Differential Responses of Corticotropin-Releasing Hormone Receptor Type 1 Variants to Protein Kinase C Phosphorylation

Danijela Markovic, Nikolleta Papadopoulou, Thalia Teli, Harpal Randeva, Michael A. Levine, Edward W. Hillhouse, and Dimitris K. Grammatopoulos

Endocrinology and Metabolism, Division of Clinical Sciences, Warwick Medical School, University of Warwick, Coventry, United Kingdom (D.M., N.P., T.T., H.R., D.K.G.); Division of Pediatrics, The Children’s Hospital of the Cleveland Clinic Foundation, Cleveland, Ohio (M.A.L.); and The Leeds Institute of Health, Genetics and Therapeutics, University of Leeds, Leeds, United Kingdom (E.W.H.)

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ABSTRACT
Corticotropin-releasing hormone (CRH) regulates diverse biological functions in mammals, through activation of two types of specific G protein-coupled receptors that are expressed as multiple mRNA spliced variants. In most cells, the type 1α CRH receptor (CRH-R1α) preferentially activates the Gαs-adenylyl cyclase signaling cascade. CRH-R1α-mediated signaling activity is impaired by insertion of 29 amino acids in the first intracellular loop, a sequence modification that is characteristic of the human-specific CRH-R1β variant. In various tissues, CRH signaling events are regulated by protein kinase C (PKC). The CRH receptors contain multiple putative PKC phosphorylation sites that represent potential targets. To investigate this, we expressed recombinant CRH-R1α or CRH-R1β in human embryonic kidney 293 cells and analyzed signaling events after PKC activation. Agonist (oxytocin) or phorbol 12-myristate 13-acetate-induced activation of PKC led to phosphorylation of both CRH-R1 variants. However, CRH-R1α and CRH-R1β exhibited different functional responses to PKC-induced phosphorylation, with only the CRH-R1β susceptible to cAMP signaling desensitization. This was associated with a significant decrease of accessible CRH-R1β receptors expressed on the cell surface. Both CRH-R1 variants were susceptible to homologous desensitization and internalization following treatment with CRH; however, PKC activation increased internalization of CRH-R1β but not CRH-R1α in a β-arrestin-independent manner. Our findings indicate that CRH-R1α and -R1β exhibit differential responses to PKC-induced phosphorylation, and this might represent an important mechanism for functional regulation of CRH signaling in target cells.

The diverse actions of corticotropin-releasing hormone (CRH) in mammals are mediated through activation of two classes of specific heptahelical G protein-coupled receptors (GPCRs), termed CRH-R1 and CRH-R2 (Chen et al., 1993; Liaw et al., 1995). These are encoded by unique genes that generate multiple variant forms and may encode different receptor isoforms, sometimes in a tissue specific manner (Grammatopoulos and Chrousos, 2002). In human tissues, several CRH-R1-derived mRNA splice variants have been described (R1α, R1β, and R1c-h). Protein sequences of these splice variants predict potential receptors containing various amino acid insertions or deletions, with varying degrees of agonist binding efficiency and signaling as well as truncated or soluble proteins.

Gene knockout studies in mice deficient for the fully active CRH-R1 receptor as well as all potential splice variants have demonstrated that it is principally responsible for mediating the CRH stress response (Timpl et al., 1998). The human homolog CRH-R1α can interact with multiple G proteins to relay signals to diverse intracellular effectors (Grammatopoulos et al., 1999, 2001; Aggelidou et al., 2002). In most tissues, signal transduction of CRH-R1 primarily involves coupling to Gαs-adenylyl cyclase system with subsequent cAMP generation and protein kinase A (PKA) activation. The
human-specific CRH-R1β receptor variant, which is identical to the CRH-R1α except for a 29-amino acid insert in the first intracellular loop, interacts with CRH and Gs with significantly reduced agonist affinity compared with CRH-R1α (Xiong et al., 1995). Like many other splice variants, the CRH-R1β mRNA expression exhibits tissue-specific characteristics and has been identified in anterior pituitary, myometrial smooth muscle cells, and endometrium and human umbilical cord blood mast cells (Chen et al., 1993; Grammatopoulos et al., 1998; Slominski et al., 2001; Karteris et al., 2004; Cao et al., 2005) but not in the placenta, adrenal, and synovium (Karteris et al., 1998, 2001; McEvoy et al., 2001). The function(s) of CRH-R1β and the potential CRH-R1-derived receptor variants is currently unknown. Recent studies investigating the function of soluble CRH-R1 variants such as R1e and R1h suggest that they can modulate CRH-R1 activity and agonist cellular responses (Pisarchik and Slominski, 2004).

Similar to many GPCRs, protein phosphorylation by Ser/Thr kinases can regulate CRH-R1 signaling. PKA-induced phosphorylation of CRH-R1α seems to reduce receptor coupling efficiency to specific G proteins and thus modifies cross-talk between distinct signaling cascades (Papadopoulou et al., 2004). By contrast, multiple G protein-coupled receptor kinases (GRKs) are involved in receptor homologous desensitization and internalization via phosphorylation at specific residues in the C terminus of CRH-R1α (Teli et al., 2005). Protein kinase C (PKC), which is also involved in homologous and heterologous desensitization of GPCRs (Smyth et al., 1998; Caunt et al., 2004), seems to modulate CRH actions in various tissues. For example, oxytocin (OT)-induced PKC activation inhibits CRH-R activity in the human pregnant myometrium at term (Grammatopoulos and Hillhouse, 1999), and recent studies in human neuroblastoma Y79 cells have shown that PKC (possibly α and β variants) is indeed involved in the heterologous, but not homologous, desensitization of the CRH-induced cAMP response (Hauger et al., 2003). However, in other tissues such as the anterior pituitary, AVP-induced PKC activation augments CRH-induced cAMP responses and the transcription of the proopiomelanocortin gene (Bilezikjian et al., 1987; Carvallo and Aguilara, 1989).

