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Cell-type specific calcium signaling by corticotropin-releasing factor type 1 (CRF_1) and 2a ($CRF_{2(a)}$) receptors: phospholipase C-mediated responses in human embryonic kidney 293 but not SK-N-MC neuroblastoma cells

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Abstract

The human corticotropin-releasing factor (hCRF) receptors CRF_1 and $CRF_{2(a)}$ couple to the G_s protein. It has been postulated that CRF receptors may also signal through phospholipase C (PLC). To test this hypothesis, binding and signaling properties were determined for both receptor subtypes stably expressed in human embryonic kidney 293 (HEK293) and human SK-N-MC neuroblastoma cells. CRF receptors were highly expressed and strongly coupled to G_s in HEK293 and SK-N-MC cells. However, when the calcium mobilization pathway was investigated, marked differences were observed. In SK-N-MC cells, neither CRF receptor stimulated calcium mobilization in the fluorometric imaging plate reader (FLIPR) assay, whereas activation of orexin type 1 and 2 receptors stably expressed in SK-N-MC cells revealed robust calcium responses. In contrast, intracellular calcium was strongly mobilized by agonist stimulation of $hCRF_1$ and $hCRF_{2(a)}$ receptors in HEK293 cells. In HEK293 cells, potency rank orders for calcium and cAMP responses were identical for both receptors, despite a rightward shift of the dose–response curves. Complete inhibition of calcium signaling of both $hCRF_1$ and $hCRF_{2(a)}$ receptors was observed in the presence of the PLC inhibitor U-73,122 whereas ryanodine, an inhibitor of calcium release channels and the protein kinase A inhibitor Rp-cAMPS were ineffective. Finally, CRF agonists produced a small but significant stimulation of inositol 1,4,5-triphosphate (IP_3) accumulation in $hCRF_1$-and $hCRF_{2(a)}$-transfected HEK293 cells. These data clearly show that phospholipase C-mediated signaling of CRF receptors is dependent upon the cellular background and that in HEK293 cells human CRF receptors robustly respond in the FLIPR format.

Keywords: Ligand binding; Cyclic AMP; Ca^{2+} mobilization; Phospholipase C; Inositol 1,4,5-triphosphate; CRF receptor signaling

1. Introduction

Corticotropin-releasing factor (CRF) and its structurally related analogs urocortins 1–3 control neuroendocrine, autonomic and behavioral responses to stress by interacting with two high-affinity CRF receptors: CRF_1 and CRF_2 [1,2]. Both receptor subtypes, which are ~70% homologous, belong to the class B1 subfamily of G protein-coupled receptors (GPCR) [3,4]. Three biologically active

Abbreviations: CRF, corticotropin-releasing factor; CRF_1, CRF type 1 receptor; CRF_2, CRF type 2 receptor; h, human; oCRF, ovine CRF; FLIPR, fluorimetric imaging plate reader; GPCR, G protein-coupled receptor; G_s, cAMP stimulatory G protein; G_q, phosphoinositide- and calcium-stimulating G protein; IP_3, inositol 1,4,5-triphosphate; PLC, phospholipase C

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splice variants, $CRF_{2(a–c)}$, have been identified for the CRF_2 receptor [5].

Despite their high degree of sequence homology, the specificity of CRF agonist and antagonist binding to CRF_1 and CRF_2 proteins differs considerably. Binding and functional studies using cell lines recombinantly or endogenously expressing CRF_1 receptors revealed a distinct ligand-selective profile: human and ovine CRF, urocortin 1, and the non-mammalian CRF agonists fish urotensin 1, and frog sauvagine bind with high affinity to the mammalian CRF_1 receptor and activate the cyclic AMP signaling pathway [6–9]. In contrast, urocortin 2 and urocortin 3 do not bind to or activate CRF_1 receptors [10–12]. Pharmacological characterization of the CRF_2 receptor splice variants revealed no major differences between $CRF_{2(a)}$, $CRF_{2(b)}$ and $CRF_{2(c)}$ receptors [6,13,14]. However, the binding profiles of these three CRF_2 receptors markedly diverge from the binding profile of the CRF_1 receptor [6,8–12]. Urotensin 1, sauvagine and urocortins 1–3 bind with up to 1000-fold higher affinities to the CRF_2 receptor than species homologues of CRF (see [5]). In agreement with the binding data, a similar rank order of potency is typically observed when these five agonists are used to stimulate intracellular cyclic AMP (cAMP) accumulation [6,8,10–12]. Therefore, urocortin 2 and urocortin 3 are generally considered to represent endogenous ligands for mammalian CRF_2 receptor variants, whereas urocortin 1 is thought to be an endogenous ligand for both CRF_1 and CRF_2 receptors.

As members of the GPCR subfamily B1, CRF_1 and CRF_2 receptors couple to the stimulatory G protein G_s [4] thereby promoting accumulation of the intracellular second messenger cAMP (see above). Because other members of the B1 subfamily have the capability to signal through activation of phospholipase C (PLC) in certain cell systems [15–17], CRF receptors may also activate PLC, and transiently mobilize calcium (Ca^{2+}) depending on the cellular background. Indeed, preliminary evidence suggests that at least the CRF_1 receptor, when recombinantly expressed may interact with G_q proteins [18–21]. In addition, the CRF_1 receptor also appears to increase inositol 1,4,5-triphosphate (IP_3) accumulation in Leydig cells and placenta [18,20]. For the CRF_2 receptor, however, this phenomenon has not been observed.

In this study, we stably expressed the human CRF_1 ($hCRF_1$) and $CRF_{2(a)}$ ($hCRF_{2(a)}$) receptors in human embryonic kidney 293 (HEK293) and neuroblastoma SK-N-MC cells to determine if G protein coupling and second messenger signaling differed in brain- and peripheral-derived cell lines. Accordingly, in addition to the conventional cAMP measurements, we also determined in HEK293 and SK-N-MC cells if both receptor subtypes can stimulate transient Ca^{2+} mobilization in the fluorometric imaging plate reader (FLIPR), which is a format allowing for real-time agonist activation measurement [22,23]. Our study establishes cell-type specific direct coupling of CRF receptors to the PLC pathway.

2. Materials and methods

2.1. Materials, peptides and reagents

All cell culture media and reagents were purchased from Life Technologies. All peptides (purity >95) were obtained from Bachem Corporation.

2.2. Radiochemicals

^{125}I-astressin (2200 Ci/mmol) was purchased from NEN while ^{125}I-Tyr^0-sauvagine (^{125}I-sauvagine; 2000 Ci/mmol) was purchased from Amersham Pharmacia Biotech.

2.3. Cell culture, transfections and radioreceptor binding assays

The HEK293 cells stably expressing the $hCRF_1$ ($hCRF_1$-HEK) and $hCRF_{2(a)}$ ($hCRF_{2(a)}$-HEK) receptors were established as previously described [7,24]. The human neuroblastoma SK-N-MC line (American Type Culture Collection No. HTB-10) was maintained in ISCOVE's medium (Life Technologies) supplemented with 5% fetal bovine serum and 4 mM L-glutamine. cDNAs of $hCRF_1$ and $hCRF_{2(a)}$ receptors and orexin type 1 (OX_1) and type 2 (OX_2) receptors (kindly provided by Dr. Philippe Samama, Roche Biosciences), were inserted into the pcDNA3 vector (2 μg each), and then stably transfected into SK-N-MC cells using the Geneporter™ reagent (Axonlab) as reported previously [24]. Two days after transfection, geneticin selection (500 μg/ml) was initiated to select receptor-expressing clones.

