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Molecular Mechanisms of Corticotropin-Releasing Factor Receptor-Induced Calcium Signaling

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ABSTRACT

The molecular mechanisms governing calcium signal transduction of corticotropin-releasing factor (CRF) receptors CRF_1 and $CRF_{2(a)}$ stably expressed in human embryonic kidney (HEK) 293 cells were investigated. Calcium signaling strictly depended on intracellular calcium sources, and this is the first study to establish a prominent contribution of the three major G-protein families to CRF receptor-mediated calcium signaling. Overexpression of $G\alpha_{q/11}$ and $G\alpha_{16}$ led to leftward shifts of the agonist concentration-response curves. Blockade of $G\alpha_{q/11}$ proteins by the small interfering RNA (siRNA) technology partially reduced agonist-mediated calcium responses in CRF_1- and $CRF_{2(a)}$-expressing HEK293 cells, thereby proving a contribution of the G_q protein family. A small but significant inhibition of calcium signaling was recorded by pharmacological inhibition of $G_{i/o}$ proteins with pertussis toxin treatment. This effect was mediated by direct binding of $G\beta\gamma$ subunits to phospholipase C. $G_{i/o}$ inhibition also elevated cAMP responses in CRF receptor-overexpressing HEK293 cells and in Y79 retinoblastoma cells endogenously expressing human CRF_1 and $CRF_{2(a)}$ receptors, thereby demonstrating natural coupling of G_i proteins to both CRF receptors. The strongest reduction of CRF receptor-mediated calcium mobilization was noted when blocking the G_s signaling protein either by cholera toxin or by siRNA. It is noteworthy that simultaneous inhibition of two G-proteins shed light on the additive effects of G_s and G_q on the calcium signaling and, hence, that they act in parallel. On the other hand, G_i coupling required prior G_s activation.

Corticotropin-releasing factor (CRF) and its related analogs urocortins 1 to 3 (UCN1–3) control a wide range of central and peripheral physiological effects, including neuroendocrine, autonomic, and behavioral responses to stress, in mammals and vertebrate species. CRF peptides interact with two CRF receptors, CRF_1 and CRF_2 (Perrin and Vale, 1999), which are ~70% homologous and belong to the class B1 subfamily of G-protein-coupled receptors (Dautzenberg and Hauger, 2002).

Despite their high sequence homology, the CRF_1 and the three functional splice variants $CRF_{2(a\text{-}c)}$ of the CRF_2 receptor differ in their ligand selectivity profile (Kostich et al., 1998; Perrin and Vale, 1999; Dautzenberg et al., 2001). Although all species homologs of CRF, UCN1, and the non-mammalian CRF peptides sauvagine and urotensin I bind and activate the CRF_1 receptor, UCN2 and UCN3 show no physiological relevant affinity for this receptor (Vaughan et al., 1995; Lewis et al., 2001; Reyes et al., 2001). In contrast, the CRF_2 receptor variants display high affinity for UCN1–3, sauvagine, and urotensin I but substantially lower affinity (10- to 200-fold) for human/rat (h/r) and ovine (o) CRF (Dautzenberg et al., 2001). This differential ligand-selectivity profile of the two CRF receptors has largely contributed to their characterization in vitro and to a lesser extent in vivo. CRF_1 and CRF_2 receptors mainly couple to the stimulatory G_s protein, which activates adenylate cyclases and subsequently the cAMP second-messenger cascade (Dautzenberg et al., 2000). We and others have reported that, despite their coupling to G_s, CRF receptors also stimulate transient calcium (Ca^{2+}) mobilization in certain cell types by phospholipase C (PLC) activation in vitro (Dautzenberg et al., 2004) and protein kinase C activation in vivo (Blank et al., 2003). PLC activation through G-protein-coupled receptors is generally

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ABBREVIATIONS: CRF, corticotropin-releasing factor; 2-APB, 2-aminoethoxyphenyl borate; CTX, cholera toxin; GRK2ct, carboxyl terminus of the G-protein kinase 2; IP_3R, inositol 1,4,5 triphosphate receptor; PKA, protein kinase A; PLC, phospholipase C; PTX, pertussis toxin; UCN, urocortin; ANOVA, analysis of variance; HEK, human embryonic kidney; siRNA, small interfering RNA; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; bp, base pair; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PBS, phosphate-buffered saline; BSA, bovine serum albumin; QT-PCR, quantitative reverse transcriptase-polymerase chain reaction; PBS-T, phosphate-buffered saline/Tween 20.

believed to be mediated by coupling to the G_q protein, but the involvement of the $G\beta\gamma$ subunits of the inhibitory G_i and G_o proteins has been reported as well (Rhee, 2001).

In a previous study (Dautzenberg et al., 2004), we reported on the ability of the two CRF receptors $hCRF_1$ and $hCRF_{2(a)}$ to mobilize Ca^{2+} in recombinant HEK293 cells but not in recombinant SK-N-MC neuroblastoma cells. This sheds light on the importance of the cellular background for the specificity of CRF receptor signaling. In this study, we elucidated the contribution of the various G-protein components in CRF-stimulated Ca^{2+} mobilization in HEK293 cells stably overexpressing $hCRF_1$ and $hCRF_{2(a)}$ receptors. Besides the contribution of G_q and $G_{i/o}$ proteins, we show for the first time a critical and robust contribution of G_s proteins in CRF receptor-mediated Ca^{2+} signal transduction.

Materials and Methods

Materials, Peptides, and Reagents. All cell culture media and reagents were purchased from Invitrogen (Merelbeke, Belgium). All peptides and chemicals were obtained from Sigma-Aldrich (Bornem, Belgium). Pertussis toxin was obtained from Calbiochem (Nottingham, UK).

Cell Culture. The stable $hCRF_1$-HEK and $hCRF_{2(a)}$-HEK cell lines were established as described previously (Dautzenberg et al., 2004). Cells were kept in DMEM supplemented with 10% fetal bovine serum (FBS) and 500 μg/ml Geneticin as selection marker (Invitrogen). The Y79 retinoblastoma cells (HTB-19; American Type Culture Collection, Manassas, VA) were grown as suspension culture in RPMI 1640 medium (Invitrogen) supplemented with 15% FBS as described previously (Dautzenberg and Hauger, 2001).

RNA Isolation and cDNA Synthesis. RNA was isolated from nontransfected HEK293B cells, and the stably transfected $hCRF_1$-HEKand $hCRF_{2(a)}$-HEK cells with the RNeasy kit from Qiagen (Venlo, The Netherlands). First-strand cDNA was prepared using 1 μg of total RNA with Superscript II (Invitrogen) according to the manufacturer's protocol.

Semiquantitative PCR. A fragment encoding 511 bp of the $G\alpha_{16}$ cDNA (NM_002068, nucleotides 713-1223) was amplified with following primer pairs: $G\alpha_{16fwd}$ (5′-GGGAATTCCACCTGCTCGA-3′), and $G\alpha_{16rev}$ (5′-CCGGTGTACATCCTCGTGTA-3′). Human thymus first-strand cDNA (Clontech, Westburg, The Netherlands) was used as a positive control for $G\alpha_{16}$ expression in native tissue. $G\alpha_q$ cDNA (NM_002072, nucleotides 888-1115) amplification was conducted with the following primers: $G\alpha_{qfwd}$ (5′-ATCATGTATTCCCATCTAGTCG-3′), and $G\alpha_{qrev}$ (5′-CAGATTGTACTCCTTCAGGT-3′) to obtain a 228-bp fragment. $G\alpha_q$ and $G\alpha_{16}$ cDNA fragments were amplified for 30 and 35 cycles, respectively. A 395-bp fragment of the Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA (NM_002046, nucleotides 208–602) was amplified for 20 cycles with the different cDNAs using the following primer pairs: $GAPDH_{fwd}$ (5′-CCTTCATTGACCTCAACTAC-3′), and $GAPDH_{rev}$ (5′-TGTCATGGATGACCTTGG-3′). The fragments were amplified with Taq polymerase (Invitrogen) under the following conditions: denaturation at 94°C for 20 s, annealing at 55°C for 30 s, and elongation at 72°C for 1 min.

cAMP Assays. $hCRF_1$-HEK and $hCRF_{2(a)}$-HEK cells were rinsed with PBS, detached with enzyme-free cell dissociation buffer (Invitrogen), and resuspended in assay buffer (Hanks' buffered salt solution supplemented with 1 mM 3-isobutyl-1-methylxanthine, 10 mM $MgCl_2$, 5 mM HEPES, and 0.1% BSA). Forty thousand cells were plated in 96-well black plates (Costar; Corning Life Sciences, Acton, MA). Cells were incubated for 30 min at 22°C with CRF receptor agonists and lysed following the Homogenous Time Resolved Fluorescence cAMP Dynamic kit protocol (Cis Bio International, Bagnol-sur-Ceze, France) for the determination of cAMP production. Fluorescence resonance energy transfer was measured in a Discovery reader (PerkinElmer Life and Analytical Sciences, Zaventem, Belgium). For the siRNA experiments, the cells were transfected under the same conditions in 10-cm dishes, coated with poly(D-lysine), and cAMP was measured in 384-well black plates with the Dynamic 2 kit (Cis Bio International) in 5000 cells.