The CRH-R1 receptor is a potential target for PKC actions, since it contains several potential PKC phosphorylation sites (Chen et al., 1993) that are identical in CRH-R1 variants with intact intracellular loops and C terminus (R1α, R1β, R1c, and R1d). Given that most tissues endogenously express multiple CRH-R1 mRNA variants, it is possible that PKC exerts distinct effects on different CRH-R1 variants. To test this hypothesis, we expressed recombinant CRH-R1α and CRH-R1β in HEK293 cells (transiently or stably), and we investigated their functional responses following PKC activation.

### Materials and Methods

**Materials.** Radiolabeled ovine (o) CRH and human/rat (h/r) CRH were obtained from Peninsula Laboratories (Bachem Ltd., Merseyside, UK). The mammalian expression vector pcDNA3.1(−) and Lipofectamine were obtained from Invitrogen (Paisley, UK). Dithiothreitol (DTT), GDP, forskolin, MES, 1,4-dioxane, triethylamine, 4-azidoanilide-HCl, 1-(3-dimethylaminopropyl)-3-ethylenecarbodiimide hydrochloride, 3-(aminopropyl)trimethoxysilane, and all other chemicals were purchased from Sigma Chemical (Gillingham, Dorset, UK). Waters Sep-Pak C18 columns were obtained from Millipore (UK) Ltd. (Watford, Hertfordshire, UK). Cyclic AMP assay kits were obtained from DuPont-NEN (Stevenage, Hertfordshire, UK).

**TABLE 1**

<table>
<thead>
<tr>
<th>Type of HEK293 Cells Expressing CRH-R1 Variants</th>
<th>Bmax (nmol/mg protein)</th>
<th>Kd (nM)</th>
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<tbody>
<tr>
<td>293-R1α (transient)</td>
<td>8.5 ± 2.7</td>
<td>1.35 ± 0.4*</td>
</tr>
<tr>
<td>293-R1β (transient)</td>
<td>11.1 ± 2.2</td>
<td>4.75 ± 0.6</td>
</tr>
<tr>
<td>OTR/CHO-R1α (trans)</td>
<td>0.4 ± 1.5</td>
<td>2.7 ± 0.5*</td>
</tr>
<tr>
<td>OTR/CHO-R1β (trans)</td>
<td>1.2 ± 2.1</td>
<td>7.6 ± 0.9</td>
</tr>
<tr>
<td>st293-R1α (stable)</td>
<td>430 ± 87</td>
<td>2.1 ± 0.5*</td>
</tr>
<tr>
<td>st293-R1β (stable)</td>
<td>520 ± 112</td>
<td>5.1 ± 0.8</td>
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</table>

* P < 0.05 compared with CRH-R1β expressed in HEK293 or OTR/CHO cells.

**Fig. 1.** Effects of protein kinase activation on CRH-induced cAMP production in 293-R1α and 293-R1β cells. HEK293 cells transiently expressing CRH-R1α or -R1β receptors were pre-treated with 100 nM CRH for 45 min, 200 nM PMA, or 100 nM indolactam V for 30 min, before subsequent stimulation with various concentrations (0.1–1000 nM) of CRH for 15 min. Cyclic AMP production was determined by RIA. Results are expressed as the mean ± S.E.M. of three estimations and are representative from four individual transfections.
bol 12-myristate 13-acetate (PMA), H89, PKC inhibitors, and the anti-G α polyclonal antibody, raised in rabbits immunized with synthetic peptides corresponding to the C terminus of G α-protein, were obtained from Calbiochem (Merck Biosciences, Nottingham, UK). Protein A-Sepharose (CL-4B) was purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). α-[32P]GTP and reagents for enhanced chemiluminescence were obtained from GE Healthcare. CRH-R1 antibody (polyclonal antibody raised against a peptide mapping at amino acid residues 384 to 397 of human CRH-R1) was purchased from Boehringer Mannheim (East Sussex, UK). Synthetic oligonucleotide probes, polymerase chain reaction, and cloning reagents, Dulbecco’s modified Eagle’s medium culture media and enzymes were purchased from Invitrogen.

Transfection of CRH-R1s and HEK293 Cell Culture. Complementary DNAs for CRH-R1α and -R1β cloned in pcDNA3.1(−) were transiently expressed in HEK293 cells (293-R1α or 293-R1β cells) using the Lipofectamine method as described previously (Grammatopoulos et al., 1999). Using the same method CRH-R1α and -R1β were transiently expressed in Chinese hamster ovary cells (CHO) stably expressing human oxytocin receptors (a gift from Dr. A. Jackson, University of Warwick, Coventry, UK).