Membranes were prepared from stably transfected HEK293 or SK-N-MC cells as previously described [7,25]. Scatchard and saturation-binding analyses with the Xlfit software program (IDBS) were utilized for calculating the dissociation constant (K_d) and maximal binding (B_{max}) values for equilibrium binding of ^{125}I-astressin or ^{125}I-sauvagine to membrane proteins (0.5–5 μg) measured using the SPA format as described previously [8,24]. Under these conditions, less than 10% of the total radioactivity was specifically bound by the various receptor constructs and the binding data conformed with a one-site model for CRF receptors expressed in both cell lines. The inhibition constant K_i was also calculated with the Xlfit program.

2.4. cAMP assays

$hCRF_1$-HEK, $hCRF_1$-SK-N-MC, $hCRF_{2(a)}$-HEK and $hCRF_{2(a)}$-SK-N-MC cells were plated at 50,000 cells per well in 96-well dishes. Transfected cells were exposed to

CRF peptides for a 10-min stimulation period at 37 °C (5% CO_2) as previously described [26].

2.5. Calcium mobilization assays

HEK293 or SK-N-MC cells stably expressing $hCRF_1$ or $hCRF_{2(a)}$ receptors were seeded at a density of 100,000 cells into poly-D-lysine coated 96-well blackwall, clear-bottom microtiter plates (Corning). One day later, the medium was removed and 100 μl loading medium [DMEM high glucose, without serum, supplemented with 10 mM HEPES-acid, 0.1% BSA, 5 mM probenecid and 2 μM Fluo-3AM (Molecular Probes)]. Cells were loaded for 1 h at 37 °C, washed twice with 100 μl assay buffer (5 mM HEPES-acid, 140 mM NaCl, 1 mM $MgCl_2$, 5 mM KCl, 10 mM glucose) and then 150 μl assay buffer was added. Cells were further pre-incubated at room temperature before adding agonists in 50 μl assay buffer and then measured on a T-channel fluorometric imaging plate reader (FLIPR, Molecular Devices). Maximum change in fluorescence over baseline was used to determine agonist response.

2.6. Stimulation of inositol 1,4,5-triphosphate (IP_3) production and data reduction

For IP_3 experiments, $hCRF_1$- and $hCRF_{2(a)}$-HEK cells were metabolically labeled with myo-[2-3*H*]inositol (5 μCi/ml) overnight (18 h). The next day labeled cells were washed twice with a large volume (40 ml) of myoinositol-free DMEM medium, and then pre-incubated in myoinositol-free Medium 199(E) with 10 mM lithium chloride for 30 min at 37 °C. After cells were again washed, and resuspended in myoinositol-free Medium 199(E) with 10 mM lithium chloride, they were maximally stimulated with 1–10 μM CRF receptor agonists for 20 min. The reaction was then stopped by adding 10 mM formic acid. Formation of IP_3 was measured by anion exchange chromatography using Bio-Rad AG 1-X8 columns, as previously described [27,28]. After Packard Ultima Flow scintillation fluid was added to collected fractions, [^{3}H] radioactivity was measured in a beta-counter. The data were analyzed by two-way ANOVA and significance between groups was determined by post hoc analysis using Dunnett's test.

3. Results

3.1. Binding properties of $hCRF_1$ and $hCRF_{2(a)}$ receptors stably expressed in HEK293 and SK-N-MC cells

A detailed characterization of CRF receptor binding and signaling properties of the HEK293 cell clones stably expressing $hCRF_1$ ($hCRF_1$-HEK) and $hCRF_{2(a)}$ ($hCRF_{2(a)}$-HEK) receptor has been previously reported [7,8,24]. After cDNAs encoding the $hCRF_1$ and $hCRF_{2(a)}$ receptors [7,24] were stably transfected into SK-N-MC neuroblastoma cells to generate the $hCRF_1$-SK-N-MC and $hCRF_{2(a)}$-SK-N-MC lines, we determined the characteristics of CRF receptor binding and agonist-stimulated cyclic AMP accumulation. Saturation-binding experiments were performed in order to quantify the receptor number (B_{max}) for the $hCRF_1$-HEK, $hCRF_1$-SK-N-MC, $hCRF_{2(a)}$-HEK and $hCRF_{2(a)}$-SK-N-MC cell clones. Non-selective agonist (^{125}I-sauvagine) and antagonist (^{125}I-astressin) radioligands were used to measure G protein-coupled and -uncoupled receptor sites (Table 1). In the $hCRF_1$-HEK cell line, the B_{max} was approximately three-fold greater for ^{125}I-astressin ($P < 0.01$) compared to ^{125}I-sauvagine binding, indicating that ~35% of CRF_1 receptor sites are coupled to G protein(s) in $hCRF_1$-HEK cells. When the same analysis was applied to the $hCRF_1$-SK-N-MC clone, a slightly higher G protein coupling rate was observed for the $hCRF_1$ in this cell line. Because $hCRF_1$-SK-N-MC membranes bound approximately two-fold more ^{125}I-astressin ($P < 0.01$) than ^{125}I-sauvagine, ~45% of CRF_1 receptors are coupled to G proteins. The total number of binding sites was significantly higher for the $hCRF_1$-SK-N-MC line compared to $hCRF_1$-HEK cells when either ^{125}I-sauvagine (80% greater; $P < 0.01$) and ^{125}I-astressin (30% greater; $P < 0.001$) were used. Only minimal differences were observed for K_d values measured for binding of the two radioligands in $hCRF_{2(a)}$-HEK and $hCRF_{2(a)}$-SK-N-MC cells (Table 1).

Table 1
Saturation-binding analyses of radioligand binding to human CRF_1 and $CRF_{(2a)}$ receptors stably expressed in HEK293 and SK-N-MC cells

Receptor	^{125}I-Sauvagine		^{125}I-Astressin	
	K_d (nM)	B_{max} (pmol/mg)	K_d (nM)	B_{max} (pmol/mg)
$hCRF_1$-HEK	0.16 ± 0.04	1.05 ± 0.06	0.56 ± 0.09	3.11 ± 0.11
$hCRF_1$-SK-N-MC	0.12 ± 0.02	1.91 ± 0.07[a]	0.51 ± 0.12	4.07 ± 0.23[b]
$hCRF_{2(a)}$-HEK	0.23 ± 0.05	2.45 ± 0.07[c]	0.26 ± 0.05	4.79 ± 0.13[d]
$hCRF_{2(a)}$-SK-N-MC	0.17 ± 0.04	2.76 ± 0.14[e]	0.22 ± 0.03	5.08 ± 0.19[f]

The data are average from at least two independent saturation-binding assays performed in triplicate. By ANOVA, there were significant differences across the groups for B_{max} values ($F = 105.5$, $P < 0.0001$). The B_{max} in each cell line was significantly higher in experiments using ^{125}I-astressin ($P < 0.01$) compared to ^{125}I-sauvagine as the radioligand. The following additional post hoc B_{max} differences were found to be statistically significant between cell groups: [a]$P < 0.01$ vs. $hCRF_1$-HEK-sauvagine; [b]$P < 0.001$ vs. $hCRF_1$-HEK-astressin; [c]$P < 0.001$ vs. $hCRF_1$-HEK-sauvagine; [d]$P < 0.001$ vs. $hCRF_1$-HEK-astressin; [e]$P < 0.01$ vs. $hCRF_1$-SK-N-MC-sauvagine; [f]$P < 0.001$ vs. $hCRF_1$-SK-N-MC-astressin.

