Regulation of the Coupling to Different G Proteins of Rat Corticotropin-releasing Factor Receptor Type 1 in Human Embryonic Kidney 293 Cells*

Received for publication, May 13, 2004, and in revised form, July 1, 2004
Published, JBC Papers in Press, July 12, 2004, DOI 10.1074/jbc.M405335200

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The regulation of G protein activation by the rat corticotropin-releasing factor receptor type 1 (rCRFR1) in human embryonic kidney (HEK)293 (HEK-rCRFR1) cell membranes was studied. Corresponding to a high and low affinity ligand binding site, sauvagine and other peptidic CRFR1 ligands evoked high and low potency responses of G protein activation, differing by 64-fold in their EC_{50} values as measured by stimulation of [35S]GTP \gamma S binding. Contrary to the low potency response, the high potency response was of lower GTP \gamma S affinity, pertussis toxin (PTX)-insensitive, and homologously desensitized. Distinct desensitization was also observed in the adenylate cyclase activity, when its high potency stimulation was abolished and the activity became low potently inhibited by sauvagine. From these results and immunoprecipitation of [35S]GTP \gamma S-bound Go_{alpha} and Go_{beta} subunits it is concluded that the high and low potency [35S]GTP \gamma S binding stimulation reflected coupling to Go_{alpha} and G_{i}, respectively, only Go_{alpha} coupling being homologously desensitized. Immunoprecipitation of [35S]GTP \gamma S-bound Go_{alpha/11} revealed additional coupling to G_{alpha/11} which also was homologously desensitized. Although Go_{alpha/11} coupling was PTX-insensitive, half of the sauvagine-stimulated accumulation of inositol phosphates in the cells was PTX-sensitive, suggesting involvement of G_{i} in addition to Go_{alpha/11} in the stimulation of inositol metabolism. It is concluded that CRFR1 signals through at least two different ways, one leading to G_{alpha} and G_{alpha/11}-mediated signaling steps and desensitization and another leading to G_{i}-mediated signals without being desensitized. Furthermore, the concentrations of the stimulans ligand and GTP and desensitization may be part of a regulatory mechanism determining the actual ratio of the coupling of CRFR1 to different G proteins.

The hypothalamic peptide corticotropin-releasing factor (CRF) does not only regulate the stress response in mammals by activation of the pituitary adrenal axis (1) but is also involved in the control of the immune response, cardiovascular, reproductive, and cognitive function, ingestive behavior, pregnancy and labor (for a review, see Refs. 2 and 3). The multiple actions of CRF are mediated by two classes of specific CRF receptors, CRFR1 (4–6) and CRFR2 (7, 8), which are encoded by unique genes and of which some variants exist, produced by alternative processing of the transcripts from each of the genes (for review, see Ref. 3). Further mammalian endogenous ligands of the receptors, urocortin (9), stresscopin-related peptide/urocortin II (10, 11), and stresscopin/urocortin III (10, 12), were detected. The different expressions of the CRF receptor types and their ligands in tissues (for review, see Ref. 3) suggest that they are involved differently in the manifold physiological functions of the CRF receptor system.

The CRF receptors belong to the G protein-coupled receptors (GPCRs). So far, CRFR1 and CRFR2 have been shown to couple to G_{alpha} proteins, leading to the stimulation of adenylate cyclase in native tissues and cells, in various brain-derived and peripheral cell lines, and in cells transfected with the receptors (for review, see Refs. 2 and 3). Additionally, by using the non-hydrolyzable GTP analog (α,γS)GTPγS-azidoalilide to label the G proteins when activated by the receptor, followed by immunoprecipitation with specific G protein antibodies, it was shown that the human CRFR1 is able to activate, in addition to G_{alpha}, also G_{i} and G_{alpha} in HEK293 and Chinese hamster ovary cells expressing the receptor (13, 14) as well as in the rat cerebral cortex (15). From these results second messengers other than cAMP or even inhibition of cAMP levels might be additionally implicated in CRF signaling. Indeed, urocortin was found to activate the G_{i}/phospholipase C/inositol triphosphate/protein kinase C pathway in HEK293 cells expressing the human subtype CRFR1α (14).