Transient Transfection of the α-Subunits of $G\alpha_q$ and $G\alpha_{16}$. The transfection mix was prepared with 0.1 μg of plasmid DNA encoding either $G\alpha_q$ or $G\alpha_{16}$ in the pcDNA3 vector in 25 μl of Optimem medium and 20 μl/ml Lipofectamine 2000 (Invitrogen) in 25 μl of OptiMEM (Invitrogen). The reaction mixture was incubated for 15 min in 96-well plates. Finally 30,000 cells in 50 μl of growth medium without antibiotics were plated per well and incubated for 4 h at 37°C and 5% CO_2. The transfection medium was then removed and replaced with normal growth medium until the cells were prepared for Ca^{2+} measurements.

Knockdown of $G\alpha_q$ and $G\alpha_s$ Proteins with siRNA. $hCRF_1$- and $hCRF_{2(a)}$-HEK cells were transfected with 100 nM siRNA and 10 μl/ml RNAiMAX (Invitrogen) in a final volume of 50 μl of OptiMEM. Ten thousand cells in 50 μl of medium without antibiotics were added to the transfection mix. After 4-h incubation at 37°C and 5% CO_2, the medium was removed and replaced with fresh growth medium and incubated for 72 h before Ca^{2+} measurements in the fluorimetric imaging plate reader. The scrambled siRNA AllStars Negative control (Qiagen) was transfected under the same conditions and used as a control. Silencer Predesigned siRNA (16704; Ambion, Lennik, Belgium) was used as control for $G\alpha_s$ activity. The siRNA 5′-AAGAUGUUGGUGGACCUGAAC-3′ (Eurogentec, Seraing, Belgium) located in the vicinity of the start codon, as reported previously, was used against $G\alpha_{q/11}$(Barnes et al., 2005; Atkinson et al., 2006).

Western Blot. After treatment, the cell culture medium was removed, and the cells were rinsed shortly with cold PBS without Ca^{2+} and Mg^{2+}, lysed with 30 μl of Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Erembodegem, Belgium) containing a protease inhibitor cocktail (Thermo Fisher Scientific). The lysates were sonicated on ice for 1 min and centrifuged for 10 min at 10,000*g* at 4°C in an Eppendorf tabletop 5417R centrifuge (Eppendorf AG, Hamburg, Germany). The supernatant fraction was transferred to a fresh tube, and the amount of protein was determined by the Bio-Rad Protein assay kit (Bio-Rad Laboratories, Hercules, CA). For each sample, 3 μg of protein in 10-μl sample buffer was loaded on Novex NuPAGE 4–12% Bis-Tris Gel (Invitrogen) and blotted on a nitrocellulose membrane with the I-Blot Machine (Invitrogen) according to the manufacturer's instructions. Membranes were washed with PBS-T [PBS containing 0.05% (v/v) Tween 20], blocked with PBS-T containing 3% BSA, and incubated overnight at 4°C with gentle agitation in the presence of primary antibody targeting $G\alpha_s$ (Upstate, Huissen, The Netherlands) or $G\alpha_{q/11}$ (Santa Cruz Biotechnology, Huissen, The Netherlands) at a dilution of 1:1000. Primary antibodies were detected using an horseradish peroxidase-labeled secondary antibody (GE Healthcare, Brussel, Belgium) at a dilution of 1:10,000. The signal was detected with the chemiluminescence horseradish peroxidase kit (Thermo Fisher Scientific), and the signals were captured on a Lumi-imager (Roche, Vilvoorde, Belgium). As control, the same blots were stripped with 200 mM NaOH for 5 min, washed three times with PBS-T, blocked with 3% BSA in PBS-T, and incubated with the antibody for β-actin at a dilution of 1:10,000 (Calbiochem, Nottingham, UK) overnight at 4°C. Protein levels of β-actin, as control, were then detected with the anti-mouse antibody at a dilution 1:10,000 (GE Healthcare).

Calcium Measurements. HEK293 cells stably expressing $hCRF_1$ and $hCRF_{2(a)}$ receptors at a confluence of 60 to 70% were seeded at a density of 75,000 cells into poly(lysine)-coated 96-well black-well clear-bottomed microtiter plate (Costar; Corning Life Sciences). The following day, cells were washed with DMEM and labeled for 1 h with loading medium containing Fluo-3/acetoxymethyl ester (DMEM without FBS, supplemented with 10 mM HEPES, 0.1%

BSA, 2.5 mM probenecid, and 2 μM Fluo-3/acetoxymethyl ester) (Invitrogen). Cells were washed once with assay buffer (5 mM HEPES, 140 mM NaCl, 1 mM $MgCl_2$, 5 mM KCl, 10 mM glucose, 2.5 mM probenecid, and 1.25 mM $CaCl_2$ at a pH of 7.4) and tested after 20-min incubation at 37°C. The agonists, dissolved in assay buffer, were added to the cells, and the change of fluorescence was monitored in the Fluorimetric imaging plate reader (Hamamatsu Photonics, Tokyo, Japan).

Quantitative PCR. Quantitative reverse transcriptase-PCR (QT-PCR) was performed on an ABI Prism 7700 cycler (Applied Biosystems, Foster City, CA) using qPCR Core Kit without dUTP (Eurogentec). The Taqman inventoried assays Hs00183449_m1, Hs00199754_m1, and Hs00275279_m1 (Applied Biosystems, Warrington, UK) were used for the determination of mRNA of Epac1, Epac2, and PLCε, respectively. The Taqman endogenous control assay was used to quantify the expression of the GAPDH and β-actin (Applied Biosystems). Serial dilutions of human kidney cDNA, human brain cDNA, and $hCRF_1$-HEK cells cDNA were used to generate standard curves for the threshold cycles for Epac1, Epac2, and PLCε. A linear regression line calculated from the standard curve allowed the determination of the expression of the different targets in the different cell lines.

Data Analysis. Calcium responses are expressed as the percentage of responses obtained in the treated cells compared with the full response (E_{max}) obtained in control cells (no treatment) or in mock-transfected cells. For statistical and graphical analysis, the Prism software package was used (GraphPad Software, Inc., San Diego, CA). Statistical analyses of the various experimental settings were achieved by one- and two-way analysis of variance (ANOVA). The post hoc analysis were performed using the Bonferroni test.

Results

Influence of Intra- and Extracellular Ca^{2+} on CRF Receptor-Mediated Ca^{2+} Mobilization in $hCRF_1$ and $hCRF_{2(a)}$ Receptor-Expressing HEK293 Cells. In a first experimental setting, we addressed the question of whether the cytoplasmic Ca^{2+} signal after CRF receptor stimulation involved intracellular Ca^{2+} stores or Ca^{2+} influx from the extracellular environment. In cells derived from hamster or human skin, a critical contribution of extracellular Ca^{2+} has been reported when the cells were stimulated with CRF agonists (Fazal et al., 1998; Wiesner et al., 2003). We were therefore interested to determine whether the cytoplasmic Ca^{2+} signal after application of a CRF receptor agonist to the $hCRF_1$ and $hCRF_{2(a)}$ receptor-expressing HEK293 cells depends on intracellular Ca^{2+} stores or on Ca^{2+} influx from the extracellular environment. To this end, the cells were stimulated with a submaximal (EC_{80}) concentration (300 nM) of the CRF_1/CRF_2 equipotent agonist sauvagine. The subsequent Ca^{2+} mobilization was recorded in the absence or presence of 2 mM concentration of the Ca^{2+} chelator EGTA to obtain full depletion of extracellular Ca^{2+}. In the presence of EGTA, the measured Ca^{2+} mobilization was indistinguishable from the control responses (Fig. 1, A and B). However, when cells were incubated with 1 μM thapsigargin, a sarcoendoplasmic reticulum Ca^{2+}-ATPase inhibitor, to deplete intracellular Ca^{2+} stores, agonist stimulation failed to induce a Ca^{2+} mobilization (Fig. 1, A and B).