Table 2
Effects of GTPγS on agonist- or antagonist radioligand binding to human CRF_1 and $CRF_{(2a)}$ receptors stably expressed in HEK293 and SK-N-MC cells

Receptor	^{125}I-Sauvagine		^{125}I-Astressin	
	IC_{50} (nM)	I_{max} (%)	IC_{50} (nM)	I_{max} (%)
$hCRF_1$-HEK	44 ± 6	31 ± 4	N.D.	3 ± 1
$hCRF_1$-SK-N-MC	49 ± 5	45 ± 3[a]	N.D.	2 ± 1
$hCRF_{2(a)}$-HEK	36 ± 4	50 ± 4[a]	N.D.	5 ± 3
$hCRF_{2(a)}$-SK-N-MC	41 ± 7	53 ± 5[a]	N.D.	5 ± 1

The data are average from at least two independent binding experiments performed in triplicate. N.D.: the inhibition of ^{125}I-astressin binding by GTPγS did not follow a dose–response relationship and thus, an IC_{50} value could not be calculated. By ANOVA, there were significant differences across the groups for the I_{max} values of GTPγS-mediated ^{125}I-sauvagine binding ($F = 35.1$, $P < 0.01$). The following additional post hoc I_{max} difference was found to be statistically significant between cell groups: [a]$P < 0.01$ vs. $hCRF_1$-HEK-sauvagine.

In these two cell lines, ~50% of the ^{125}I-astressin binding sites were labeled with ^{125}I-sauvagine. The B_{max} values did not significantly differ between $hCRF_{2(a)}$-HEK and $hCRF_{2(a)}$-SK-N-MC cells. In addition, the total number of CRF binding sites in the two $hCRF_2$-expressing cell lines was consistently higher than the B_{max} values measured in the two $hCRF_1$-expressing cell lines. Thus, we concluded that $hCRF_{2(a)}$ receptors in HEK293 and SK-N-MC cells were expressed at higher levels and were more strongly coupled to G proteins than $hCRF_1$ receptors expressed in these two cellular backgrounds. However, the B_{max} values for $hCRF_1$ receptors was significantly greater in SK-N-MC compared to HEK293 cells ($P < 0.001$). In agreement with the saturation-binding analyses a smaller receptor proportion being sensitive to GTPγS inhibition was found for $hCRF_1$-HEK cells in comparison to $hCRF_1$-SK-N-MC and the two $hCRF_{2(a)}$ receptor preparations with the agonist ^{125}I-sauvagine as radioligand (Table 2).

Competitive binding studies using five different agonists (oCRF, urocortins 1–3, and sauvagine) against ^{125}I-sauvagine were next completed in $hCRF_1$-HEK, $hCRF_1$-SK-N-MC, $hCRF_{2(a)}$-HEK and $hCRF_{2(a)}$-SK-N-MC lines. Agonist binding properties were significantly different for $hCRF_1$ and $hCRF_{2(a)}$ receptors in agreement with previous studies [10–12] (Table 3). An identical rank order binding profile was observed for the $hCRF_1$ receptor expressed in HEK293 and SK-N-MC cells. Urocortin 1, sauvagine and oCRF bound with subnanomolar to low nanomolar affinities, whereas urocortin 2 displaced the radiolabeled antagonist with >1 μM affinities (Table 3). Because urocortin 3 exhibited the weakest affinity for the $hCRF_1$ receptor, an IC_{50} value could not be calculated in both cell lines. In contrast, a different binding profile was observed for the $hCRF_{2(a)}$ receptor expressed in HEK293 and SK-N-MC cells. The $hCRF_{2(a)}$ receptor in both lines exhibited subnanomolar binding affinity for urocortin 1 and sauvagine. The binding affinities for urocortins 2 and 3 were in the low nanomolar range (Table 3). In contrast, oCRF was significantly less potent in competing with ^{125}I-sauvagine in both $hCRF_{2(a)}$-expressing cell lines based on its IC_{50} values being ~100–200 nM (Table 3). However, there were no significant differences between IC_{50} values for each agonist acting at CRF_1 receptors expressed in HEK293 and SK-N-MC cells. Likewise, agonist IC_{50} values did not differ for $hCRF_{2(a)}$-expressing HEK293 and SK-N-MC cells (Table 3).

3.2. cAMP accumulation in $hCRF_1$-HEK, $hCRF_1$-SK-N-MC, $hCRF_{2(a)}$-HEK and $hCRF_{2(a)}$-SK-N-MC cells

Next, we analyzed the functional properties of both CRF receptor subtypes stably expressed in HEK293 and SK-N-MC cells by measuring agonist-stimulated accumulation of intracellular cAMP, which is the second messenger normally associated with CRF receptor signaling. In agreement with the binding studies, the $hCRF_1$ and the $hCRF_{2(a)}$ receptor expressed in both cell lines revealed an identical subtype-specific potency rank order. For the $hCRF_1$-HEK and $hCRF_1$-SK-N-MC cells, oCRF, urocortin 1 and sauvagine were highly potent agonists (Fig. 1A; Table 4). In contrast, urocortin 2 was less potent by three orders of magnitude in the two $hCRF_1$-expressing cell lines. Finally, urocortin 3 concentrations of 10 μM and more were required to stimulate cAMP production in $hCRF_1$-HEK and $hCRF_1$-SK-N-MC cells (Fig. 1A; Table 4). In contrast, in $hCRF_{2(a)}$ receptor-expressing lines, the following potency rank order profile for agonist-stimulated cAMP accumulation was observed (Fig. 1B; Table 3): sauvagine

Table 3
Competitive binding of CRF analogs to $hCRF_1$ and $hCRF_{(2a)}$ receptors stably expressed in HEK293 and SK-N-MC cells

Ligand	$hCRF_1$-HEK, IC_{50} (nM)	$hCRF_1$-SK-N-MC, IC_{50} (nM)	$hCRF_{2(a)}$-HEK, IC_{50} (nM)	$hCRF_{2(a)}$-SK-N-MC, IC_{50} (nM)
oCRF	1.24 ± 0.26	1.18± 0.25	229 ± 36[a]	137 ± 38[b]
Urocortin 1	0.31 ± 0.09	0.29 ± 0.11	0.26 ± 0.07	0.45 ± 0.12
Urocortin 2	4600 ± 624[c]	3700 ± 501[c]	2.41 ± 0.51	3.11 ± 0.63
Urocortin 3	>10000[c,d]	>10000[c,d]	12.9 ± 2.6	10.1 ± 2.9
Sauvagine	0.78 ± 0.15	0.64 ± 0.11	0.52 ± 0.12	0.61 ± 0.14

The data are means ± S.E.M. of three to four independent experiments performed in triplicate. The human versions of urocortins 1–3 were used.
[a] $P < 0.0001$ vs. urocortin 1, urocortin 2, urocortin 3 and sauvagine.
[b] $P < 0.0005$ vs. urocortin 1, urocortin 2, urocortin 3 and sauvagine.
[c] $P < 0.0001$ vs. oCRF, urocortin 1 and sauvagine.
[d] $P = 0.0001$ vs. UCN 2.