From the above mentioned findings it is suggested that the CRFR1 adds to the growing list of GPCRs that simultaneously couple to unrelated G proteins and show multiple signaling (16). To come to conclusions on the regulation of the G protein coupling of CRFR1, in this investigation we studied the conditions for the coupling of the CRFR1 to different G protein classes as well as the functional consequences and relations to the receptor activation, using HEK cells stably transfected with the rat receptor as cellular model.

EXPERIMENTAL PROCEDURES

Materials—All peptidic ligands of CRFR1 used (sauvagine, 3-I-Tyr\(^{2}\)-Gln\(^{3}\)-sauvagine, urotensin, urotensin I, oCRF, rhCRF, \(\alpha\)-helical CRF(9–41)) were synthesized in our laboratory. [35S]GTP\(\gamma\)S (1,250 Ci/mmol), [\(\alpha\)H]Tyr\(^{2}\)-sauvagine (2200 Ci/mmol), and [\(\alpha\)H]AMP were purchased from PerkinElmer Life Sciences. [\(\alpha\)32Pi]ATP and myo-[2,3]H]inositol were from Amersham Biosciences. cDNA encoding for rCRFR1 was a gift of U. B. Kaupp (Julich, Germany). LipofectAMINE
was obtained from Invitrogen; G418 from Calbiochem; and Dulbecco’s modified Eagle’s medium, PTx, and protein A-Sepharose CL-4B from Sigma. Three affinity-purified rabbit polyclonal anti-G protein antibodies from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) were used: Gαi5 (C-18), raised against a peptide mapping at the carboxyl terminus of Gαi of rat origin; Gαi2 (C-10), raised against a peptide mapping at the carboxyl terminus of Gαi of rat origin, which reacts with Gαi5, Gαo, and to a lesser extent with Gαs of mouse, rat, human, and bovine origin; and Gαq11 (C-19), raised against a peptide mapping within a domain common to Gαi and Gαq of mouse origin which reacts with Gαi5 and Gαq11 of mammalian origin.

HEK293 Cell Culture and Transfection with rCRFR1—HEK293 cells were maintained at 37 °C under 5% CO2 in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. For stable expression, HEK293 cells were plated in 60-mm culture dishes at a density of 4 × 104 cells/dish, grown overnight, and transfected with cDNA encoding for rCRFR1 in the expression vector pcDNA3, using LipofectAMINE. G418-resistant cells were selected in Dulbecco’s modified Eagle’s medium containing 400 μg/ml G418. Clones of G418-resistant cells were examined for [35S]Tyr0-sauvagine binding to detect cells that expressed CRFR1. Nontransfected and stably transfected HEK293 cells were seeded in 100-mm culture dishes at a density of 1–2 × 106 cells/dish and grown at 37 °C to about 90% confluence in Dulbecco’s modified Eagle’s medium, containing additionally 400 μg/ml G418 for the stably transfected cells. The cells were harvested 96 h after seeding. In some cases, 100 μg/ml Ptx was added to part of the stably transfected cells 24 h before harvesting the cells to inactivate the G proteins. When desensitization of the receptor was studied, the cells were incubated with 1 μM sauvagine for 24 h followed by extensive washing (eight times with cell culture medium over 2 h at 37 °C) to allow for total clearance of the ligand.

HEK Cell Membrane Preparation—Cells were washed with and collected by scraping into phosphate-buffered saline (8.1 mM Na2HPO4, 1.5 mM KH2PO4, 137 mM NaCl, 2.7 mM KCl, pH 7.4). After centrifugation at 400 × g for 5 min, the cells were suspended in buffer A (20 mM HEPES, pH 7.8, containing 1 mM EDTA and 27% sucrose) and homogenized by a Teflon-glass homogenizer (10 strokes, 750 rpm). The homogenate was centrifuged at 10,000 × g for 10 min. The membrane pellet was resuspended in buffer B (20 mM HEPES, pH 7.8, 1 mM EDTA) and stored at −70 °C. Protein concentrations were determined according to Bradford (17). Membranes obtained from HEK293 cells stably transfected with rCRFR1 are designated HEK-rCRFR1 cell membranes.