For generation of HEK293 cell lines stably expressing CRH-R1α or -R1β, each receptor variant cDNA, cloned in pcDNA3.1(−), was transfected using Lipofectamine reagent (Invitrogen). The cells were grown in DMEM in the presence of 500 μg/ml G418, and those survival were subcultured. A number of these cell lines (st293-R1α or st293-R1β) were selected for characterization of their binding and signaling properties.

Binding, CAMP Assays, and Receptor Desensitization Studies. Binding affinity and maximal binding site concentrations (Bmax) of CRH-R1α and -R1β receptors were assessed in cell membrane preparations from 293-R1α, 293-R1β, st293-R1α, or st293-R1β by Scatchard analysis using [32P]GTP-γ-S. In brief, cells were resuspended in ice-cold CRH binding buffer (50 mM Tris-HCl, pH 7.4, 5 mM EGTA, 10 mM MgCl2, 0.5% (v/v) PMSF, 1 mM DTT, and 100 mM aprotinin) and membrane-rich fractions were prepared as described previously (Hauger et al., 1997). The binding data were analyzed using the computer program EDBDA (McPherson, 1983), which provides initial estimates of equilibrium binding parameters by Scatchard and Hill analyses and then produces a file for the nonlinear curve-fitting program Ligrand (Munson and Rodbard, 1980).

Cyclic AMP stimulation assays of HEK293 cells transiently or stably expressing CRH-R1α or -R1β receptors were carried out as described previously (Grammatopoulos et al., 1999). Cyclic AMP production was measured using a cAMP RIA kit. For desensitization studies, HEK293 cells transiently expressing CRH-R1α or -R1β were plated in 12-well plates, when up to 80% confluent the cells were pretreated with 100 nM hCRH or 200 nM PMA in stimulation buffer (DMEM containing 1 mg/ml 3-isobutyl-1-methylxanthine and 10 mM MgCl2) for 45 or 30 min, respectively. At the end of the incubation period, the medium was removed. The cells were then rinsed with fresh DMEM and incubated with various concentration of CRH (0.1–1000 nM) in the stimulation buffer for 15 min at 37°C. Following extensive washing of cells in 20 volumes of DMEM and centrifugation at 200 g for 10 min (twice) to ensure that excess CRH added during the preincubation period was removed (Hauger et al., 1997). Intracellular cAMP was extracted and measured by RIA as described previously (Grammatopoulos et al., 1999). In some experiments, results were calculated and expressed as percentage of maximal adenylyl cyclase (AC) stimulation (by forskolin) to correct for differences in the AC stimulation between various 293-R1α or 293-R1β cell preparations used.

**In Vitro Phosphorylation of CRH-Rs.** 293-R1α or 293-R1β cells (~5 × 10⁶) were incubated in phosphate-free DMEM containing 300 μCi/ml [32P]orthophosphate for 3 h at 37°C, before the addition of vehicle or 200 nM PMA for 30 min at 37°C. At the end of the incubation period, cells were scraped into ice-cold buffer containing 10 mM Tris, pH 7.4, 5 mM EGTA, 5 mM EDTA, 1 mM PMSF, 10 mg/ml benzamidine, 5 mg/ml leupeptin, 10 mM sodium pyrophosphate, 10 mM NaF, 0.1 mM sodium orthovanadate, and 100 mM okadaic acid, followed by centrifugation at 40,000g for 1 h. The resulting pellet was resuspended in 1 ml of PBS containing 1% Triton X-100, 0.05% SDS, 1 mM EGTA, 1 mM EDTA, 1 mM PMSF, 10 mg/ml benzamidine, 5 mg/ml leupeptin, 10 mM sodium pyrophosphate, 10 mM NaF, 0.1 mM sodium orthovanadate, and 100 mM okadaic acid; the samples were solubilized for 2 h on ice. Solubilized material was preincubated with preimmune serum (1:200) for 1 h, and CRH-Rs were immunoprecipitated with 25 μl of CRH-R1 antibody and 100 μl of protein A-Sepharose beads (4°C overnight). Samples were resuspended in SDS-loading buffer and were subjected to 12% SDS-PAGE and autoradiography (~70°C, 10–14 days) using intensifying screens. Untransfected HEK293 cells were used as negative controls. The specificity of the primary antibody was shown by preabsorption of the primary antibody with a synthetic peptide (1 μM).

**Activated G α-Protein Labeling.** 293-R1α or 293-R1β cells (~5 × 10⁶) were pretreated with vehicle or 200 nM PMA for 30 min at 37°C and exposed to 100 nM CRH for 15 min in the presence of [α-32P]GTP-γ-azidoanilide followed by UV cross-linking. Cell membranes were prepared as describe above, and agonist-induced Gα labeling was carried out as described previously (Grammatopoulos et al., 1999). In brief, [α-32P]GTP-azidoanilide-labeled G proteins were precipitated by centrifugation and solubilized in 120 μl of 2% SDS. Then, 360 μl of 10 mM Tris-HCl buffer, pH 7.4, containing 1% (v/v) Triton X-100, 1% (v/v) deoxycholate, 0.5% (w/v) SDS, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 0.2 mM PMSF, and 10 μg/ml aprotinin was added, and insoluble material was removed by centrifugation. Aliquots of solubilized membranes (100 μl) were incubated with 10 μl of undiluted Gα-protein antisera at 4°C for 2 h under constant rotation. Then, 50 μl of protein A-Sepharose beads (10% (w/v) in the above-mentioned buffer) was added, and the incubation was continued at 4°C for...
Fig. 3. Effect of PKC activators on forskolin-induced cAMP production, CRH-induced Gs-protein activation, and CRH-Rs in vitro phosphorylation, in HEK293 cells expressing CRH-R1 receptor variants. a, treatment of 293-R1α or -R1β cells for 30 min in the presence or absence of PKC activators (PMA or indolactam V) as described under Material and Methods. Results are expressed as the mean ± S.E.M. of five estimations from four individual transfections. 