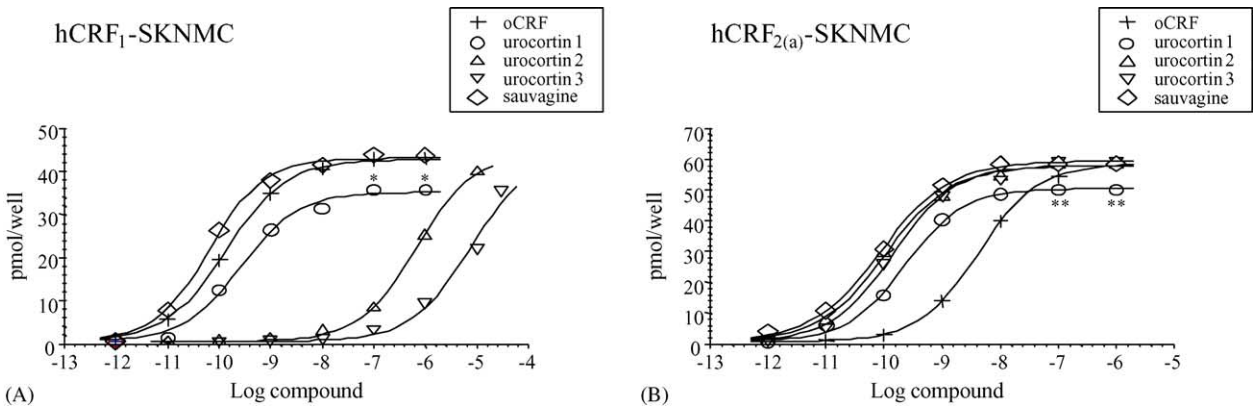

Fig. 1. Stimulation of cAMP accumulation by CRF agonists in SK-N-MC cells stably expressing $hCRF_1$ (A) and $hCRF_{2(a)}$ (B) receptors. Cells were incubated for 10 min at 37 °C with increasing concentrations (1 pM–10 μM) of CRF agonists as indicated in Section 2. The results are representatives of five independent experiments performed in triplicate. The E_{max} values elicited by urocortin 1-stimulation differed significantly from the E_{max} values obtained by stimulation with oCRF, urocortin 2, urocortin 3 and sauvagine. $hCRF_1$-SK-N-MC: $F(4,12) = 9.4$, $P < 0.005$; $hCRF_{2(a)}$-SK-N-MC: $F(4,12) = 17.99$, $P < 0.0001$.

∼ urocortin 2 > urocortin 3 ∼ urocortin 1 ≫ oCRF. Notably, urocortin 1 was significantly less efficacious in stimulating cAMP accumulation in $hCRF_1$-SK-N-MC and $hCRF_{2(a)}$-SK-N-MC cells (Fig. 1). A similar situation has been observed between $hCRF_1$-HEK and $hCRF_{2(a)}$-HEK cells (see [8]). In addition, the cAMP response magnitude for urocortin 1 was ∼85% of the maximal cAMP response produced by the other agonists. Importantly, although the potency rank order for agonist-stimulated cAMP accumulation was identical for $hCRF_1$-HEK versus $hCRF_1$-SK-N-MC and $hCRF_{2(a)}$-HEK versus $hCRF_{2(a)}$-SK-N-MC cells, all CRF agonists were five-fold more potent in the $hCRF_1$-SK-N-MC and $hCRF_{2(a)}$-SK-N-MC cells compared to the HEK293 lines (Table 4).

3.3. Transient Ca^{2+} mobilization in $hCRF_1$-HEK and $hCRF_{2(a)}$-HEK but not $hCRF_1$-SK-N-MC and $hCRF_{2(a)}$-SK-N-MC cells

Because in initial reports CRF_1 receptors have been postulated to signal via the PLC cascade [19–21], we tested the ability of recombinant CRF_1 and $CRF_{2(a)}$ receptors expressed in HEK293 and SK-N-MC cells to couple to G_q protein and stimulate transient Ca^{2+} mobilization using the real-time FLIPR assay [22,23]. Since the ability of SK-N-MC cells to functionally respond in the FLIPR format has not been tested previously to our knowledge, we also measured FLIPR responses activated by recombinantly expressed orexin OX_1 and OX_2 receptors which typically couple to G_q proteins [29] thereby serving as positive controls. In the first experimental setting, we used the most potent CRF_1/CRF_2 agonist sauvagine (see cAMP experiments) for the various CRF receptor expressing lines and orexin A, which is equally potent at the OX_1 and OX_2 receptors [29].

Sauvagine stimulated transient Ca^{2+} mobilization in $hCRF_1$-HEK and $hCRF_{2(a)}$-HEK cells in a concentration-dependent manner (Fig. 2; Table 4). In $hCRF_1$-HEK cells, sauvagine-stimulated Ca^{2+} mobilization reached a peak of ∼14,000 relative fluorescence units (RFU) within 40 s after agonist application and then rapidly returned to baseline levels over the next 90 s (Fig. 2). In $hCRF_{2(a)}$-HEK cells, a strong Ca^{2+} mobilization with a peak of ∼8000 RFU was observed during a ∼40-s sauvagine incubation. The $hCRF_{2(a)}$-HEK cell Ca^{2+} response returned to baseline levels with a slightly slower kinetic (Fig. 2). However, Ca^{2+} mobilization did not significantly increase in $hCRF_1$-SK-N-MC and $hCRF_{2(a)}$-SK-N-MC cells

Table 4
Stimulation of cAMP production by various CRF peptides in HEK293 and SK-N-MC cells stably expressing human CRF_1 and $CRF_{(2a)}$ receptors

Ligand	$hCRF_1$-HEK, EC_{50} (nM)	$hCRF_1$-SK-N-MC, EC_{50} (nM)	$hCRF_{2(a)}$-HEK, EC_{50} (nM)	$hCRF_{2(a)}$-SK-N-MC, EC_{50} (nM)
oCRF	0.54 ± 0.10[a]	0.10 ± 0.01	19.9 ± 3.6[b,c]	4.28 ± 0.59[b]
Urocortin 1	1.41 ± 0.21[a]	0.22 ± 0.08	1.28 ± 0.12[c]	0.24 ± 0.02
Urocortin 2	2270 ± 186[a,d]	637 ± 120[d]	0.46 ± 0.07[c]	0.09 ± 0.02
Urocortin 3	>10000[d,e]	9400 ± 689[d,e]	0.96 ± 0.12[c]	0.24 ± 0.04
Sauvagine	0.38 ± 0.07[a]	0.06 ± 0.01	0.31 ± 0.04[c]	0.05 ± 0.02

The data are means ± S.E.M. of five independent experiments performed in triplicate. The human versions of urocortins 1–3 were used.
[a] $P < 0.02$ vs. $hCRF_1$-SK-N-MC.
[b] $P < 0.0001$ vs. urocortin 1, urocortin 2, urocortin 3 and sauvagine.
[c] $P <$ vs. $hCRF_{2(a)}$-SK-N-MC.
[d] $P < 0.0001$ vs. oCRF, urocortin 1 and sauvagine.
[e] $P = 0.0001$ vs. urocortin 2.