Receptor/G Protein Coupling Estimated by Binding of [35S]GTPγS to HEK-rCRFR1 Cell Membranes—3–10 μg of membrane protein was incubated in triplicate at 25 °C with generally 100 pm [35S]GTPγS (37 kBq/ml) in a total volume of 500 μl of medium consisting of 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 μM GDP, 1 mM GTP, 100 mM NaCl, 100 μM MgCl2, 10 mM KCl, 2.7 mM MgCl2, 100 μM DTT, 0.75 mM 3-I-Tyr0,Gln1-sauvagine. The samples were filtered through GF/C filters using a Brandel harvester. From the data of the two separate filters were counted for 35S activity.

Inositol 1,4,5-Triphosphate Accumulation in HEK-rCRFR1 Cells—The assay was performed according to Ref 20. Briefly, HEK-rCRFR1 cells (100,000/well) were grown in 24-well plates in culture medium. After preincubation with 74 kBq/ml [3H]Inositol for 20 h in the absence and presence of 200 ng/ml Ptx, the cells were stimulated with different concentrations of sauvagine for 60 min in culture medium containing 10% dialyzed calf serum containing additionally 10 mM HEPES, 0.5% bovine serum albumin, and 10 mM LiCl. The cells were lysed with 150 μl/well 0.1% NaOH, and subsequently 50 μl of 0.2% formic acid, 1,000 μl of 5 mM sodium tetraborate, and 1,000 μl of 0.5 mM EDTA were added. The lysates were centrifuged, and the supernatants were subjected to anion exchange chromatography on SepPak Vac 3-ml Waters Accel® cartridges. [3H]Inositol 1,4,5-trisphosphate was eluted with 0.1 M formic acid and 0.4 M ammonium formate and counted. Concentration-response curves were fitted by nonlinear regression using the program PRISM.

**RESULTS**

**Optimum Conditions for rCRFR1-activated [35S]GTPγS Binding to HEK-rCRFR1 Cell Membranes—Stimulation by sauvagine of [35S]GTPγS binding to HEK-rCRFR1 cell membranes, used as a measure of total G protein activation, was systematically optimized by examining the effects of factors known to be critical in GPCR-evoked GTP binding, GDP, MgCl2, NaCl, DTT, [35S]GTPγS, temperature, and time. The optimum incubation conditions for a maximum ratio of stimulated/basal binding and, at the same time, high bound activities, were selected to be 0.1 μM GDP, 10 mM MgCl2, 25 mM NaCl, and 100 μM [35S]GTPγS tracer, without DTT, which decreased at low concentrations (0.1–1 mM) selectively the stimulation of binding by sauvagine and at higher concentra-
tion basal and stimulated binding uniformly. The influence of DTT is in line with findings showing that disulfide bonds in the extracellular amino-terminal part of the CRFR1 are important for the formation of the active receptor state (21). Basal and stimulated binding increased time-dependently both with half-lives of about 30 min at, consequently, constant relative stimulation over basal at 25 °C. Under the optimum conditions, during incubation over 2 h about 230 fmol [35S]GTPγS/mg of protein was bound in the absence of a stimulating CRF agonist, and this amount bound was increased by 80–160% by the CRF agonists. No stimulation of [35S]GTPγS binding by sauvagine to membranes obtained from nontransfected HEK cells was observed.