Mainly two types of intracellular receptor Ca^{2+} channels, IP_3 receptors (IP_3R), and ryanodine receptors mediate intracellular Ca^{2+} mobilization (Sitsapesan et al., 1995; Patel et al., 1999). Earlier (Dautzenberg et al., 2004), we demonstrated that ryanodine receptors are not involved in CRF agonist-mediated Ca^{2+} mobilization. To show the direct involvement of the IP_3Rs, HEK293 cells stably expressing $hCRF_1$ and $hCRF_{2(a)}$ receptors were incubated with 100 μM concentration of the IP_3R antagonist 2-aminoethoxydiphenyl borate (2-APB). 2-APB treatment almost completely abolished sauvagine-mediated Ca^{2+} mobilization: the remaining mobilization was 11.6 ± 4.0 and 13.7 ± 4.6% of the control response for $hCRF_1$- and $hCRF_{2(a)}$-HEK cells, respectively (Fig. 1, A and B), demonstrating the critical contribution of the IP_3Rs in CRF receptor-mediated Ca^{2+} signal transduction.

Role of the Different α-Subunits of the G_q Family. The G_α subunits of the G_q family are known as the main G proteins mediating the activation of PLC-β isoforms and subsequent stimulation of the IP_3Rs, which trigger the release of Ca^{2+} from intracellular stores. $G\alpha_{q/11}$ and $G\alpha_{16}$ are the main members of this family (Hubbard and Hepler, 2006). To elucidate the role of this G protein class, we transiently transfected $hCRF_1$- and $hCRF_{2(a)}$-HEK cells with $G\alpha_q$, $G\alpha_{11}$, and $G\alpha_{16}$ cDNA constructs. Cells were incubated for 48 h at 37°C before the Ca^{2+} assay. No difference in Ca^{2+} mobilization was observed when other CRF agonists were used instead of sauvagine (data not shown). Overexpression of $G\alpha_q$ subunits in $hCRF_1$- and $hCRF_{2(a)}$-HEK cells significantly increased the efficacy and potency of sauvagine in the Ca^{2+} mobilization assay compared with mock-transfected cells (Fig. 2 and Table 1). The EC_{50} value of sauvagine decreased from 146.6 ± 3.3 to 30.3 ± 5.6 nM (CRF_1) and from 119.4 ± 2.9 to 13.0 ± 3.3 nM [$CRF_{2(a)}$], and the response E_{max}

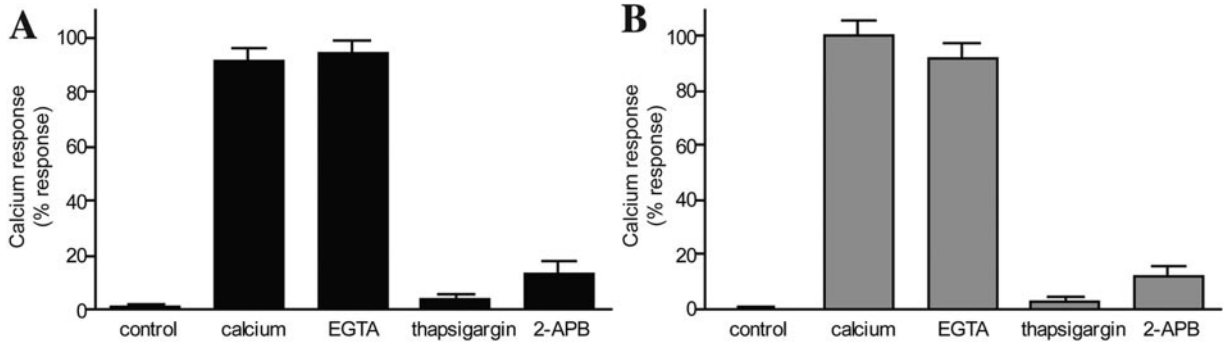

Fig. 1. Effect of intra- and extracellular Ca^{2+} on agonist mediated transient Ca^{2+} mobilization in HEK293 cells stably expressing $hCRF_1$ (A) and $hCRF_{2(a)}$ (B) receptors. The cells were incubated with assay buffer containing 1.25 mM Ca^{2+} as control, 2 mM EGTA, 1 μM thapsigargin, or 100 μM 2-APB for 30 min in the absence of extracellular Ca^{2+} and stimulated with 300 nM sauvagine. The results are expressed as the percentage of Ca^{2+} response in the treated cells in the absence of extracellular Ca^{2+} compared with the control cells in the presence of extracellular Ca^{2+}. The results are the average ± S.E.M. of three independent experiments performed in triplicate.

increased by approximately 25% for both cell lines (Table 1). Overexpression of $G\alpha_{11}$ resulted in similar effects in both cell lines (data not shown). Transfection of the $G\alpha_{16}$ subunit did not modify the efficacy of sauvagine (no significant alterations of the maximal responses were observed); its potency was increased more than 100-fold in both cell lines. EC_{50} values were in the nanomolar (1.3 ± 0.1 nM for CRF_1) and even subnanomolar [0.3 ± 0.05 nM for $CRF_{2(a)}$] range. This potency shift after $G\alpha_{16}$ expression was identical with the one observed in the cAMP stimulation experiments (Dautzenberg et al., 2004), thereby indicating a replacement of the $G\alpha_s$ protein by $G\alpha_{16}$. The overexpression of the $G\alpha$ subunits of the other G-proteins did not change the potency of the Ca^{2+} signal (data not shown).

We next verified whether endogenous $G\alpha_q$ and $G\alpha_{16}$ proteins were expressed at the RNA level in the wild-type and the $hCRF_1$- and $hCRF_{2(a)}$-expressing HEK293 cells. Inputs of cDNA were adjusted to obtain a similar signal for the GAPDH expression. Although $G\alpha_q$ proteins were expressed in all cell lines, no amplification of $G\alpha_{16}$ was measured in HEK293 background. Instead, the expression of $G\alpha_{16}$ was clearly observed in thymus cDNA (Fig. 3), which has been reported to express this gene (Wilkie et al., 1991). To further quantify the contribution of endogenous $G\alpha_q$ and $G\alpha_{11}$ proteins in the Ca^{2+} mobilization assay, we used an siRNA sequence targeting both of them. This approach has been widely described in the literature for successfully knocking down these proteins (Barnes et al., 2005; Atkinson et al., 2006). Remaining protein levels were verified by Western blot and revealed an almost complete knockdown of total $G\alpha_{q/11}$ protein 72 h after transfection (Fig. 4A). Under these conditions, a strong decrease of the sauvagine-mediated Ca^{2+} signal was observed (i.e., a reduction by 54.5 ± 4.4 and 49.0 ± 2.5% in $hCRF_1$- and $hCRF_{2(a)}$-HEK cells, respectively; Fig. 4, B and C), indicating a partial contribution of $G\alpha_q$ proteins in CRF receptor-mediated Ca^{2+} signal.

Effect of Blocking of the $G_{i/o}$ Signaling Pathway. We were interested to identify other G-proteins that might be involved in CRF receptor-mediated Ca^{2+} mobilization. One potential candidate is the G_i protein family, which has been reported to stimulate Ca^{2+} mobilization through binding of the $G\beta\gamma$ subunits to PLC-β (Dorn et al., 1997). Pertussis toxin (PTX) inhibits $G_{i/o}$ signaling through ADP ribosylation of $G\alpha_{i/o}$ and subsequent inhibition of the various $G_{i/o}$ subunits (Katada and Ui, 1982). To analyze the role of the potential coupling of the CRF_1 and $CRF_{2(a)}$ receptors to this family of G-proteins, we incubated the HEK293 cells stably expressing both receptors with 100 ng/ml PTX for 18 h. The sauvagine-mediated Ca^{2+} signal was reduced by 31.6 ± 1.4 and 18.7 ± 0.8% in CRF_1 and $CRF_{2(a)}$, respectively (Fig. 5). Although the inhibition by PTX was rather modest, it was highly significant ($p < 0.01$) in all experiments ($n = 6$). To further substantiate the involvement of $G\beta\gamma$, we transiently transfected $hCRF_1$- and $hCRF_{2(a)}$-HEK cells with a cDNA construct encoding the carboxyl terminus of G-protein kinase 2 (GRK2ct), a well known $G\beta\gamma$ scavenger (Koch et al., 1994). Forty-eight hours after GRK2ct transfection, the cells were tested in the Ca^{2+} mobilization assay. GRK2ct transfection resulted in a significant decrease of the sauvagine-mediated Ca^{2+} signal (i.e., a reduction by 31.9 ± 1.2 and 15.7 ± 1.2% compared with mock-transfected $hCRF_1$ and $hCRF_{2(a)}$-HEK cells, respectively) (Table 2). This reduction was quite similar to the one obtained by PTX treatment. Thus, the small contribution of G_i proteins is likely to be achieved by the $G\beta\gamma$ complex, which directly binds to and stimulates PLC-β isoforms.