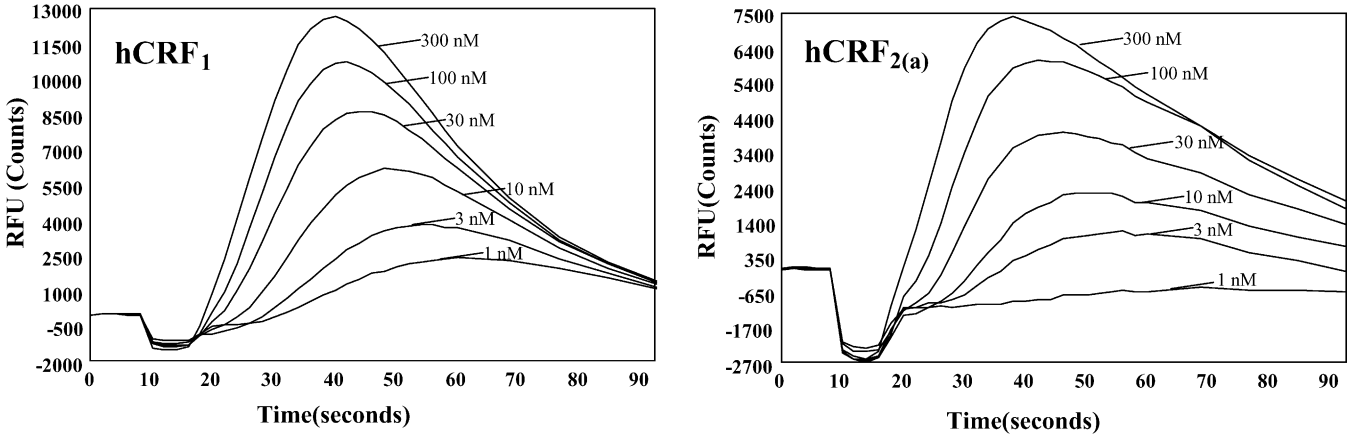

Fig. 2. Real time dose–response curves for sauvagine-mediated transient Ca^{2+} mobilization in $hCRF_1$-HEK and $hCRF_{2(a)}$-HEK cells. Cells (100,000 per well) were incubated with increasing sauvagine concentrations (1–300 nM) for the indicated time. The results are representatives of 11 independent experiments performed in quadruplicate.

exposed to sauvagine. Because orexin A stimulated robust Ca^{2+} mobilization responses in SK-N-MC cells stably expressing OX_1 and OX_2 receptors (Table 5), we ruled out the possibility that the SK-N-MC cellular background has defective G_q signaling. Thus, we concluded that $hCRF_1$ and $hCRF_{2(a)}$ receptors signal through different pathways in HEK293 and SK-N-MC cells.

Next, we determined if $hCRF_1$ and $hCRF_2$ receptors expressed in HEK293 cells could stimulate intracellular Ca^{2+} release in response to other CRF ligands. When $hCRF_1$-HEK cells were incubated with oCRF, urocortin 1 and sauvagine, a nearly equipotent stimulation of transient Ca^{2+} mobilization was observed with EC_{50} values in the low nanomolar range (Fig. 3; Table 6). In contrast, no Ca^{2+} responses were observed when $hCRF_1$-HEK cells were exposed to two selective CRF_2 receptor agonists urocortins 2 and 3. However, as observed in cAMP experiments, urocortin 1 was less efficacious than oCRF and sauvagine in stimulating Ca^{2+} mobilization with the maximum only reaching ~9500 RFU compared to ~14,000 RFU resulting from oCRF and sauvagine stimulation (Fig. 3). As observed in the initial experiments, stimulation of Ca^{2+} mobilization in $hCRF_{2(a)}$-HEK cells by various CRF agonists produced maximal responses of only ~60% of the responses observed in the $hCRF_1$-HEK cells. However, in contrast to the $hCRF_1$-HEK cells, all agonists were able to mobilize Ca^{2+} transients in the $hCRF_{2(a)}$-HEK line, albeit with different potencies. While sauvagine, urocortins 1 and 2 only differed by a factor of ~2.5 from each other, urocortin 3 was almost 10-fold less potent than sauvagine (Fig. 3; Table 6). Finally, oCRF only increased Ca^{2+} transients at a concentration in the low micromolar range (Table 6). As observed with the $hCRF_1$-HEK cells, urocortin 1 was less efficacious than the four other agonists to stimulate Ca^{2+} mobilization in $hCRF_{2(a)}$-HEK cells (Fig. 3).

Table 5
Maximal FLIPR responses of CRF_1 and $CRF_{2(a)}$ receptors in HEK293 and SK-N-MC cells, and comparison to OX1 and OX2 receptors in SK-N-MC cells

Cell line	Receptor	E_{max}; ΔRFU
HEK293	$hCRF_1$	13900 ± 560
	$hCRF_{2(a)}$	7600 ± 480
SK-N-MC	$hCRF_1$	230 ± 200
	$hCRF_{2(a)}$	210 ± 170
	OX_1	12200 ± 500
	OX_2	14800 ± 800

The data are means ± S.E.M. of two (OX1 and OX_2) to six (CRF receptors in HEK293 and SK-N-MC cells) independent experiments performed in quadruplicate using a maximally stimulating concentration of 1 (M sauvagine (CRF receptors) or orexin A (OX_1 and OX_2).

In another experimental setting, we tested the ability of the nonselective CRF_1/CRF_2 peptide antagonist astressin and the CRF_2-selective antagonist antisauvagine to inhibit agonist-induced Ca^{2+} mobilization. Increasing concentrations of astressin or antisauvagine (0.1 nM–10 μM) were used in the presence of a submaximal sauvagine concentration (50 nM) to measure the inhibitory potency of both antagonists. In $hCRF_1$-HEK cells, astressin efficiently inhibited sauvagine-stimulated Ca^{2+}-responses with an IC_{50} of ~60 nM, while antisauvagine, even at the highest dose, was without effect (Fig. 4). In contrast, both antagonists potently inhibited sauvagine-stimulated Ca^{2+} transients in $hCRF_{2(a)}$-HEK cells (Fig. 4). The IC_{50} values for astressin (~20 nM) and antisauvagine (~12 nM) only differed minimally from each other and were in good agreement with their inhibitory potency in cAMP stimulation experiments [26].

3.4. Mechanism of transient Ca^{2+} mobilization in $hCRF_1$-HEK and $hCRF_{2(a)}$-HEK cells

In order to determine the exact mechanism of agonist-induced Ca^{2+} mobilization in HEK293 cells stably expressing both human CRF receptor subtypes we measured FLIPR responses in the presence of various inhibitors.

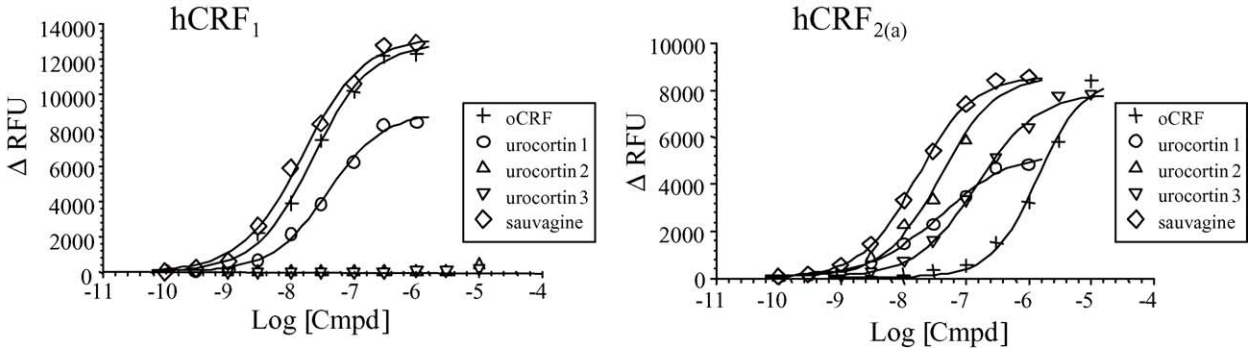

Fig. 3. Stimulation of transient Ca^{2+} mobilization in $hCRF_1$-HEK and $hCRF_{2(a)}$-HEK cells by various CRF agonists. Cells (100,000 per well) were incubated with increasing agonist concentrations (0.1 nM–10 μM) for up to 2 min. Maximal relative fluorescence units (RFU) at the peak of the transient Ca^{2+} mobilization curve were taken for quantification. The results are representatives of up to eight independent experiments performed in quadruplicate. For the $hCRF_1$ receptor significant differences [$F(4,28) = 2967.661$, $P < 0.0001$] in the potency of the various agonists were obtained. Similarly, the stimulation experiments with the $hCRF_{2(a)}$ receptor [$F(4,30) = 147,211$, $P = 0.0001$] also revealed significant differences. Furthermore, the E_{max} values elicited by urocortin 1-stimulation differed significantly from the E_{max} values obtained by stimulation with the other agonists and reached only ~70%. $hCRF_1$-HEK: $F(2,10) = 299.4$, $P < 0.0001$; $hCRF_{2(a)}$-HEK: $F(4,30) = 647.8$, $P < 0.0001$.