**rCRFR1/G Protein Coupling Estimated by CRFR1 Agonist-stimulated [35S]GTPγS Binding to HEK-rCRFR1 Cell Membranes and to Goα, Goα, and Goα Subunits in the Membranes**—Using intact membranes, concentration-response curves of all peptide CRFR1 agonists studied were clearly biphasic, resulting in two EC50 values corresponding to high potency and low potency responses around EC50(h) 5 × 10−11 m and EC50(l) 3 × 10−9 m, respectively (Fig. 1 and Table I). On average, low and high potencies differed in their EC50 values by 64.2 ± 9.3-fold, and the part of high potency response was calculated to be 27.76 ± 1.54% of the maximum activity for the sum of both response phases at 100 pm tracer (from all peptides, n = 21). All agonists stimulated the binding to the same maximum activity (Fig. 2). When the concentration of [35S]GTPγS was increased from 100 to 1,000 pm, the EC50 values for sauvagine did not significantly change; however, the part of high potency phase increased from 27.8% to more than 50% (inset in Fig. 1), at a reduced stimulation over basal of 35.2% compared with about 105% at 100 pm tracer.

The antagonist α-helical CRF(9–41) showed no activity on its own (Figs. 1 and 2) but competitively antagonized the stimulated binding as shown for sauvagine in Fig. 3. The Schil plot (not shown) from these data resulted in Schil constants Ks (1) of 9.21 × 10−9 m (± 2.98 × 10−7) and Ks (2) of 4.77 × 10−9 m (± 1.25 × 10−10) for the high and low potency responses, respectively, which showed that the compound antagonized both responses equally.

As expected from the biphasic concentration-response curves obtained with the membranes, CRFR1 did not couple only to Goα protein as generally functionally observed. Anti-Goα, anti-Goα, and anti-Goα antibodies precipitated solubilized [35S]GTPγS-bound protein obtained from the membranes in significantly higher amounts after stimulation with sauvagine as well as compared with native HEK cell membranes (Fig. 4).

**Affinity and Dissociation of [35S]GTPγS Binding to HEK-rCRFR1 Cell Membranes—GTPγS binding isotherms for basal binding and binding stimulated by sauvagine were fitted according to a one-site and two-site binding model, respectively (Fig. 5A and Table II).** The results show clearly that sauvagine stimulated binding by increasing the apparent affinity of the nucleotide binding site to GTPγS by more than 10-fold (Kd 5 × 10−10 m). This increase in affinity should not only be the result of accelerated dissociation of GDP from the binding site but also because of a real increase in affinity of the site because even in the absence of GDP the affinity was increased by receptor stimulation (data not shown). Nevertheless, the nonstimulated binding sites had a rather high affinity (Kd about 1 × 10−8 m) discriminating them from any nonspecific binding. It must be noted that the parameters given in Table II (including those after pretreatment of the cells with PTX, see below) are, although exact with respect to the models used, rather rough because the continuous displacement curves (Fig. 5) did not allow a clear definition of nonspecific binding for calculations. For this reason, the nonspecific binding had to be tested to give good fits. More realistically, the binding curves seem to reflect a continuum of several binding sites, which is deduced further from the dissociation experiments (Fig. 6). Dissociation
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TABLE I

Concentration-response curves as given in Fig. 1 (untreated cells) were fitted according to a two-site response model, resulting in EC_{50}(h) and EC_{50}(l) for the high and low potency coupling to G_s and G_i proteins, respectively. Results are expressed as the mean ± S.E. of more than three independent experiments performed in triplicate.

| Ligand       | EC_{50}(h) | EC_{50}(l) | EC_{50}(l)/EC_{50}(h) |
|--------------|------------|------------|------------------------|
| Urocortin    | 9.98 × 10^{-12} ± 2.87 × 10^{-12} | 6.10 × 10^{-10} ± 1.73 × 10^{-10} | 61.2 |
| Urotensin I  | 2.56 × 10^{-11} ± 5.21 × 10^{-12} | 1.44 × 10^{-9} ± 5.76 × 10^{-10} | 56.0 |
| Sauvagine    | 3.15 × 10^{-11} ± 7.50 × 10^{-12} | 1.40 × 10^{-9} ± 3.86 × 10^{-10} | 44.5 |
| 3-1-Tyr^b,Gln^i-sauvagine | 3.24 × 10^{-11} ± 7.08 × 10^{-12} | 3.23 × 10^{-9} ± 4.76 × 10^{-10} | 99.7 |
| rhCRF       | 4.89 × 10^{-11} ± 6.72 × 10^{-12} | 4.02 × 10^{-9} ± 1.26 × 10^{-10} | 82.2 |
| oCRF        | 1.50 × 10^{-11} ± 1.01 × 10^{-10} | 6.20 × 10^{-9} ± 1.40 × 10^{-9} | 41.2 |