Finally, to better understand the coupling of CRF receptors to $G_{i/o}$ proteins, we tested both HEK293 cell lines in cAMP accumulation assays. Eighteen hours of incubation with PTX resulted in a significant increase in sauvagine-mediated cAMP responses in both cell lines (Fig. 6, A and B), thereby demonstrating $G_{i/o}$ coupling capability of both CRF receptors. To further demonstrate that $G_{i/o}$ coupling is not only restricted to recombinant CRF_1 and $CRF_{2(a)}$ receptor expression, the effect of PTX treatment on cAMP accumulation was also investigated in human Y79 retinoblastoma cells, which endogenously express CRF_1 (Hauger et al., 1997) and $CRF_{2(a)}$ receptors (Gutknecht et al., 2008). When Y79 cells were incubated with PTX, an almost 2-fold increase in sauvagine-mediated cAMP responses was observed (Fig. 6C). Thus, it is evident that under native conditions, CRF_1 and $CRF_{2(a)}$ couple to the G_i protein family.

Effect of Blocking the G_s Signaling Pathway. Because the above-described experiments do not argue for the sole contribution of G_i and G_q proteins to CRF receptor-mediated

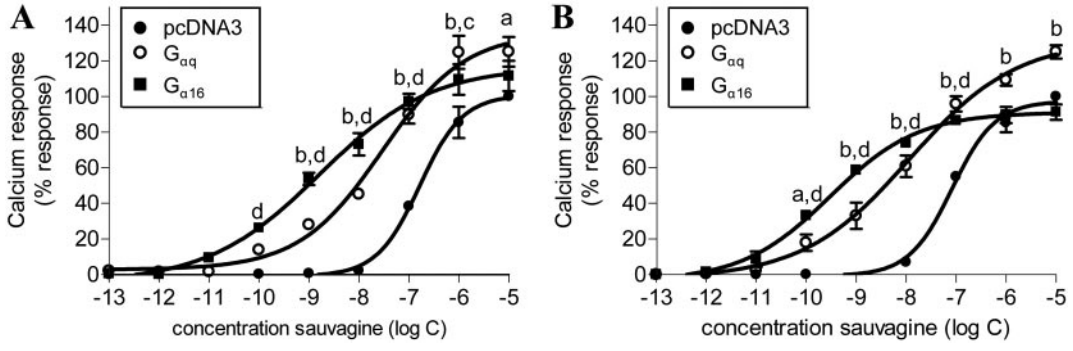

Fig. 2. Effect of transient expression of $G\alpha_q$ and $G\alpha_{16}$ proteins on agonist-mediated Ca^{2+} responses in HEK293 cells stably expressing $hCRF_1$ (A) and $hCRF_{2(a)}$ (B) receptors. Cells were stimulated in a concentration-dependent manner with sauvagine. The results are expressed as the percentage of Ca^{2+} response in cells transfected with $G\alpha_q$ or $G\alpha_{16}$ compared with the mock-transfected cells. The results are the average ± S.E.M. of three independent experiments performed in triplicate. By the two-way ANOVA analysis, there were significant differences between the mock-transfected cells and the cells transfected with $G_{\alpha q}$ ($F = 152$, $p < 0.0001$) and $G\alpha_{16}$ ($F = 207$, $p < 0.0001$) for CRF_1-HEK and $G\alpha_q$ ($F = 207$, $p < 0.0001$) and $G\alpha_{16}$ ($F = 365$, $p < 0.0001$) for $CRF_{2(a)}$-HEK cells. The following post hoc differences were found to be statistically different for efficacious concentrations in mock-transfected cells versus cells transfected with $G\alpha_q$ or $G\alpha_{16}$: $G\alpha_q$ versus pcDNA3: a, $p < 0.01$; $G\alpha_q$ versus pcDNA3: b, $p < 0.001$; $G\alpha_{16}$ versus pcDNA3: c, $p < 0.01$; $G\alpha_{16}$ versus pcDNA3: d, $p < 0.001$.

Ca^{2+} mobilization, we further examined the potential contribution of other G-proteins. Because CRF receptors mainly couple to G_s proteins, we assessed the role of $G\alpha_s$ in $hCRF_1$- and $hCRF_{2(a)}$-HEK cells. Cholera toxin (CTX) irreversibly activates and thereby depletes $G\alpha_s$ protein stores by blockade of the intrinsic phosphodiesterase activity of $G\alpha_s$ (Chang and Bourne, 1989). We first tested the effect of an 18-h CTX treatment on cAMP accumulation in $hCRF_1$- and $hCRF_{2(a)}$-HEK cells after 18-h incubation. The effect of CTX on both cell lines was identical: the basal cAMP levels were increased ~7-fold compared with control cells, and sauvagine failed to further elicit outspoken concentration-dependent increases in cAMP production such as that seen for the control cells (Fig. 7A). We then tested the CTX-treated cells in the Ca^{2+} mobilization assay. Surprisingly, a strong signal reduction in the sauvagine-mediated Ca^{2+} signal [by 59.1 ± 7.6 and 60.3 ± 3.8% in CRF_1- and $CRF_{2(a)}$, respectively] took place (Fig. 7, B and C). This suggests that G_s contributes to the Ca^{2+} signaling of both receptors.

To further support this interpretation, we also used an siRNA approach to knock down the $G\alpha_s$ proteins without alteration of basal cAMP levels. $hCRF_1$- and $hCRF_{2(a)}$-expressing HEK293 cells were transfected with a scrambled control siRNA and the G_s-specific siRNA. Control siRNA transfection did not affect the sauvagine-mediated Ca^{2+} mobilization compared with nontransfected cells and furthermore did not affect $G\alpha_s$ protein levels either (data not shown). Instead, transfection of G_s siRNA almost completely abolished $G\alpha_s$ protein levels after 72-h incubation without any effect on intrinsic $G\alpha_q$ protein levels (Fig. 8A). Under these conditions, sauvagine was completely unable to stimulate cAMP production in $hCRF_1$- (Fig. 8B) and $hCRF_{2(a)}$-HEK cells (data not shown). Knockdown of the $G\alpha_s$ proteins also induced a substantial decrease of the sauvagine-mediated Ca^{2+} mobilization in both $hCRF_1$- and $hCRF_{2(a)}$-HEK cells (Fig. 8, C and D). This signal reduction (to 28.7 ± 4.2 and 42.1 ± 1.0% for $hCRF_1$-HEK and $hCRF_{2(a)}$-HEK cells, respectively) even exceeded the ones obtained after CTX treatment and was at least to the same extent as that of $G\alpha_q$ knockdown. In contrast, control siRNA treatment had no effect on sauvagine-mediated Ca^{2+} mobilization.

Simultaneous Knockdown of Two G-Proteins. The above results have demonstrated the contribution of G_q, G_i, and G_s proteins to CRF receptor-mediated Ca^{2+} signaling. To further analyze the contribution of those proteins, and especially to determine whether the G_q signaling depends on the G_s signaling as suggested previously (Berger et al., 2006), the effect of simultaneous blockade of two G-proteins was investigated. When $hCRF_1$-HEK cells were transfected with a combination of G_s and G_q siRNA, the sauvagine-mediated Ca^{2+} signal was almost completely abolished (13.1 ± 1.2% of control levels observed in the cells transfected with the scrambled siRNA), whereas single transfection decreased the Ca^{2+} responses by ~50% (G_q siRNA) or 60 to 70% (G_s siRNA) (Fig. 9A). Similar results were observed for the $CRF_{2(a)}$-HEK cells (Fig. 9B).

We further investigated the outcome of the simultaneous $G\alpha_s/G\alpha_i$ and $G\alpha_q/G\alpha_i$ blockade by combining the siRNA (for

TABLE 1

Overexpression of $G\alpha_q$ and $G\alpha_{16}$ proteins in $hCRF_1$- and $hCRF_{2(a)}$-HEK cells

Medium effective concentration (EC_{50}) and maximal concentration (E_{max}) were determined in the Ca^{2+} mobilization in $hCRF_1$- and $hCRF_{2(a)}$ cells transfected with $G\alpha_q$ and $G\alpha_{16}$. The results are expressed as the percentage of the Ca^{2+} response in cells transfected with either $G\alpha_q$ or $G\alpha_{16}$ compared with cells transfected with empty vector pcDNA3 (mock). The data are means ± S.E.M. of three to six independent stimulation experiments performed in triplicate. The values are expressed as the percentage of response to the maximal response measured on the mock-transfected cells.