### TABLE 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>293-R1α</th>
<th>293-R1β</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Maximum AC Stimulation (100 nM CRH)</td>
</tr>
<tr>
<td>Control cells + CRH</td>
<td>3 ± 2.2</td>
<td>61 ± 12.3</td>
</tr>
<tr>
<td>+ PMA/CRH</td>
<td>4.8 ± 0.2</td>
<td>145 ± 21.4*</td>
</tr>
<tr>
<td>+ IndoV/CRH</td>
<td>6 ± 1.2</td>
<td>132 ± 32.3*</td>
</tr>
<tr>
<td>H89-treated cells + CRH</td>
<td>4 ± 0.2</td>
<td>57 ± 7.6</td>
</tr>
<tr>
<td>+ PMA/CRH</td>
<td>3.1 ± 1.5</td>
<td>146 ± 12.1*</td>
</tr>
<tr>
<td>+ IndoV/CRH</td>
<td>3.8 ± 0.9</td>
<td>139 ± 25.1*</td>
</tr>
<tr>
<td>Calphostin C-treated cells + CRH</td>
<td>5 ± 1.2</td>
<td>50 ± 8.9</td>
</tr>
<tr>
<td>+ PMA/CRH</td>
<td>4 ± 0.2</td>
<td>65 ± 12.9</td>
</tr>
<tr>
<td>+ IndoV/CRH</td>
<td>4.7 ± 0.8</td>
<td>59 ± 7.2</td>
</tr>
<tr>
<td>Bisindolymaleimide I-treated cells + CRH</td>
<td>3.2 ± 1.4</td>
<td>53 ± 9.9</td>
</tr>
<tr>
<td>+ PMA/CRH</td>
<td>6 ± 0.7</td>
<td>55 ± 7.6</td>
</tr>
<tr>
<td>+ IndoV/CRH</td>
<td>6.5 ± 0.8</td>
<td>60 ± 5.9</td>
</tr>
</tbody>
</table>

* p < 0.05 compared with cAMP response of cells without any pretreatment.

Effects of PKC and PKA inhibitors on CRH-induced cAMP production
The 293-R1α and 293-R1β cells were treated with various PKC (100 nM calphostin C or 100 nM bisindolylmaleimide I) or PKA (10 μM H89) inhibitors to investigate the effect on CRH-induced cAMP production in the presence or absence of PKC activators (PMA or indolactam V) as described under Material and Methods. Results are expressed as the mean ± S.E.M. of five estimations from four individual transfections.

**TABLE 2**
The 293-R1α and 293-R1β cells were treated with various PKC (100 nM calphostin C or 100 nM bisindolylmaleimide I) or PKA (10 μM H89) inhibitors to investigate the effect on CRH-induced cAMP production in the presence or absence of PKC activators (PMA or indolactam V) as described under Material and Methods. Results are expressed as the mean ± S.E.M. of five estimations from four individual transfections.
overnight under constant rotation. The beads were collected by centrifugation, washed twice with 1 ml of a 50 mM Tris-HCl buffer, pH 7.4, containing 10% Nonidet P-40, 0.5% SDS, and 600 mM NaCl, and then further washed twice with 1 ml of a 100 mM Tris-HCl buffer, pH 7.4, containing 300 mM NaCl and 10 mM EDTA and dried under vacuum in a Speed-Vac microcentrifuge. The immune complexes were dissociated from protein A by reconstitution in 100 μl of Laemmli’s buffer and boiling for 5 min. Samples were then subjected to gel electrophoresis using discontinuous SDS-PAGE slab gels (10% running; 5% stacking). Molecular weight markers dissolved in solubilization buffer were also electrophoresed. The gels were then stained with Coomassie Blue, dried using a slab gel dryer, and exposed to Fuji X-ray film at −70°C for 2 to 5 days for determination of the incorporation of [α-32P]GTP-γ-s-phosphate into stimulated Gs proteins.