Fig. 2. Maximum stimulation over basal of [^{35}S]GTP\_S binding to HEK-rCRFR1 cell membranes by CRF receptor ligands. Data were estimated from the concentration-response curves as given in Fig. 1. Data are the means ± S.D. calculated from a series of more than three curves; data for PTX-treated cells were combined from all curves obtained (three for sauvagine and each one for the other agonists).

Fig. 3. Effect of \( \alpha \)-helical CRF(9-41) on the concentration-response curves for sauvagine-stimulated binding of [^{35}S]GTP\_S to HEK-rCRFR1 cell membranes. The response curves were determined in the absence and presence of fixed concentrations of \( \alpha \)-helical CRF(9-41), using 11 \( \mu \)g of protein and 113 pm [^{35}S]GTP\_S. For incubation conditions, see Fig. 1. Data points represent the mean ± S.D. of triplicate experiments.

Fig. 4. Immunoprecipitation of [^{35}S]GTP\_S-bound G_{\alpha_s}, G_{\alpha_i}, and G_{\alpha_{21}} subunits in solubilized membranes obtained from native HEK and HEK-rCRFR1 cells. The membranes were incubated with [^{35}S]GTP\_S in the absence (basal) and presence of 1 \( \mu \)M sauvagine (stimulated) and solubilized (for conditions, see “Experimental Procedures”). The G_{\alpha_s}[^{35}S]GTP\_S complexes were immunoprecipitated using antibodies directed against G_{\alpha_s}, G_{\alpha_i}, and G_{\alpha_{21}}, and the [^{35}S]GTP\_S activities in the precipitates were determined. Included are data gained with membranes obtained from HEK-rCRFR1 cells after treatment with PTX or sauvagine for 24 h at 37 °C. Data are shown as the mean ± S.E. of the values for the stimulation over basal for at least three separate experiments carried out at least twice. Statistically significant changes in [^{35}S]GTP\_S binding caused by sauvagine are indicated as *, \( p < 0.05 \); significant changes in the stimulation of the binding after cell treatment with sauvagine or PTX as \( p < 0.05 \).

pretreatment of the cells, known to inactivate G_i proteins, totally abolished the low potency response to all peptides seen with untreated cell membranes (Fig. 1), and the curves could only be fitted according to a normal one-site fit. At 100 pm [^{35}S]GTP\_S, the remaining activity comprised 23.9 ± 0.94% (Fig. 2) of that obtained with untreated cells (from three experiments with sauvagine and each one with the other peptides), which closely corresponded to 27.76% activity as found for the high potency response with untreated cell membranes. These results suggested that the low potency PTX-sensitive phase represented coupling to G_i, the high potency phase coupling to G_s. PTX treatment also abolished the sauvagine-evoked increase of G_{\alpha_s}-[^{35}S]GTP\_S immunoprecipitate, but not that of G_{\alpha_i} and G_{\alpha_{21}} (Fig. 4).

From GTP\_S binding isotherms as shown in Fig. 5 it was determined that of [^{35}S]GTP\_S from basal and stimulated binding sites proceeded similarly, but the basal activity dissociated more rapidly. The curves could not be fitted to simple models, and, furthermore, after a relatively rapid dissociation phase about 70% of occupied sites remained dissociating only very slowly after more than 2 h.