To this end we chose the following inhibitors: ryanodine, an inhibitor of Ca^{2+} release channels [30,31], Rp-cAMPS an inhibitor of protein kinase A [32] and U-73,122 a potent inhibitor of PLC and G_q signaling [30]. At a 100-nM sauvagine concentration ryanodine (45 μM) and Rp-cAMPS (15 μM) failed to block transient Ca^{2+} mobilization in $hCRF_1$-HEK and $hCRF_{2(a)}$-HEK cells, whereas U-73,122 (10 μM) blocked sauvagine-stimulated FLIPR responses by more than 95% in both $hCRF_1$ and $hCRF_{2(a)}$ receptor expressing HEK293 cells (Fig. 5A). The inhibitory effect of 10 μM U-73,122 on agonist-mediated Ca^{2+} mobilization was further investigated in $hCRF_1$- and $hCRF_{2(a)}$-HEK cells by performing full dose–response curves for sauvagine and in case of the $hCRF_{2(a)}$ receptor also for urocortin 3. U-73,122 potently inhibited FLIPR responses in both cell lines (Fig. 5B). At maximally stimulating sauvagine and urocortin 3 concentrations U-73,122 inhibited transient Ca^{2+} mobilization by ~92–95%, whereas the structurally related but inactive U-73,343 was without significant effect (<10% inhibition) on CRF receptor-stimulated FLIPR responses (not shown).

Finally, to confirm functionally that CRF receptors stably expressed in HEK293 cells activated the PLC-PKC signaling pathway, we determined if IP_3 accumulation was increased by submaximal CRF receptor agonist concentrations. In $hCRF_1$-HEK cells, oCRF and sauvagine (100 nM each) but not urocortin 2 (10 μM) stimulated a significant ($P < 0.01$) ~70% increase IP_3 formation over basal levels (Fig. 6). A significant increase in IP_3 levels was also observed in HEK cells transiently transfected with $hCRF_1$ and stimulated with hCRF (data not shown) in agreement with previous reports [19,20]). In $hCRF_{2(a)}$-HEK cells, all agonists at 100 nM concentrations also stimulated small (~50–60%) but significant ($P < 0.01$) elevation of IP_3 production over basal values (Fig. 6). As expected from the FLIPR experiments no stimulation of IP_3 production by CRF agonists was observed in $hCRF_1$-SK-N-MC and $hCRF_{2(a)}$-SK-N-MC cells (not shown).

Table 6
Stimulation of intracellular calcium by various CRF peptides in HEK293 cells stably expressing CRF_1 and $CRF_{(2a)}$ receptors

Ligand	$hCRF_1$, EC_{50} (nM)	$hCRF_{2(a)}$, EC_{50} (nM)
oCRF	23.2 ± 5.3	1640 ± 378[a]
Urocortin 1	33.1 ± 6.7	40.4 ± 5.9
Urocortin 2	>10000[b]	43.1 ± 6.4
Urocortin 3	>10000[b]	156 ± 45
Sauvagine	20.8 ± 4.5	21.9 ± 3.6

The data are means ± S.E.M. of at least seven independent experiments performed in quadruplicate. The human versions of urocortins 1–3 were tested.

[a] $P = 0.0001$ vs. urocortin 1, urocortin 2, urocortin 3 and sauvagine.

[b] $P < 0.0001$ vs. oCRF, urocortin 1 and sauvagine.

4. Discussion

This study establishes that coupling of CRF_1 and $CRF_{2(a)}$ to the PLC pathway is governed by cellular background whereas CRF receptor coupling to G_s occurred in both brain- and peripheral-cell lines. In a series of experiments, oCRF-, urocortin 1- and sauvagine-induced activation of $hCRF_1$ receptors markedly increased cAMP accumulation in HEK293 or SK-N-MC cells consistent with a high efficiency G_s-mediated signaling. When the $hCRF_{2(a)}$ receptor was activated by agonists, cAMP accumulation in both cell lines was increased with the following potency rank order profile: sauvagine ~ urocortin 2 ~ urocortin 3 > urocortin 1 ≫ oCRF. When CRF receptor signaling through PLC was assessed, oCRF, urocortin 1, and sauvagine were found to stimulate transient Ca^{2+} mobilization in both $hCRF_1$-HEK and $hCRF_{2(a)}$-HEK cells in a concentration-dependent and nearly equipotent manner. Using CRF_2 receptor-selective agonists, urocortin 2 was ~10-fold more potent than urocortin 3 in mobilizing

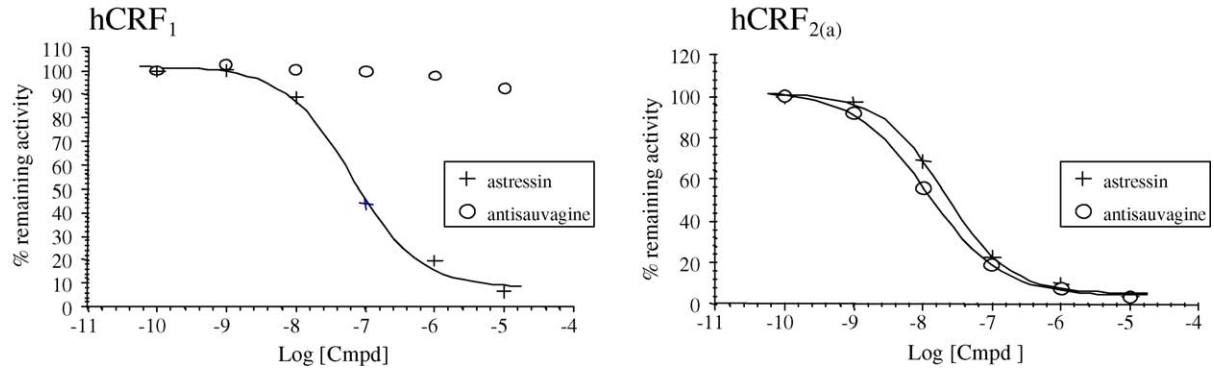

Fig. 4. Antagonist-mediated inhibition of Ca^{2+} mobilization in $hCRF_1$-HEK and $hCRF_{2(a)}$-HEK cells. Cells were incubated with increasing concentrations of astressin and antisauvagine (0.1 nM–10 μM each) in the presence of 50 nM sauvagine. The results are representatives of three independent FLIPR experiments performed in quadruplicate.

calcium in HEK293 cells. However, in $hCRF_1$-SK-N-MC and $hCRF_{2(a)}$-SK-N-MC cells, Ca^{2+} mobilization was not increased by sauvagine and other agonists. Therefore, cell-type specific factors may account for specificity of CRF receptor-G protein interactions.

In all species, the CRF_1 and the $CRF_{2(a)}$ receptor are mainly found in the central nervous system [1,2,5]. However, pharmacological characterizations of vertebrate CRF_1 and CRF_2 receptor variants have been conducted in various peripheral cells [6,7,9,14,19,20,24] including the HEK293 line [7,14,21,24].