Receptor/β-Arrestin Immunofluorescence and Internalization Studies. HEK293 cells transiently or stably expressing CRH-R1α or CRH-R1β receptor variants, seeded on glass cover slips pretreated with 3-(aminopropyl)triethoxy silane, were grown in six-well plates until 70 to 80% confluent. Following treatment with 100 nM CRH (for 45 min at 37°C) and 200 nM PMA (for 30 min at 37°C), cells were fixed with 4% paraformaldehyde in PBS. Cellular distribution of CRH-R immunoreactivity was determined as described previously (Teli et al., 2005). For double immunostaining, after 1-h incubation with CRH-R1 antibody in the presence or absence of blocking peptide (10-fold molar excess) and 15-min wash, the slides were incubated overnight at 4°C with a rabbit polyclonal β-arrestin antiserum (1:50) with or without blocking peptide (10-fold molar excess); following the 15-min wash and incubation with donkey anti-rabbit Alexa-Fluor 488 antibody and donkey anti-goat Alexa-Fluor 594 antibody (1:400 in PBS (0.01%)-Triton X-100), the slides were mounted. The cells were examined under an oil immersion objective (63×) using a Leica DMRE laser scanning confocal microscope with TCS SP2 scan head (Leica Microsystems, Inc., Deerfield, IL). Alexa-Fluor 488 was excited with 488-nm Ar laser at 25% power, and the fluorescence signal was collected with a 500- to 555-nm emission filter. For Alexa-Fluor 594-nm detection, the 543-nm Green HeNe laser at 50% power was used with a 555- to 620-nm emission filter. Optical sections (0.5 μm) were taken, and representative sections corresponding to the middle of the cells are presented. Images were collected in 1026 × 1026 pixels with a scan speed of 400 Hz. The images were manipulated with Leica (5× zoom) and Adobe Photoshop software (Adobe Systems, Mountain View, CA).

For each treatment, between 20 and 30 individual cells in five random fields of view were randomly selected and examined. Fluorescence intensity profiles were generated along multiple line axes, analyzed, and quantified using ImageJ software developed at the National Institutes of Health (http://rsb.info.nih.gov/ij). Relative quantification of intracellular (internalized) CRH-R1 was carried out by measuring the amount of total fluorescence along the longitudinal axis corresponding to the intracellular space (average 4–15 μm). The activated β-arrestin that was translocated to the plasma membrane was quantified by measuring fluorescence along the area corresponding to the cell membrane (1–3 and 19–21 μm). In addition, qualitative (visual) examination of images and manual scoring of protein movement also were carried out in a blinded manner by an independent biomedical laboratory officer of the Molecular Pathology Laboratory (Division of Pathology, University Hospitals Coventry and Warwickshire, NHS Trust, Coventry, UK).

Statistics. The results obtained are presented as the mean ± S.E.M. of each measurement. Data were tested for homogeneity, and comparison between group means was performed by one- or two-way analysis of variance. Probability values of p < 0.05 are considered to be significant.

Results

Effect of PKC Activation on CRH-Induced cAMP Production in CRH-R1α- and -R1β-Expressing Cells. Although capable of activating multiple signaling pathways, the CRH-R1 couple preferentially to Gs to activate the adenyl cyclase signaling cascade. Therefore, we focused on cAMP production following treatment of HEK293 cells transiently expressing CRH-R1α or CRH-R1β receptor variants, with various concentrations of h/rCRH (0.1–1000 nM). CRH-radioreceptor assays using [125I]-labeled CRH showed that the maximum binding site concentrations (B_max) was similar for both receptors (Table 1), confirming that transfection efficiencies were not much different for the two receptors. In agreement with previous data (Xiong et al., 1995), we found that the CRH-R1α isoform was able to bind CRH with 3- to 4-fold greater affinity (K_d for CRH-R1α was 1.35 ± 0.4 nM and for CRH-R1β was 4.75 ± 0.6 nM, p < 0.05). Furthermore, CRH-R1α was significantly more potent than the R1β isoform in stimulating adenyl cyclase activity (maximal cAMP response 61 ± 12.3 and 15 ± 1.3 pmol/ml, respectively). To confirm that CRH-R1α and -R1β signaling is susceptible to homologous desensitization under the experimental conditions used, we assessed the effect of CRH pretreatment on subsequent CRH-induced accumulation of intracellular cAMP. Pretreatment of 293-R1α and 293-R1β cells for 45 min with a single dose of 100 nM CRH resulted in a significant attenuation (maximum inhibition of 68 ± 5 % and 78 ± 9% for the R1α and R1β, respectively) of the subsequent cAMP response to increasing concentrations of CRH (Fig. 1). PKC-dependent effects on CRH-R1 variants were also investigated in 293-R1α and 293-R1β cells, by measuring the effects of PKC activators PMA (200 nM) or indolactam V (100 nM) on CRH-induced cAMP production. Interestingly, PKC activation (by PMA or indolactam V) produced very different effects on signaling by the two CRH-R1 variants without affecting receptor binding characteristics. The CRH-R1α-mediated...
cAMP production was enhanced (170–240%), whereas R18/H9252-mediated cAMP production was significantly reduced (50–70%) (Fig. 1). The phorbol ester 4a-phorbol-12,13-didecanoate (100–500 nM), which does not activate PKC, had no effect on either CRH-R1α- or CRH-R1β-dependent activation of adenylyl cyclase (data not shown). Similar experiments were carried out in HEK293 cells stably expressing CRH-R1α or -R1β receptors, with significantly greater concentration of cell-surface binding sites than the transient expression cellular systems and comparable affinity for agonist binding (Table 1). The different cAMP functional responses of CRH-R1α or -R1β to PMA pretreatment were also evident in st293-R1α or st293-R1β cells (data not shown).

