRK6 (up to 7-fold) were found in stimulated immune cells (De Blasi et al., 1995; Loudon et al., 1996). Regulation of GRK5 in cultured vascular smooth muscle cells and rat aorta by angiotensin II and hypertension was recently reported (Ishizaka et al., 1997).

The relatively low levels of overexpression of GRK2 allowed us to address the issue of selectivity that could be lost in cells transiently transfected due to the high levels of regulatory proteins. For example, in our experimental conditions, TSHr appears to be potently regulated by GRK2, whereas $\alpha_{1A}$-AR was not affected by GRK2 overexpression; another study (Diviani et al., 1996) performed in transiently transfected COS7 cells demonstrated the $\alpha_{1A}$-AR is regulated by GRK2.

Based on the present data and on those by Nagayama et al. (1996), it is likely that both GRK2 and GRK5 are involved in the regulation of TSHr and that a certain level of redundancy between these kinases is likely to occur. The TSHr appears to be tonically regulated by these kinases, as documented by the use of GRK2-K220R dominant negative (present study) and by the GRK5 antisense treatment (Nagayama et al., 1996). The effect of these kinases can, however, be complementary in regulating TSHr. Overexpression of GRK2 desensitizes the rapid (~20- to 30-min) TSH-induced cAMP response, whereas overexpression of GRK5 desensitized the receptor-mediated response after 2 to 24 h of exposure to the agonist. In addition, the action of intracellular regulators, such as calcium/CaM, which selectively inhibit GRK subtypes (Iacovelli et al., 1999), can play a role in regulating the effects of different GRKs on TSHr. The present results together with those of previous investigations further support the notion that the TSHr-mediated signaling, which in thyroid cells transduces fundamental messages important for cell proliferation and differentiation, is selectively and actively controlled by GRK/arrestin regulatory mechanisms.

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