Influence of Pretreatment of HEK-rCRFR1 Cells with PTX on the rCRFR1-activated [^{35}S]GTP\_S Binding to Their Membranes and to Different G\_s Subunits in the Membranes—PTX
calculated that the affinity of the stimulated binding sites after PTX pretreatment of the cells was decreased from \( K_{d1} 5 \times 10^{-10} \) to \( 1.25 \times 10^{-9} \) M and that the amounts of stimulated binding sites before and after PTX treatment did not significantly differ (\( B_{\text{max1}} 1.90 \) and \( 2.07 \) pmol/mg protein, respectively, Table II). The last fact may be explained by the limitations of the fits of the binding curves (Fig. 5) as discussed above.

Influence of Long Term Incubation of HEK-rCRFR1 Cells with Sauvagine on the rCRFR1-activated \[^{35}\text{S}]\text{GTP}\gamma\text{S} Binding to Their Membranes and to Different Gα Subunits in the Membranes—Incubation of the cells for 24 h with 1 \( \mu \)M sauvagine and extensive washing over 2 h to allow for the washout of the peptide from 1 \( \mu \)M to at least 1 pm, totally desensitized the high potency phase of the sauvagine-stimulated \[^{35}\text{S}]\text{GTP}\gamma\text{S} binding but left the low potency \( G\gamma \)-coupled phase unchanged (Fig. 7). In this series of experiments (\( n = 7 \)) the potencies of sauvagine in stimulating monophosphatically the binding of \[^{35}\text{S}]\text{GTP}\gamma\text{S} to membranes obtained from sauvagine-pretreated cells and in stimulating the low potency binding to membranes obtained from control cells were identical (\( EC_{50} 3.47 \times 10^{-9} \pm 3.54 \) versus \( EC_{50}(l) 3.70 \times 10^{-9} \pm 1.32 \times 10^{-9} \)). The combined treatment of the cells with sauvagine and PTX abolished the whole stimulation (Fig. 7). Sauvagine treatment also significantly diminished sauvagine-evoked increase of \( G\alpha_{i1} \) and \( G\alpha_{q11} \).
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The inhibition curves given in Fig. 5 were fitted according to a one-site and two-site model for the basal and stimulated binding, respectively. Given are the $K_d$ (m) and $B_{max}$ (pmol/mg of protein) as the mean ± S.E. from three experiments. $K_{f1}$ and $B_{max1}$ refer to the stimulated part of the binding isotherms in presence of sauvagine.

| Pretreatment | $K_d$ (m) | $K_{f1}$ (m) | $K_{f2}$ (m) | $B_{max}$ (pmol/mg) | $B_{max1}$ (pmol/mg) | $B_{max2}$ (pmol/mg) |
|--------------|-----------|--------------|--------------|--------------------|----------------------|--------------------|
| None         | 6.52 × 10^{-9} ± 1.20 × 10^{-9} | 5.00 × 10^{-10} ± 1.68 × 10^{-10} | 2.59 × 10^{-8} ± 1.13 × 10^{-8} | 13.17 ± 0.75 | 1.90 ± 0.15 | 18.04 ± 3.00 |
| PTX          | 1.06 × 10^{-8} ± 1.53 × 10^{-8} | 1.25 × 10^{-9} ± 4.71 × 10^{-11} | 3.74 × 10^{-8} ± 1.02 × 10^{-8} | 15.18 ± 2.99 | 2.07 ± 0.19 | 24.81 ± 2.63 |

Fig. 6. Dissociation kinetics of [35S]GTPγS binding to HEK-rCRFR1 cell membranes. About 100 pm [35S]GTPγS was incubated with the membranes at 25 °C for 90 min in binding medium without (basal) and with 0.1 μM sauvagine (stimulated). Dissociation was initiated after centrifuging the membranes from the chilled preincubations by resuspending them with 1 μM GTPγS at 25 °C. Data points represent the percentage of remaining bound activity (mean ± S.D. of triplicate experiments).