The effect of agonist-dependent PKC activation on CRH-R1α or -R1β activity was tested by transient expression of each CRH-R1 variant in CHO cells stably expressing the oxytocin receptor (OTR) (OTR/CHO-R1α or OTR/CHO-R1β). Scatchard analysis using radiolabeled oCRH, for each of the CRH-R1 receptor variants, confirmed that transient expression in the OTR/CHO cellular system did not significantly alter the binding affinity of each CRH-R1 receptor variant. The maximum binding site concentrations ($B_{max}$) were also found to be similar, confirming that transfection efficiencies were similar for both receptors. However, we noticed consistently lower levels of either CRH-R1α or -R1β expression compared with the HEK293 expression system (Table 1).

Pretreatment with OT at concentrations greater than 10 nM, sufficient to stimulate PKC activity (data not shown), increased CRH-induced cAMP production by 60 to 80% in OTR/CHO-R1α but desensitized the CRH response in OTR/CHO-R1β cells by 45 to 60% (Fig. 2). These results demonstrated the ability of agonists that activate PKC to differentially modulate CRH-R1 isoform function.

Phorbol esters can induce various biological effects in addition to PKC activation (Caloca et al., 2001); therefore, the specificity of PMA actions on the CRH-R1 variants was evaluated by the use of PKC inhibitors. Preincubation of 293-R1α and 293-R1β cells with 100 nM calphostin C or 100 nM bisindolylmaleimide I, but not with the PKA inhibitor H89 (10 μM), markedly inhibited the PMA or indolactam V (PKC activators) effects on CRH-R1α and -R1β function as measured by dose-dependent increase of cAMP levels following...
CRH stimulation (Table 2). No significant difference was found in the potency of the two inhibitors. Similar results were obtained when st293-R1α or st293-R1β cells were used (data not shown).

**Effect of PKC Activation on CRH-R1α and -R1β Phosphorylation and Receptor Coupling to Gα,β,γ.** PKC can modulate GPCR signaling by phosphorylation of specific isoforms of AC (Yoshimasa et al., 1987). The effect of PMA-induced PKC activation on AC was analyzed directly using forskolin, a diterpene activator of AC. In both 293-R1α and 293-R1β, PMA caused a modest (44 ± 10%) increase in forskolin-stimulated cAMP production (Fig. 3a). Because the direct effect of PKC on AC activity could not explain the differential response of the CRH-R1α and -R1β, activation of Gα-protein was determined by measurement of CRH-dependent binding of [α-32P]GTP-γ-azidoanilide to Gα for each CRH-R1 variant. PMA treatment for 30 min increased CRH-induced Gα activation by 180 ± 10% in 293-R1α cells, but it decreased Gα activation by 65 ± 6% in 293-R1β cells (Fig. 3b). Immunoprecipitation of CRH-R1α or CRH-R1β, after PMA treatment in the presence of 32P, demonstrated that both R1 variants were phosphorylated following PKC activation (Fig. 3c), suggesting that phosphorylation of the receptors might explain their contrasting behavior. In the absence of PMA pretreatment, some phosphorylation was evident for both CRH-R1 variants, indicative of either basal protein kinase activity targeting CRH-R1. Untransfected HEK293 cells were used as a negative control to confirm the specificity of the anti-CRH-R1 antibodies.

**Effect of PKC Activation on CRH-R1α and CRH-R1β Internalization Characteristics.** To investigate further the differential response of CRH-R1 variants to PKC phosphorylation and potential changes in the receptor internalization characteristics, indirect immunofluorescence was used with specific CRH-R1 and -R1β-antibodies to monitor distribution of the transfected receptor and endogenous β-arrestin. In some experiments, antibodies were coincubated with synthetic blocking peptides corresponding to the immunizing peptides (Fig. 4). Results showed almost complete inhibition of fluorescent signal, confirming the specificity of fluorescent immunostaining (Fig. 4). Under basal conditions, both CRH-R1α and -R1β receptors were exclusively localized on the cell surface of HEK293 cells (Fig. 5a). This was demonstrated by the peak of red fluorescence at the point where the line demarcated the cell membrane (1–3 and 19–21 μm, respectively). Treatment of HEK293 cells transiently expressing recombinant CRH-R1α with 100 nM CRH for 45 min elicited a significant redistribution of cellular immunostaining, indicative of receptor internalization (Fig. 5b). This was illustrated by increased amount of red fluorescence throughout the intracellular space (4–18 μm). Identical results were shown in cells expressing CRH-R1β (Fig. 5b). In addition, in the absence of agonist activation, PMA treatment induced receptor internalization only in HEK293 cells transiently expressing recombinant CRH-R1β receptor variant but not R1α (Fig. 5c). These observations were confirmed by quantification of intracellular fluorescence spectra of 20 individual cells that were randomly selected (Fig. 6). These results were additionally confirmed by using a manual scoring of protein movement (0, no staining to 5, substantial cytoplasmic staining) by an independent observer (data not shown).

**Fig. 6.** Relative quantification of CRH-R1α and CRH-R1β endocytosis following CRH and PMA treatment. For each treatment, 20 individual cells in five random fields of view, were examined and CRH-R1 fluorescence intensity measurements generated. Cytoplasmic fluorescence intensity of CRH-R immunostaining (red) was measured, by summing the spectral measurement (distance 4–18 μm).