Transfection	$hCRF_1$-HEK		$hCRF_{2(a)}$-HEK	
	EC_{50}	E_{max}	EC_{50}	E_{max}
	nM	%	*nM*	%
Mock	146.6 ± 3.31	100 ± 3.9	119.4 ± 2.9	100 ± 2.3
$G\alpha_q$	30.3 ± 5.61*	125.13 ± 8.3*	13.0 ± 3.3*	125.0 ± 3.7*
$G\alpha_{16}$	1.3 ± 0.1†	111.6 ± 8.5	0.3 ± 0.1†	91.3 ± 4.3

* $P < 0.01$, statistical difference versus mock transfection.
† $P < 0.001$, statistical difference versus mock transfection.

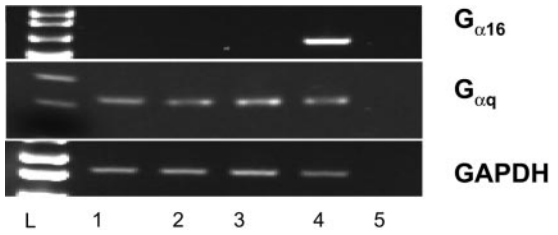

Fig. 3. Semiquantitative RT-PCR amplification of $G\alpha_q$ and $G\alpha_{16}$ in cDNA of nontransfected HEK293 cells. Amplification for 30 cycles of $G\alpha_q$ and $G\alpha_{16}$ was performed using primer sets described under *Materials and Methods*. In control reactions, GAPDH fragments were amplified for 20 cycles: L, ladder (DNA 1 kb Plus DNA ladder; Invitrogen); 1, HEK293 wild-type cells; 2, $hCRF_1$-HEK cells; 3, $hCRF_{2(a)}$-HEK cells; 4, thymus cDNA as positive control; 5, no cDNA control as negative control.

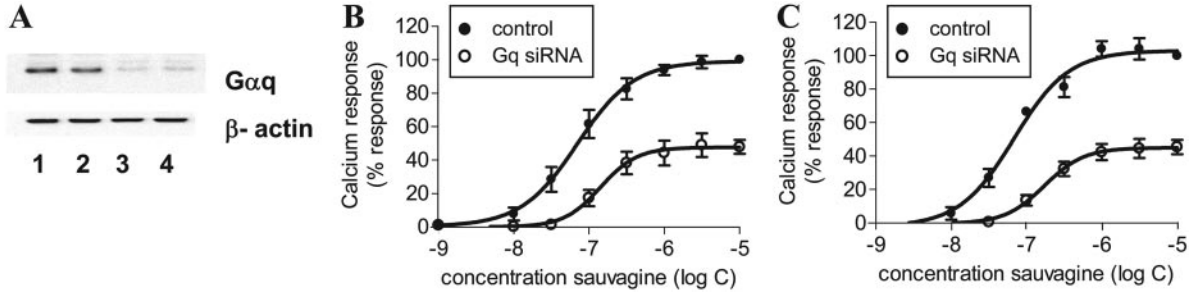

Fig. 4. Effect of $G\alpha_{q/11}$ knockdown by siRNA. The siRNA treatment was conducted as described under *Materials and Methods*. A, representative blot of endogenous $G\alpha_q$ and β-actin in cells after transfection of control or $G_{q/11}$ siRNA (G_q siRNA) for 72 h. 1, no siRNA; 2, siRNA control; 3, G_q siRNA in $hCRF_1$-HEK cells; and 4, G_q siRNA in $hCRF_{2(a)}$-HEK cells. B, effect of the knockdown of $G\alpha_{q/11}$ proteins on the Ca^{2+} mobilization induced by sauvagine in $hCRF_1$-HEK and $hCRF_{2(a)}$-HEK cells (C). The results are expressed as the percentage of Ca^{2+} response in cells transfected with G_q siRNA compared with cells transfected with a scrambled siRNA (AS). The results are the average ± S.E.M. of three independent experiments performed in triplicate. By two-way ANOVA analysis, there were significant differences between cells transfected with the scrambled siRNA (AS) versus G_q siRNA ($F = 191$, $p < 0.0001$) for the $hCRF_1$-HEK cells and for $hCRF_{2(a)}$-HEK cells ($F = 368$, $p < 0.0001$).

$G\alpha_s$ and $G\alpha_q$) and PTX (for G_i) treatments. Control siRNA transfection and incubation with PTX produced a similar decrease of the sauvagine-mediated Ca^{2+} mobilization as obtained by the PTX treatment alone. Knockdown of $G\alpha_q$ (siRNA treatment) and depletion of G_i (PTX treatment) proteins resulted in a synergistic reduction of Ca^{2+} signals in the $hCRF_1$- and $hCRF_{2(a)}$-expressing HEK293 cells (Fig. 10, A and B). In contrast, the Ca^{2+} signal reduction after $G\alpha_s$ knockdown was not further enlarged by cotreatment with PTX (Fig. 10, C and D). Thus, it seems likely that G_i proteins act downstream of the G_s signal.

Expression of Epac1, Epac2, and PLCε. We have shown previously that in contrast to a profound Ca^{2+} signal in CRF_1- and $CRF_{2(a)}$-HEK cells, no Ca^{2+} was monitored in SK-N-MC cells stably transfected with both CRF receptor cDNAs (Dautzenberg et al., 2004). To further analyze the differences between the two cell lines, we quantified the expression of Epac1, Epac2, and PLCε, which have been shown previously to mediate Ca^{2+} release from intracellular stores downstream of cAMP production (Schmidt et al., 2001). To this end, RNA was isolated from the different cell lines, first-strand cDNA synthesis was completed, and the relative expression of Epac1, Epac2 and PLCε was determined by QT-PCR versus the two internal controls, GAPDH and β-actin, which revealed similar results. The expression of Epac1 was nearly undetectable in both cell lines (Fig. 11A), whereas a strong amplification was observed from kidney cDNA, which was used for the linear regression of Epac1 mRNA quantification (data not shown). Total brain cDNA was used for the linear regression for Epac2 quantification. Epac2 mRNA was present in both cell lines, but its expression was significantly higher (~5 fold) in HEK293 cells stably expressing CRF receptors versus SK-N-MC cells stably expressing CRF receptors ($p < 0.01$) (Fig. 11B). Finally, PLCε mRNA expression was only detectable in the various HEK cell lines, whereas no amplification could be observed in the SK-N-MC lines (Fig. 11C).

Discussion

This study established that the $hCRF_1$ and $hCRF_{2(a)}$ receptors couple to different G-proteins in stably transfected HEK293 cells, leading to transient Ca^{2+} mobilization on top of the natural increase in cAMP stimulation. Calcium triggers a variety of cellular responses, including gene transcription and neuronal modification. The resulting neuroplasticity is important for cognitive brain functions like learning and memory (Mattson, 2007). Because of its potential (patho) physiological relevance, it is important to better understand the relationship between CRF action and the elicited Ca^{2+} responses.

CRF signaling has been widely studied in transiently or stably transfected HEK293 cells to elucidate G-protein coupling and downstream signaling events such as mitogen-activated protein kinase signaling. The system has been further applied to studying CRF receptor internalization and β-arrestin recruitment (Oakley et al., 2007; Markovic et al., 2008), as well as Ca^{2+} mobilization (Dautzenberg et al., 2004). To better understand the underlying mechanisms of this mobilization, we checked the involvement of the different potential Ca^{2+} sources and the contribution of different signaling pathways.

The presented data show that, in $hCRF_1$- and $hCRF_{2(a)}$-expressing HEK293 cells, CRF agonists stimulate transient Ca^{2+} mobilization from intracellular stores via the activation of IP_3Rs (Fig. 1), whereas ryanodine receptors are not involved (Dautzenberg et al., 2004). Comparison of the present findings with ones published previously (Dautzenberg et al., 2004; Soares et al., 2005) support the notion that the source in which Ca^{2+} is mobilized largely depends on the cellular context. Depletion of the extracellular Ca^{2+} strongly decreases CRF-induced adrenocorticotropin hormone release in anterior pituitary (Soares et al., 2005). Furthermore, in AtT20 cells, adrenocorticotropin hormone secretion requires intracellular Ca^{2+} and activation of ryanodine receptors (Soares et al., 2005). Otherwise, we have been unable previously to detect Ca^{2+} signaling in recombinant SK-N-MC cells stably expressing both CRF receptors subtypes. In these cells, no CRF receptor-mediated IP_1 or IP_3 production could be measured, whereas a small signal was obtained in HEK293 cells (Dautzenberg et al., 2004). The lack of CRF receptor-mediated Ca^{2+} responses in SK-N-MC cells could, at least in part, be related to the expression of the various IP_3Rs. Three IP_3Rs, which differ in their sensitivity to IP_3, are present in mammalian species, and their expression differs from one cell type to another. The type 2 (the most sensitive receptor) is found in glia but also in HEK293 cells, whereas its expression in neuronal cells is very low (Iwai et al., 2007).