Go$_2$-bound [35S]GTPγS (Fig. 4), and increased the stimulatory activity of sauvagine on the activity of the adenylate cyclase at low ligand concentrations (Fig. 9). In addition, when it was found that long term stimulation of the cells with sauvagine abolished specifically the high potency phase (Fig. 7) it was shown in parallel that the amount of immunoprecipitated Go$_2$-bound [35S]GTPγS stimulated by sauvagine was diminished almost to the level seen with native, nontransfected HEK cell membranes (Fig. 4), and, furthermore, the adenylate cyclase was no longer stimulated (Fig. 9, inset). On the contrary, PTX abolished the low potency phase (Fig. 1) and the stimulation of immunoprecipitated Go$_2$-bound [35S]GTPγS by sauvagine (Fig. 4) totally. Therefore it is concluded that the rCRFR1 is coupled to Gi with low ligand potency in addition to Gs with high potency (Table I), which should mean that the ligand concentration has a major regulatory function in the coupling of the receptor to different G proteins. Because the portion of Gi coupling was found to be enhanced when the [35S]GTPγS concentration was increased (Fig. 1, inset), it is further concluded that cellular GTP is not only substrate for the G proteins but, in addition to the ligand concentration, also regulates the portions of the different G proteins actually coupled to the receptor. The present results parallel in some respects those found for the activation of the h-5-hydroxytryptamine$_{1A}$ receptor in Chinese hamster ovary cells where biphasic response curves for the full agonist-stimulated [35S]GTPγS binding were also found (22). In the latter case, however, the multiple G protein subtypes involved in activation were restricted to the G$_i$ class, of which a single subunit G$_{ai3}$ was the sole component involved in the high potency phase.

In Fig. 7 Influence of pretreatment of HEK-rCRFR1 cells with sauvagine on [35S]GTPγS binding to their membranes. HEK-rCRFR1 cells were treated for 24 h without and with 1 μM sauvagine or 1 μM sauvagine together with 100 ng/ml PTX, and with PTX alone before membrane preparation. About 10 μg of the membranes were incubated in binding medium (see “Experimental Procedures”) with 100 pm [35S]GTPγS and increasing concentrations of sauvagine at 25 °C for 2 h. Data points were normalized with the maximum and basal response of the control membranes taken 100% and 0%, respectively, and represent the mean ± S.D. of triplicate experiments. Curves were fitted according to a two-site and one-site response model for untreated and sauvagine or PTX-treated cells, respectively.
coupled to G proteins. This was in line with the splitting of receptor binding sites into about 98% low and 2% high affinity sites. Taken together it is concluded that the low number of high affinity receptor sites evokes the high potency activation of Gs protein, whereas a small number of the low affinity sites couples to Gi.

Because the [35S]GTP<sub>S</sub> assay per se does not differentiate among G protein subtypes, CRFR1 coupling to G proteins other than G<sub>s</sub> and G<sub>i</sub> could also be involved. Indeed, after receptor activation G<sub>q/11</sub>-bound [35S]GTP<sub>S</sub> was immunoprecipitated in higher amounts, which were not significantly lowered after PTX pretreatment of the cells (Fig. 4). In line with these results, sauvagine stimulated the accumulation of inositol phosphates in HEK-rCRFR1 cells (Fig. 10). The α subunits of the G<sub>q/11</sub> subfamily have been shown to activate phospholipase C isozymes and to stimulate the inositol phosphate production in a PTX-independent way (23); however, about half of the sauvagine-stimulated production in the HEK-rCRFR1 cells was inhibited after pretreatment of the cells with PTX (Fig. 10). Some receptors were found to activate phospholipase Cβ isozymes also through G<sub>q/11</sub> dimers released from heterotrimer G<sub>q/11</sub> proteins (23, 24). Obviously, the coupling of CRFR1 in the HEK-rCRFR1 cells to G<sub>i</sub> proteins leads to the release of βγ subunits that contribute to the stimulation of phospholipase C in a PTX-sensitive manner. This result is contrary to findings showing that G proteins other than G<sub>q/11</sub> were not involved in urocortin-induced mitogen-activated protein kinase in HEK-rCRFR1 cells, thought to be mediated by the phospholipase C/inositol phosphate signaling pathway (14).