The involvement of endogenous β-arrestin in receptor desensitization/internalization was also investigated. In both 293-R1α and -R1β unstimulated cells, β-arrestin immunofluorescence (green) was widely distributed in the cytoplasm, whereas CRH-R1 immunofluorescence (red) was confined to the plasma membrane (Figs. 7a and 8a). In both CRH-R1α and -R1β cellular systems, CRH treatment elicited a significant and rapid (within 2 min of CRH treatment) translocation of β-arrestin to the plasma membrane, where it colocalized with CRH-R1α or -R1β, as demonstrated by a significant increase in plasma membrane immunostaining of β-arrestin signal and the appearance of yellow signal in the overlap image (Figs. 7b and 8b, top). This was confirmed by quantification of fluorescence that showed an increased green fluorescence at the point where the line demarcated the cell membrane (1–3 and 19–21 μm, respectively). Within 30 min, a significant pool of CRH-R1α and -R1β receptors was internalized. Interestingly a fraction of receptors (both CRH-R1α and -R1β) seemed to be colocalized with cytosolic β-arrestin (Figs. 6b and 7b, bottom). This was also evident in the analysis of fluorescence spectra where some (but not all) intensity peaks of green and red fluorescence could be observed at the same position.

In both 293-R1α and -R1β cellular models, PMA treatment for 2 to 30 min did not affect β-arrestin cellular distribution (Figs. 7c and 8c). As expected, PMA treatment did not alter CRH-R1α cellular distribution. In contrast, PMA induced CRH-R1β internalization demonstrated by a significant redistribution of cellular immunostaining, indicative of receptor internalization. These observations were again confirmed by quantification of intracellular fluorescence spectra of 20 individual cells which were randomly selected (Figs. 7 and 8, insets). Collectively, these results suggest that CRH-R1β internalization occurs without β-arrestin involvement. Identical results were obtained when st293-R1α and st293-R1β cells were used (data not shown).

**Discussion**

The human specific CRH-R1β receptor splice variant is identical to the CRH-R1α except for a 29-amino-acid insert in
the first intracellular loop (IC)1 of the R1β, which results in impaired agonist binding and G protein coupling (Xiong et al., 1995). Indeed, our data suggest that the CRH-R1α receptor can only weakly activate the Gs-protein/adenylyl cyclase pathway, in agreement with published data (Xiong et al., 1995), and they show no significant coupling to Gi-, Gq-, or Go proteins (unpublished data). Lack of CRH-R1α receptor isoform-specific antibodies has prevented the conclusive demonstration of CRH-R1α protein expression in native tissues. Regardless of whether this receptor transcript is significantly expressed as protein product, possible increased expression of the CRH-R1α receptor transcript at the expense of the wild-type receptor at transcription would result in reduced levels of fully functional CRH-R1 and decreased tissue responsiveness to CRH. To the best of our knowledge, no tissue has been identified where CRH-R1α variant mRNA is exclusively expressed in the absence of CRH-R1β mRNA, suggesting the presence of splicing mechanisms controlling the balance of CRH-R1α and CRH-R1β mRNA expression levels. CRH-R1β functional role is uncertain; since the binding affinity of this CRH-R1 variant is significantly lower than the circulating CRH levels, one can hypothesize that this receptor variant cannot be activated by CRH and that induction of high levels of CRH-R1β in certain tissues would render these

Fig. 7. CRH-R1α and β-arrestin subcellular distribution following CRH or 200 nM PMA: visualization by fluorescent confocal microscopy. HEK293 cells transiently expressing CRH-R1α were stimulated with 100 nM CRH or 200 nM PMA for 2 to 30 min. CRH-R1α and β-arrestin distribution was monitored over the ensuing time period by indirect double immunofluorescence using specific primary antibodies and Alexa-Fluor 594 secondary antibody for CRH-R1 (red) and Alexa-Fluor 488 secondary antibody for β-arrestin (green). Colocalization shows up as yellow in the overlap image. Identical results were obtained from four independent experiments. Scale bar, 10 μm. Representative profiles of fluorescence intensity, generated along the lines depicted in the overlap images by using ImageJ software, are also shown. Inset, for quantification of cytoplasmic CRH-R1 and plasma membrane β-arrestin distribution, 20 individual cells in five random fields of view were examined, and the sum of fluorescence intensity of either cytoplasmic (distance 4–18 μm) or plasma membrane (1–3 and 19–21 μm) fluorescence was measured. Results are expressed as the mean ± S.E.M. of three estimations from 20 individual cells. *p < 0.05 compared with untreated values.
refractory to the actions of CRH. However, local levels of CRH expression might be considerably higher than those found in peripheral blood, and under certain conditions, CRH peptide output might reach sufficiently high levels to achieve CRH-R1/H9252 activation. At present, there are no data about the ratio of expression of the R1/H9251 and R1/H9252 receptor proteins in tissues, probably reflecting the methodological difficulties mentioned above; however, preliminary data from our laboratory have identified a specific mechanism involving progesterone that regulates the ratio of CRH-R1/H9251/R1/H9252 mRNA expression in human myometrial smooth muscle cells during pregnancy (Karteris et al., 2003).