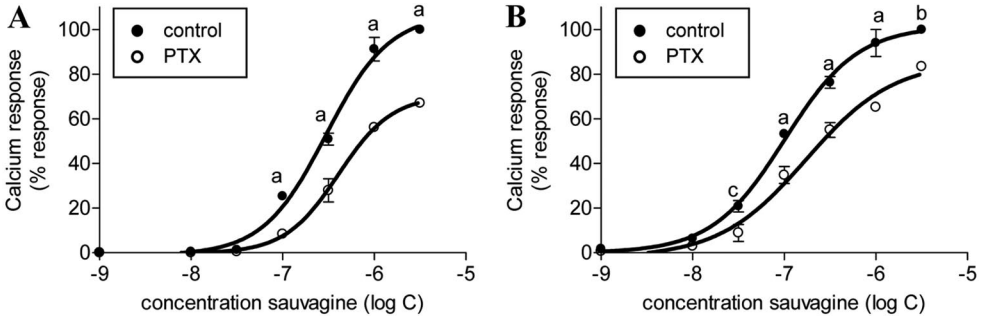

Fig. 5. PTX effects on transient Ca^{2+} mobilization in $hCRF_1$-HEK (A) and $hCRF_{2(a)}$-HEK (B) cells. Cells (75,000 cells/well in 96-well plates) were incubated for 18 h in the absence or presence of 100 ng/ml PTX. The results are expressed as the percentage of Ca^{2+} response in cells incubated with PTX compared with control cells. The results are the average ± S.E.M. of three independent experiments performed in triplicate. By two-way ANOVA analysis, there were significant differences between cells incubated with PTX versus control for $hCRF_1$-HEK cells ($F = 217$, $p < 0.0001$) and for $hCRF_{2(a)}$-HEK cells ($F = 104$, $p < 0.0001$). The following differences were found to be statistically significant in post hoc Bonferroni analysis: PTX versus control: a, $p < 0.001$; PTX versus control: b, $p < 0.01$; PTX versus control: c, $p < 0.05$.

Next, we attempted to identify the G-proteins that are responsible for the CRF receptor-mediated Ca^{2+} signal transduction. Among the different G-proteins, the Gq family is well known to stimulate PLC-β isoforms and production of IP_3. By using immunoprecipitation methods, CRF receptor interaction with $G\alpha_{q/11}$ in rat hippocampus and in HEK293 cells stably transfected with $rCRF_1$ receptors has been reported in addition to $G\alpha_s$ protein interaction (Blank et al., 2003; Berger et al., 2006). In agreement, transfection of the $hCRF_1$- and $hCRF_{2(a)}$-HEK cells with $G\alpha_q$ or $G\alpha_{11}$ cDNAs increased the potency and the efficacy of sauvagine. Conversely, when the $G\alpha_{q/11}$ were knocked down by the siRNA approach, the sauvagine-mediated Ca^{2+} signal was decreased by approximately 50%. Both results provide solid support for the functional coupling of the $G\alpha_q$ proteins in HEK293 cells expressing both CRF receptors.

Because the Ca^{2+} response could only be partly depleted by $G\alpha_q$ knockdown approaches, we investigated the potential contribution of G_i proteins. The ability of these proteins to trigger functional Ca^{2+} responses has already been documented (Dorn et al., 1997), and the data presented here strongly support their functional coupling to CRF receptors. Blockade of $G_{i/o}$ by PTX decreased CRF receptor-mediated Ca^{2+} responses to a small (~20%) but significant extent. It is noteworthy that whereas G_i impairs cAMP production by virtue of its $G\alpha$ subunits, its $G\beta\gamma$ subunits are able to trigger Ca^{2+} mobilization by directly binding to PLC-β (Koch et al., 1994). Indeed, we found that the overexpression of a $G\beta\gamma$ subunit scavenger protein (GRK2ct) blocks the Ca^{2+} signal to the same extent as PTX treatment.

Because the combined contribution of G_q and G_i proteins still did not fully account for CRF receptor-mediated Ca^{2+}mobilization, we next investigated the potential contribution of the only remaining G-protein: G_s. Although the implication of the G_s in the CRF receptor-mediated Ca^{2+} signaling has not been reported so far, there is now good evidence for such a mechanism to take place and that the contribution of G_s even exceeds that of G_q. This is based on the findings obtained by two independent experimental approaches: 1) pharmacological intervention using CTX; and 2) siRNA knock down. The first approach depleted the intracel-

TABLE 2

Effect of the blockade of the G_i proteins by PTX and the $G\beta\gamma$ subunits by GRK2ct

The results are expressed as the percentage of the maximal Ca^{2+} response (E_{max}) in the PTX-treated cells and cells transfected with the $G\beta\gamma$ scavenger GRK2ct compared with the E_{max} value of control cells. The data are means ± S.E.M. of three to six independent stimulation experiments performed in quadruplicate.

Treatment	E_{max}	
	$hCRF_1$-HEK	$hCRF_{2(a)}$-HEK
	%	
Control	100 ± 6.0	100 ± 5.0
PTX	68.4 ± 1.4*	81.3 ± 0.8*
GRK2ct	68.1 ± 1.2*	84.3 ± 1.2†

* $P < 0.01$, statistical difference versus mock transfection.
† $P < 0.001$, statistical difference versus mock transfection.

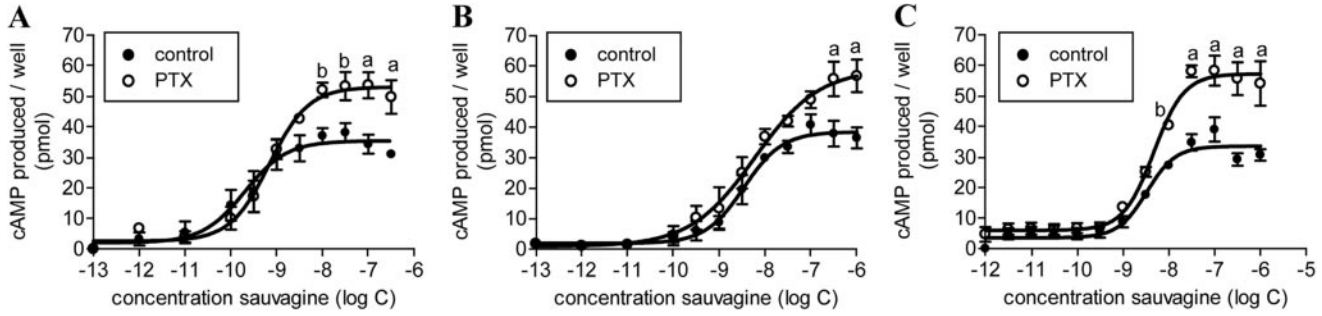

Fig. 6. PTX effects on agonist-mediated cAMP response in HEK293 cells stably expressing $hCRF_1$ (A) and $hCRF_{2(a)}$ (B) receptors, and Y79 cells endogenously expressing human CRF_1 and $CRF_{2(a)}$ receptors (C). Cells were incubated for 18 h in the absence or presence of 100 ng/ml PTX and cAMP was determined as described under *Materials and Methods*. The results are the average ± S.E.M. of three independent experiments performed in quadruplicate. By two-way ANOVA analysis, there were significant differences between the control cells and the PTX-treated cells for $hCRF_1$-HEK cells ($F = 17.94$, $p < 0.0001$), for $hCRF_{2(a)}$-HEK cells ($F = 22.06$, $p < 0.0001$), and for the Y79 cells ($F = 92.5$, $p < 0.0001$). The following differences were found to be statistically significant in Bonferroni post hoc analysis: PTX versus control: a, $p < 0.01$; PTX versus control: b, $p < 0.05$.