Previously, receptor-G protein interactions were believed to be highly selective in cell systems endogenously expressing signaling proteins in a physiologically stringent setting. A similar finding was also observed in heterologous, peripheral cell systems artificially induced to express a receptor that is not normally present in the cell [33]. This hypothesis proposed that a given receptor coupled to only G_s, G_i, or G_q. Specificity of receptor-G protein interactions was thought to depend on unique intracellular structural motifs governing a receptor's affinity for binding a specific G protein. However, recent

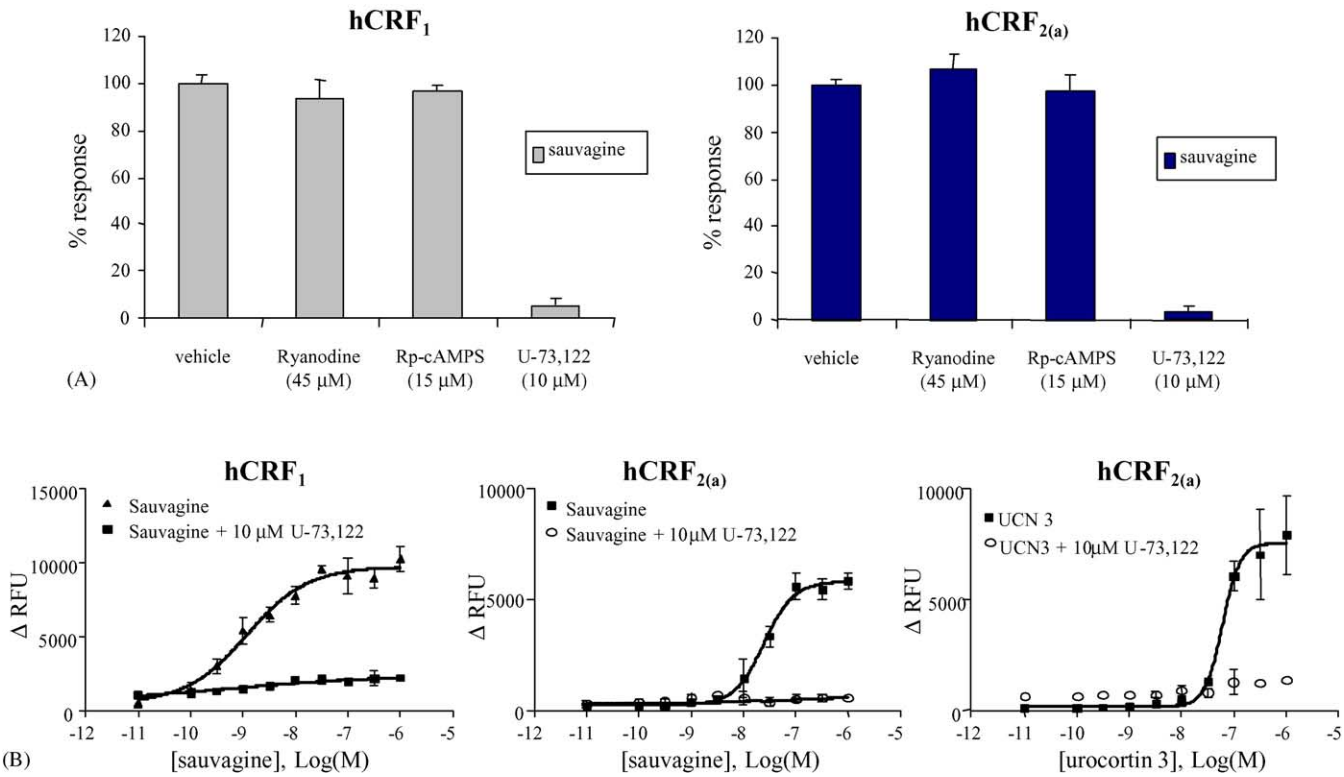

Fig. 5. Inhibition of sauvagine-mediated FLIPR responses by various inhibitors. (A) $hCRF_1$-HEK and $hCRF_{2(a)}$-HEK cells were incubated with a submaximal sauvagine concentration (100 nM) in the absence of presence of ryanodine (45 μM), RP-cAMPS (15 μM) or U-73,122 (10 μM). (B) Full dose–response stimulation curves for the agonists sauvagine and urocortin 3 were generated in the absence or presence of 10 μM U-73,122. The results are representative of three independent experiments performed in quadruplicate.

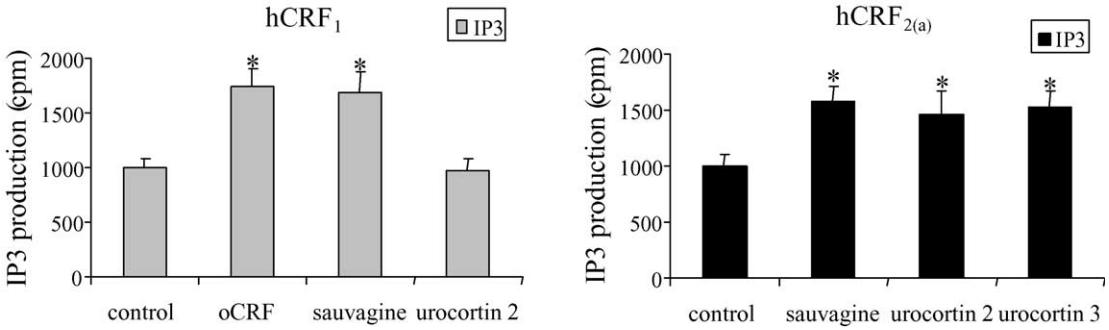

Fig. 6. Stimulation of IP_3 accumulation in $hCRF_1$- and $hCRF_{2(a)}$-expressing HEK293 cells by sauvagine. Cells were incubated at 37 °C for 5 min with sauvagine (100 nM). The results are representative of four independent experiments performed in quadruplicate. By ANOVA, there were significant differences for IP_3 stimulation in $hCRF_1$-HEK [$F(3,12) = 36.31$, $P < 0.01$] and $hCRF_{2(a)}$-HEK cells [$F(3,12) = 28.56$, $P < 0.01$]. Statistical significance: $^{*}P < 0.01$ vs. control.

studies have reported that a receptor can bind to more than one G protein. One factor that may account for a receptor binding to different G proteins is cellular background [34]. Little information is available regarding the selectivity of CRF receptor-G protein interactions in neuronal background which could play an important role in GPCR signaling in the central nervous system. For example, recombinant expression of pituitary adenylate cyclase-activating polypeptide (PACAP) receptor PAC_1 splice variants in peripheral cell lines [15,16,35] revealed important differences in the binding and cAMP signaling properties when compared to their endogenous expression in a neuronal cell line [36,37]. So far, no attempt has been made to express CRF_1 and CRF_2 receptor subtypes recombinantly in a neuronal-like background. Thus, our study represents the first characterization of recombinant $hCRF_1$ and $hCRF_{2(a)}$ receptors in a neuroblastoma line. SK-N-MC cells were chosen due to a pharmacological characterization of the recombinant histamine H3 receptor in SK-N-MC cells providing results identical to the native centrally expressed H3 receptor [38,39].