The great differences in ligand potencies for the G<sub>s</sub> and G<sub>i</sub> coupling, EC<sub>50</sub> around 5 × 10<sup>-11</sup> and 3 × 10<sup>-9</sup> M (Table I), respectively, suggest that different active receptor states are
Fig. 9. Concentration-response curves for activation/inhibition of adenylate cyclase activity by sauvagine in membranes obtained from HEK-rCRFR1 cells without and after pretreatment with PTX or sauvagine. About 20 μg of membrane protein was incubated in duplicate in a reaction mixture consisting of 50 mM Tris-HCl, pH 7.4, 4 mM MgCl₂, 2 mM EDTA, 1 mM isobutylmethylxanthine, 1 mM cAMP, 100 μM ATP, 10 μM GTP, 1 mM DTT, 0.75 μCi of [α-32P]ATP, 5.1 mg/ml phosphocreatine, 1.32 mg/ml creatine phosphokinase, and 12 mg/ml bovine serum albumin in a final volume of 100 μl at 32 °C for 20 min. [α-32P]cAMP formed was isolated by sequential chromatography. Data are the means ± S.D. Compared were the activities of membranes obtained from cells without and after pretreatment with 200 ng of PTX/ml and, in another set of experiments, without and after pretreatment with 1 μM sauvagine (inset), each for 24 h at 37 °C.

Fig. 10. Sauvagine-stimulated accumulation of inositol phosphates in HEK-rCRFR1 cells without and after pretreatment with PTX. Cells (100,000/24-well plate) were preloaded with myo-[2-3H]inositol for 20 h at 37 °C in culture medium in the absence and presence of 200 ng/ml PTX, washed, and stimulated for 60 min with increasing sauvagine concentrations in culture medium containing additionally 10 mM LiCl. After lysis of the washed cells, 3H-labeled inositol phosphates were separated by Dowex chromatography. The accumulation of labeled inositol phosphates is expressed as the percentage of 3H activity (mean ± S.D.) separated by chromatography from the total activity taken up by the cells.

The mechanism behind the differentiated homologous desensitization of the G protein coupling of CRFR1 remains to be resolved. It has been well established that GPCR kinases (GRKs) play a major role in this process. GRKs phosphorylate serine and threonine residues at intracellular domains of the agonist-activated receptor. This phosphorylation interferes with the G protein coupling of the receptor and promotes the

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interaction of the receptor with intracellular proteins that maintain the inactive state of the receptor and favor its internalization (31, 32). Y-79 and IMR-32 cells were shown to respond to CRFR1 activation with loss of receptors (28, 30). Furthermore, CRFR1 was rapidly phosphorylated in response to high CRF concentration in COS-7 cells (33). This phosphorylation was independent of protein kinase A activation, but in Y-79 cells an up-regulation of GRK3 was observed during desensitization of CRFR1 (26). Therefore, a GRK-mediated mechanism is likely to be involved also in the desensitization of CRFR1. In the last years experimental data have accumulated (34–36) to suggest that different active conformational states of one and the same receptor exist and may have differing abilities to produce diverse signaling ways. Based on this concept (37), from our results it may be speculated that activation of the CRFR1 in HEK cells results in receptor states that activate Gs and Gq/11 proteins and are subject in parallel to a mechanism that ensures a more rapid decline of the stimulatory effect on the adenylate cyclase may represent a regulatory mechanism that ensures a more rapid decline of the stimulatory effect when at the same time the inhibitory activity, not involved in desensitization, remains unchanged.

Acknowledgments—We thank U. B. Kaupp (Julich, Germany) for the gift of rat CRFR1 cDNA and M. Georgi, G. Vogelreiter, and A. Klose for technical assistance.

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*J. Biol. Chem. 2004, 279:38386-38394.*
doi: 10.1074/jbc.M405335200 originally published online July 12, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M405335200

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