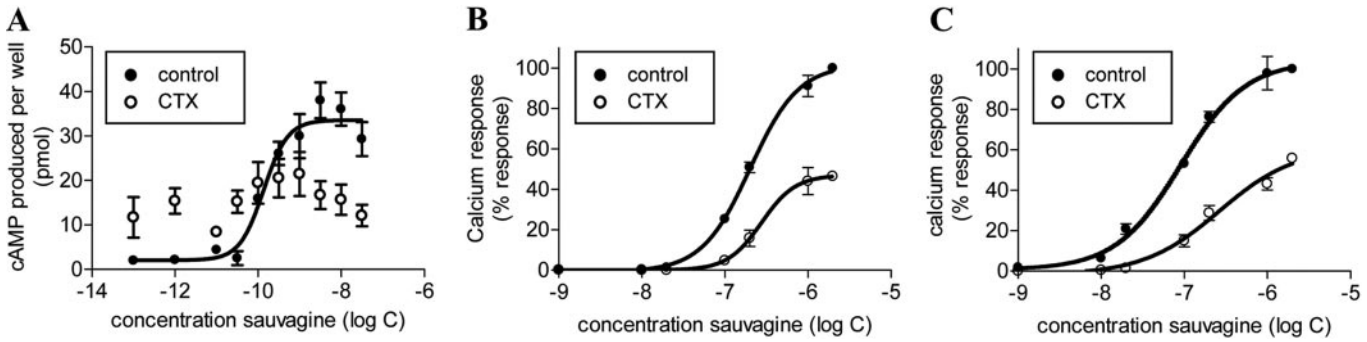

Fig. 7. CTX effects on agonist-mediated cAMP response in HEK293 cells stably expressing CRF_1 (A) and on Ca^{2+} mobilization in $hCRF_1$-HEK (B) $hCRF_{2(a)}$-HEK (C) cells. Incubation of the cells for 18 h was performed in the absence or presence of 100 ng/ml CTX, followed by cAMP production or Ca^{2+} mobilization, was measured in the presence of increasing concentrations of sauvagine. The results are expressed as the percentage of Ca^{2+} response in cells incubated with CTX compared with control cells The results are the average ± S.E.M. of at least three independent experiments performed in triplicate. By two-way ANOVA analysis, there were significant differences between the controls cells and the CTX-treated cells for $hCRF_1$-HEK cells ($F = 233$, $p < 0.0001$) and for $hCRF_{2(a)}$-HEK cells ($F = 575$, $p < 0.0001$).

lular $G\alpha_s$ protein store and reduced the sauvagine-mediated Ca^{2+} signal by approximately 40%. The siRNA knockdown decreased the Ca^{2+} signal in CRF receptor-expressing HEK293 cells (60 and >70%, respectively, for the $hCRF_{2(a)}$ and $hCRF_1$-HEK cells) even stronger.

Activation of $G\alpha_s$ protein has been reported to trigger Ca^{2+} mobilization from intracellular stores via Epac (Kang et al., 2003), a cAMP sensor that activates the ε-isoform of PLC through the activation of small GTPase Rap-1. Because of the strong contribution of the $G\alpha_s$ protein to the Ca^{2+} mobilization, we were interested to identify potential molecular differences between HEK293 and SK-N-MC neuroblastoma cells. Despite the existence of a functional $G\alpha_s$ protein in both cell lines, no CRF receptor-mediated Ca^{2+} mobilization was observed in SK-N-MC cells (Dautzenberg et al., 2004), indicating the potential lack of another component in the signal transduction cascade. Indeed, our QT-PCR data demonstrate the expression of at least one Epac gene, namely Epac2, and PLCε in HEK293 but not in SK-N-MC cells. The lack of expression of these two mRNAs in SK-N-MC cells might at least partly explain the lack of CRF receptor-mediated Ca^{2+} responses in this cell line.

Because none of the G-proteins accounted on its own for the CRF receptor-mediated Ca^{2+} mobilization, we simultaneously knocked down two distinct G-proteins. Although single treatment with either G_s or $G_{q/11}$ siRNA decreased the Ca^{2+} signal to approximately 50%, the knock down of both proteins almost completely abolished this signal. These results demonstrate that the G_q and G_s proteins are acting side by side. The blockade of G_q and G_i proteins by G_q siRNA and PTX treatment, respectively, shed light on the concurrent role of G_q and G_i in the Ca^{2+} signaling as well. However,

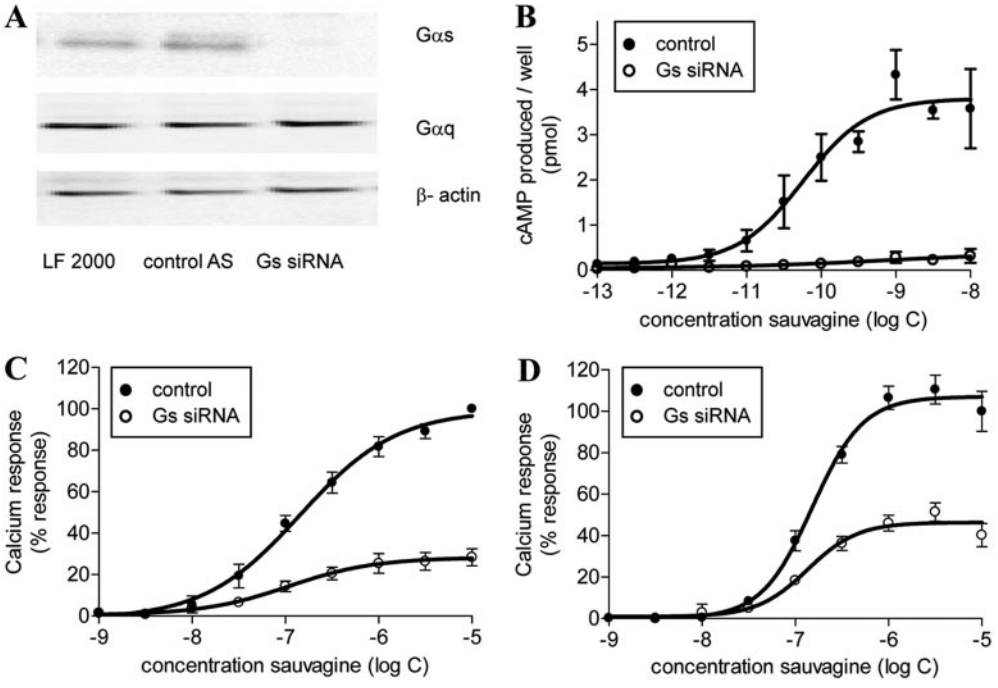

Fig. 8. Functional effect of the knockdown of the $G\alpha_s$ proteins by siRNA. A, representative blot of endogenous $G\alpha_s$ and β-actin proteins in cells transfected with scrambled siRNA or with G_s siRNA in $hCRF_1$-HEK cells. B, cAMP stimulation in $hCRF_1$-HEK cells in cells transfected with G_s siRNA. C and D, effect of G_s siRNA transfection on the Ca^{2+} mobilization induced by sauvagine in $hCRF_1$-HEK (C) and in $hCRF_{2(a)}$-HEK cells (D). The results are expressed as the percentage of Ca^{2+} response in cells transfected with G_s siRNA compared with cells transfected with scrambled siRNA (AS). The results are the average ± S.E.M. of three independent experiments with triplicate (for Ca^{2+} mobilization) or quadruplicate (for cAMP measurements). By two-way ANOVA analysis, there were significant differences between the cells treated with scrambled siRNA (AS) and those treated with G_s siRNA for $hCRF_1$-HEK cells ($F = 179$, $p < 0.0001$) and for $hCRF_{2(a)}$-HEK cells ($F = 385$, $p < 0.0001$).

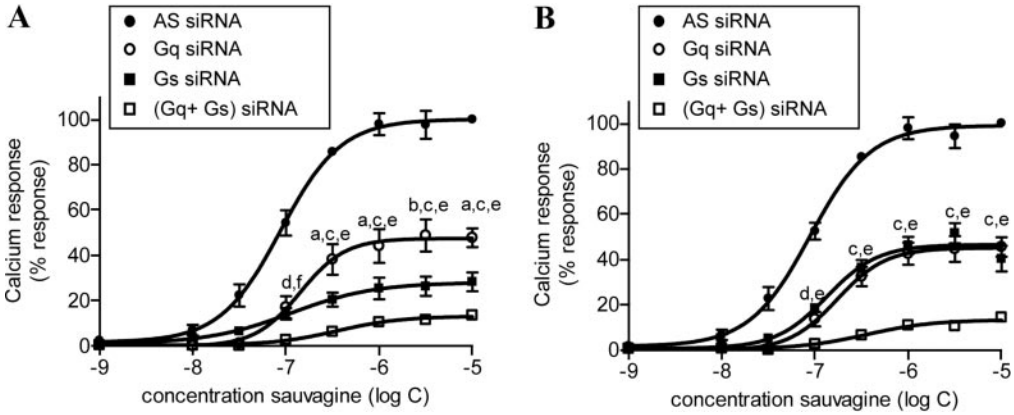

Fig. 9. Effect of a combination of G_s and G_q siRNA transfection on the Ca^{2+} mobilization induced by sauvagine in $hCRF_1$-HEK (A) and $hCR_{F2(a)}$-HEK cells (B). The results are expressed as the percentage of Ca^{2+} response in cells transfected with G_s, G_q, or (G_q + G_s) siRNA compared with cells transfected with scrambled siRNA (AS). The results are the average ± S.E.M. of three independent experiments performed in triplicate. By two-way ANOVA, there were significant differences between the cells transfected with G_s siRNA versus (G_q + G_s) siRNA $hCRF_1$-HEK cells ($F = 75.8$, $p < 0.0001$) and for $hCRF_{2(a)}$-HEK cells ($F = 218$, $p < 0.0001$). The following differences were found to be statistically significant in Bonferroni post hoc analysis: PTX versus control: a, $p < 0.01$; PTX versus control: b, $p < 0.05$.