In many cellular systems, CRH actions are modulated by PKC, and accumulating evidence suggests that the CRH-R1 is indeed a target of PKC-induced phosphorylation (Pisarchik and Slominski, 2004). Since most tissues and native cells express multiple CRH-R1 variants (Grammatopoulos et al., 1998, Pisarchik and Slominski, 2001), we created a model system in HEK293 cells to study independently PKC-mediated effects on functional activity of the CRH-R1α and -R1β. This study provides novel evidence that although both R1α and R1β CRH-R1 splice variants are susceptible to PKC-mediated phosphorylation, they exhibit differential functional responses to phorbol ester- (PMA) or agonist (oxytocin)-induced activation of PKC, with only the CRH-R1β susceptible to signaling desensitization and internalization. In contrast, CRH-R1α ability to stimulate the Gαs-AC pathway and cAMP production is enhanced in response to PKC

![Fig. 8. CRH-R1β and β-arrestin subcellular distribution following CRH or 200 nM PMA: visualization by fluorescent confocal microscopy. HEK293 cells transiently expressing CRH-R1β receptor variant were stimulated with 100 nM CRH or 200 nM PMA for 2 to 30 min. CRH R1β and β-arrestin distribution was monitored over the ensuing time period by indirect double immunofluorescence using specific primary antibodies and Alexa-Fluor 594 secondary antibody for CRH-R1 (red) and Alexa-Fluor 488 secondary antibody for β-arrestin (green). Colocalization shows up as yellow in the overlap image. Some images are presented with cell nuclei stained with the DNA-specific dye DAPI (blue). Identical results were obtained from four independent experiments. Scale bar, 10 μm. In some experiments, profiles of fluorescence intensity were generated along the lines depicted in the overlap images, by using ImageJ software. Inset, for quantification of cytoplasmic CRH-R1 and plasma membrane β-arrestin distribution, 20 individual cells in five random fields of view, were examined and the sum of fluorescence intensity of either cytoplasmic (distance 4–18 μm) or plasma membrane (1–3 and 19–21 μm) fluorescence was measured. Results are expressed as the mean ± S.E.M. of three estimations from 20 individual cells. * p < 0.05 compared with untreated values.](#)
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PKC-mediated phosphorylation can regulate the functional responsiveness of GPCRs by initiating heterologous desensitization as well as internalization and down-regulation of many GPCRs (Hipkin et al., 2000; Bhattacharyya et al., 2002). There is evidence that PKC can initiate receptor desensitization, directly or indirectly, via transactivation of specific GGR isoforms, facilitating GRK translocation to the membrane or enhancing GRR activity (Hubbard et al., 2000; Krasel et al., 2001; Mundell et al., 2004). Phosphorylation by PKC may serve as a disparate mechanism for regulating GRK activity, thus providing the cell with a mechanism by which specific homologous desensitization can be regulated heterologously (Xiang et al., 2001). Our previous studies suggest that CRH-R1α homologous desensitization involves multiple GRK isoforms (Teli et al., 2005), thus potential PKC-GRK interactions might also modulate heterologous CRH-R1β desensitization.

Confocal microscopy studies in 293-R1α and 293-R1β cells revealed that both CRH-R1 variants were susceptible to homologous desensitization and internalization, demonstrating for the first time that the CRH-R1β can retain some normal GPCR functional characteristics despite its reduced binding and signaling activity. Agonist-activation of both CRH-R1 variants was associated with initial recruitment of β-arrestin to the plasma membrane and colocalization with the CRH-R1, in agreement with previous studies (Rasmussen et al., 2004; Teli et al., 2005; Holmes et al., 2006), providing also indirect evidence that the intracellular mechanisms inducing β-arrestin translocation to the plasma membrane are independent of CRH-R1 signaling potency. Our studies also suggest that a fraction of internalized CRH-R1α and R1β receptors colocalize with β-arrestin raising the possibility of distinct pathways (β-arrestin-dependent and -independent) involved in CRH-R1 trafficking, in agreement with previous studies (Perry et al., 2005; Holmes et al., 2006). Furthermore, our data suggest that PKC activation leads to reduced expression of cell surface CRH-R1β, but not CRH-R1α, receptors available for agonist binding by inducing heterologous receptor endocytosis. This might have a significant contribution to the diminished functional response of CRH-R1β following PKC activation. In addition, the finding that PMA-induced CRH-R1β desensitization and internalization was not associated with recruitment of β-arrestin to the plasma membrane points toward the presence of alternative β-arrestin-independent pathways that are activated in response to PKC phosphorylation of the CRH-R1.

In conclusion, we identified a novel mechanism regulating CRH-R1 signaling activity using the ability of the CRH-R1 gene to generate receptor variants with distinct responses to PKC-induced phosphorylation. It seems that the response of CRH-R1 to PKC and ultimately the tissue sensitivity to CRH is dependent on the splicing pattern of the CRH-R1. Signals that promote increased CRH-R1α expression would potentially increase tissue sensitivity to CRH actions via the amplifying effect of signals activating PKC (e.g., AVP in anterior pituitary cells) and inducing CRH-R1α-Gα-protein interactions. In contrast, increased expression of CRH-R1β (e.g., in pregnant myometrium at term that is associated with inhibition of progesterone activity) will reduce tissue sensitivity to CRH actions due to the presence of signaling-impaired CRH-Rs that are susceptible to PKC-induced desensitization and internalization.

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Address correspondence to: Dr. Dimitris Grammatopoulos, Department of Biological Sciences, Sir Quinton Hazell Molecular Medicine Research Centre, The University of Warwick, Gibbet Hill Rd., Coventry CV4 7AL, UK. E-mail: d.grammatopoulos@warwick.ac.uk