When $hCRF_1$ and $hCRF_{2(a)}$ receptors were highly expressed in the SK-N-MC cells, there were substantial differences between the $hCRF_1$-HEK, $hCRF_1$-SK-N-MC, $hCRF_{2(a)}$-HEK and $hCRF_{2(a)}$-SK-N-MC lines in saturation-binding experiments where the agonist sauvagine and the antagonist astressin were used as radioligands. Agonist and antagonist labeling as well as GTPγS-inhibition studies established that $hCRF_{2(a)}$ receptors expressed in both cell lines bound the ligands with nearly identical affinities and exhibited similar B_{max} values. Furthermore, in the $hCRF_{2(a)}$-HEK and $hCRF_{2(a)}$-SK-N-MC lines, slightly more than 50% of the $hCRF_{2(a)}$ receptor proteins were coupled to G proteins. However, for the $hCRF_1$ receptor expressing lines, differences were observed. The density of expressed $hCRF_1$ receptors was 80% greater in SK-N-MC compared to HEK cells when ^{125}I-sauvagine was used as the tracer for receptor quantification but only 30% higher with ^{125}I-astressin. Furthermore, GTPγS inhibited ^{125}I-sauvagine to $hCRF_1$-HEK cells only by ∼30% but by almost 50% to $hCRF_1$-SK-N-MC cells. This data indicates that the $hCRF_1$ receptor is better coupled in SK-N-MC than in HEK293 cells. When competition-binding studies were completed using oCRF, urocortins 1–3, and sauvagine, no pharmacological differences between the two CRF_1 and CRF_2 receptor expressing lines were observed. Neither the rank order binding profile nor the affinity for the various ligands differed significantly for the $hCRF_1$ and the $hCRF_{2(a)}$ receptor expressing HEK293 and SK-N-MC lines.

Important signaling differences were demonstrated between the CRF receptor-expressing HEK293 and SK-N-MC cells in two functional assays. In cAMP experiments, all agonists were significantly more potent in stimulating cAMP accumulation in $hCRF_1$-SK-N-MC and $hCRF_{2(a)}$-SK-N-MC cells compared to their respective HEK293 counterparts, except for urocortin 3 which was relatively inactive in stimulating signaling at the $hCRF_1$-HEK and $hCRF_1$-SK-N-MC cells. Nevertheless, the CRF receptor-specific rank order potency profiles were not different in both cell lines.

More striking differences were observed when the $hCRF_1$ and $hCRF_{2(a)}$ receptor-expressing cell lines were tested for their ability to promote transient Ca^{2+} mobilization in the FLIPR system. The FLIPR system allows for real-time measurements of agonist-mediated responses. Another advantage of the FLIPR system is its ability to directly measure second messenger responses in contrast to gene reporter assays, which often do not replicate the potency rank order profiles of second messenger assays [40,41]. One disadvantage of the FLIPR assay, however, is the inability of some G_s-coupled GPCRs to respond in this system. Co-expression of the Gα16 subunit has facilitated PLC-mediated signaling for some, but not all, of these receptors [42]. The CRF receptors belong to the B1 GPCR subclass, which comprises G_s-coupled receptors [4]. However, some members of this subclass can couple to PLC in recombinant and/or endogenous expression systems [17,43]. Preliminary studies showed small PLC-signaling of recombinant mammalian, chick and fish CRF_1 but not

CRF_2 receptors [19–21]. Thus, it seemed likely, that the $hCRF_1$ receptor might couple to G_q and respond in the FLIPR system.

We observed robust agonist-stimulated Ca^{2+} transients for $hCRF_1$ and $hCRF_{2(a)}$ receptors in HEK293 but not SK-N-MC cells. This result was surprising for two reasons: (i) $hCRF_1$ receptor expression was higher in SK-N-MC cells; and (ii) a stronger G_s coupling for both receptors was observed in SK-N-MC cells. Since higher receptor expression levels has been proposed to promote coupling to additional second messenger systems [17], it was expected that recombinantly expressed CRF receptors would more readily activate the PLC signaling cascade in SK-N-MC compared to HEK293 cells. We further ruled out that SK-N-MC cells are unable to respond in the FLIPR system by demonstrating that OX_1 and OX_2 receptors strongly coupled to PLC activation when expressed in SK-N-MC cells. We, therefore, concluded that differential coupling of $hCRF_1$ and $hCRF_{2(a)}$ receptors to cAMP and PLC in HEK293 and SK-N-MC cells most likely depends on the endogenous repertoire of G proteins in these lines. $hCRF_1$ and $hCRF_{2(a)}$ receptors appear to couple to G_s in a selective manner in SK-N-MC cells. However, both receptors stimulate cAMP and PLC in HEK293 cells. The hypothesis that CRF receptor-G protein specificity is governed by cellular background is further strengthened by our observation that CRF_1 receptors appear to couple exclusively with G_s in Y79 retinoblastoma cells [28], rat hypothalamic 4B cells endogenously expressing CRF_1 receptors [44], and rat amygdalar AR5 cells endogenously expressing $CRF_{2(a)}$ receptors [45] (F.M. Dautzenberg, unpublished observation). Furthermore, it was recently shown that CRF receptors may differentially bind to either G_q or G_s in vivo in two different mouse strains [46].

When a variety of CRF agonists was tested for their potency to stimulate FLIPR responses in $hCRF_1$-HEK and $hCRF_{2(a)}$-HEK cells, we observed a rank order potency profile virtually identical with the profile obtained in cAMP assays. While oCRF, urocortin 1 and sauvagine were equipotent in stimulating Ca^{2+} transients in $hCRF_1$-HEK cells, urocortins 2 and 3 were inactive. In contrast, urocortins 1 and 2 and sauvagine were up to 10-fold more potent than urocortin 3. In addition, Ca^{2+} transients were stimulated by oCRF only at micromolar concentration in $hCRF_{2(a)}$-HEK cells. The use of high oCRF concentrations was necessary due to the lower potency of all agonists at both receptors in the FLIPR experiments compared to the cAMP assays. In other experiments, we have shown that the CRF_1/CRF_2 nonselective antagonist astressin and the CRF_2-specific antagonist antisauvagine [8] were able to antagonize agonist-mediated FLIPR responses in a receptor-specific manner. At this point it is important to note that urocortin 1 was less efficacious in the cAMP and Ca^{2+} stimulation experiments a phenomenon that we and others have frequently observed (see [8]) and which is likely reflected by its apparent high affinity for G protein-uncoupled and -coupled CRF receptors and likely restricted to recombinant systems [8].

Transient Ca^{2+} mobilization by CRF receptor activation in principle could also be mediated by mechanisms other than PLC activation. For another class B receptor, the glucagon-like peptide 1 (GLP-1) receptor such a phenomenon has been observed [30]. Ca^{2+} mobilization by activation of the receptor was mediated by ryanodine-sensitive Ca^{2+} release channels but not by direct activation of PLC [30]. However, when we tested the ability of ryanodine, Rp-cAMPS, a potent protein kinase A inhibitor and the PLC inhibitor U-73,122 [32] we observed potent inhibition of Ca^{2+} mobilization only in the presence of U-73,122. Furthermore, CRF agonists promoted a small but significant stimulation of IP_3 production in $hCRF_1$- and $hCRF_{2(a)}$-HEK cells. Because of the PLC sensitivity of the Ca^{2+} release we believe that the observed IP_3 production in CRF receptor expressing HEK293 cells accounts for transient Ca^{2+} mobilization. These results clearly demonstrate that the observed activation of Ca^{2+} transients is mediated by direct activation of PLC in HEK293 cells stably expressing CRF receptor.

In conclusion, agonist-activated $hCRF_1$ and $hCRF_{2(a)}$ receptors more potently stimulate cAMP accumulation in SK-N-MC cells compared to HEK293 cells. In contrast, dual cAMP-and PLC-mediated signaling of $hCRF_1$ and $hCRF_{2(a)}$ receptors was observed in HEK293 cells. Despite a rightward shift of the agonist dose–response curves in the FLIPR compared to the cAMP system, we believe that the FLIPR readout is a robust and reproducible system for the functional detection of CRF receptor responses. We further propose that this assay system is suitable for high throughput screening of a large number of chemical molecules.

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