compared with the decrease that was obtained of G_s by siRNA alone, simultaneous G_i inactivation by PTX did not produce a further decrease. This observation could reflect a signal switch from G_s to G_i. Such a phenomenon was observed to take place during β_2-adrenoreceptor internalization (Daaka et al., 1997), and it was explained by a model in which PKA-mediated phosphorylation of the β_2-adrenoreceptor reduces its affinity for G_s and increases its affinity for G_i. More recently, it was shown that PKA-mediated phosphorylation of the β_1-adrenoreceptor switches its signaling preference from G_s to G_i (Martin et al., 2004). However, such a model is unlikely to apply to the present observations because PKA does not play a role in the Ca^{2+} mobilization (Dautzenberg et al., 2004). Furthermore, it has been shown that the CRF_1 receptors are neither phosphorylated by PKA nor by calcium/calmodulin-dependent protein kinase in COS-7 cells (Hauger et al., 2000). The molecular mechanism underlying the nonadditive effect of G_s and G_i in the CRF receptor-mediated Ca^{2+} signaling clearly needs further research for clarification. A potential hint has been provided recently by Berger et al. (2006), who showed that different conformations of the $rCRF_1$ receptor are involved in the coupling to either G_s or G_i proteins.

Finally, we also found that $G_{\alpha 16}$ overexpression facilitated CRF agonist-induced Ca^{2+} mobilization in $hCRF_1$- and $hCRF_{2(a)}$-HEK cells and that it increased agonist potencies to the same extent as observed in cAMP assays. This finding might be of interest for the set up of screening assays (Kostenis, 2001), especially when using cells types in which $G\alpha_{16}$ proteins and CRF receptors are not naturally coexpressed. Indeed, although CRF receptors have been reported to be expressed in lymphoid organs, the main source for $G\alpha_{16}$ expression (Radulovic et al., 1999; Baigent and Lowry, 2000), we did not find any trace of $G\alpha_{16}$ expression in HEK293 cells and other cells endogenously expressing CRF receptors (E. Gutknecht and F. Dautzenberg, unpublished observations).

In summary, this study demonstrates that the three major G-protein families, G_q, G_i, and G_s, play a role in the transient Ca^{2+} mobilization that takes place upon $hCRF_1$ and $hCRF_{2(a)}$ receptor stimulation in recombinant HEK293 cells. Although G_q and G_i-mediated Ca^{2+} signaling is mediated via PLC-β, G_s-mediated Ca^{2+} signaling is likely to converge at the level

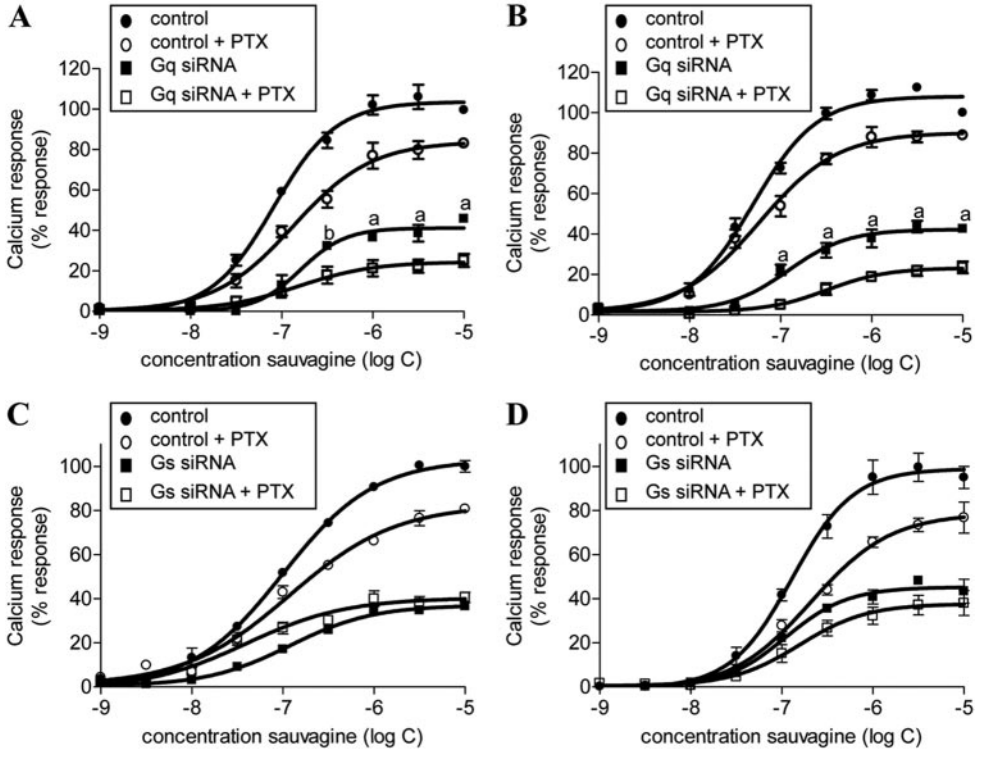

Fig. 10. Effect of a cotreatment with G_q siRNA and PTX (A and B) or G_s siRNA and PTX (C and D) on Ca^{2+} mobilization induced by sauvagine in $hCRF_1$-HEK (A and C) and $hCRF_{2(a)}$-HEK cells (B and D). The results are expressed as the percentage of Ca^{2+} response in cells transfected with the different siRNA, combined or not with the PTX treatment, compared with cells transfected with scrambled siRNA (AS). The results are the average ± S.E.M. of three independent experiments performed in triplicate. By two-way ANOVA analysis, there were significant differences between the cells transfected with G_s siRNA versus (G_q siRNA + PTX) $hCRF_1$-HEK cells ($F = 30.1$, $p < 0.0001$) and for $hCRF_{2(a)}$-HEK cells ($F = 104$, $p < 0.0001$). The following differences were found to be statistically significant in Bonferroni post hoc analysis: (G_q siRNA + PTX) versus G_q: a, $p < 0.001$, and b, $p < 0.01$.

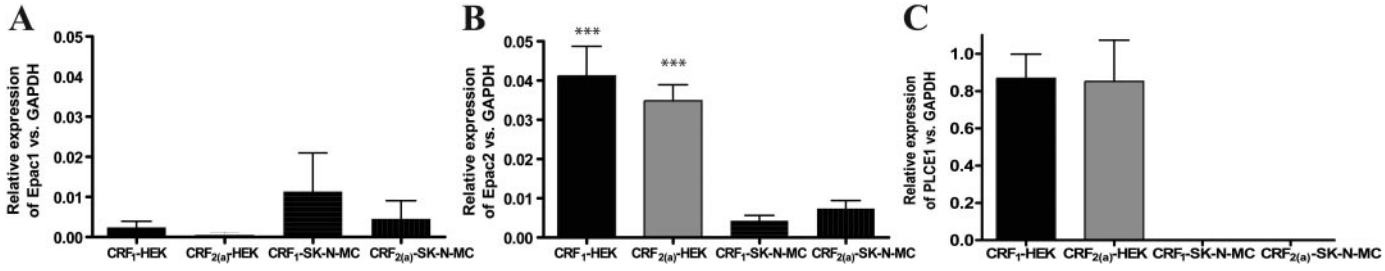

Fig. 11. QT-PCR amplification of Epac1 (A), Epac2 (B), and PLCε (C) from first-strand cDNA synthesized from total RNA isolated from $hCRF_1$-HEK, $CRF_{2(a)}$-HEK, CRF_1-SK-N-MC, and $CRF_{2(a)}$-SK-N-MC cells. The results are representative of three independent RNA isolations performed in duplicate and normalized against GAPDH. Similar results were obtained with β-actin normalization. By ANOVA, there were significant differences between the HEK cell lines and the SK-N-MC lines for the Epac 2 (***, $p < 0.01$).

of Epac and subsequently PLC-ε, whereas PKA is not involved. For both $hCRF_1$ and $hCRF_{2(a)}$ receptors, we found that G_s and G_q produce additive effects and hence act in parallel. Inhibition of these two G-proteins completely abolished CRF receptor-mediated Ca^{2+} responses. In contrast, the small contribution of G_i is likely to result from a switch in the CRF receptor-coupling preference, because blockade of G_s and G_i had no additive effect. Future studies will pinpoint on a molecular level when and how this signal switch occurs.

Acknowledgments

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