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**Differential responses of CRH receptor type 1 variants to PKC
phosphorylation**

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Abbreviations: CRH: corticotropin-releasing hormone; PKC: protein kinase C; PKA: protein kinase A; GPCR: G-protein coupled receptor; PMA: phorbol 12-myristate 13-acetate; HEK293 cells: human embryonic kidney 293 cells; CHO cells: chinese hamster ovary cells.

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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 1 α CRH receptor (CRH-R1 α) preferentially activates the G_s-adenylyl cyclase signalling cascade. CRH-R1 α -mediated signalling activity is impaired by insertion of 29-amino acids in the 1st intracellular loop, a sequence modification that is characteristic of the human-specific CRH-R1 β variant. In various tissues, CRH signalling 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-R1 α or CRH-R1 β in HEK-293 cells and analyzed signalling events after PKC activation. Agonist (oxytocin) or PMA-induced activation of PKC led to phosphorylation of both CRH-R1 variants. However, CRH-R1 α and CRH-R1 β exhibited different functional responses to PKC-induced phosphorylation, with only the CRH-R1 β susceptible to cAMP signalling desensitization. This was associated with a significant decrease of accessible CRH-R1 β 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-R1 β but not CRH-R1 α in a β -arrestin independent manner. Our findings indicate that CRH-R1 α and R1 β exhibit differential responses to PKC-induced phosphorylation and this might represent an important mechanism for functional regulation of CRH signalling in target cells.

INTRODUCTION

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 (R1 α , R1 β , 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 signalling, 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 homologue CRH-R1 α can interact with multiple G proteins to relay signals to diverse intracellular effectors (Grammatopoulos et al., 1999; Grammatopoulos et al., 2001; Aggelidou et al., 2002). In most tissues, signal transduction of CRH-R1 primarily involves coupling to G_s-adenylyl cyclase system with subsequent cAMP generation and protein kinase A (PKA) activation. The human-specific CRH-R1 β receptor variant, which is identical to the CRH-R1 α except for a 29 amino acid insert in the first intracellular loop, interacts with CRH and G_s with significantly reduced agonist affinity compared to CRH-R1 α (Xiong et al., 1995). Like many other splice variants, the CRH-R1 β mRNA expression exhibits tissue-specific characteristics and has been identified in anterior pituitary, myometrial smooth muscle cells, endometrium and human umbilical cord blood mast cells (Chen et al., 1993; Grammatopoulos et al., 1998; Karteris et al., 2004; Cao et al., 2005; Slominski et

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al., 2001) but not in the placenta, adrenal and synovium (Karteris et al., 1998; Karteris et al., 2001; McEvoy et al., 2001). The function(s) of CRH-R1 β 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 α activity and agonist cellular responses (Pisarchik and Slominski, 2004).

Similar to many GPCRs, protein phosphorylation by Ser/Thr kinases can regulate CRH-R1 signalling. PKA-induced phosphorylation of CRH-R1 α appears to reduce receptor coupling efficiency to specific G-proteins and thus modifies cross-talk between distinct signalling 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-R1 α (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), appears 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; Carvalho and Aguilera, 1989).

The CRH-R1 receptor is a potential target for PKC actions, since it contains several potential PKC phosphorylation sites (Chen et al., 1993) which are identical in CRH-R1

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variants with intact intracellular loops and C-terminus (R1 α , R1 β , R1c and R1d). Given that most tissues endogenously express multiple CRH-R1 mRNA variants, it is possible that PKC exert distinct effects on different CRH-R1 variants. To test this hypothesis, we expressed recombinant CRH-R1 α and CRH-R1 β in HEK293 cells (transiently or stably) and we investigated their functional responses following PKC activation.

MATERIALS AND METHODS

Materials: Radioiodinated ovine (o) CRH and human/rat (h/r) CRH were obtained from Peninsula Laboratories (Bachem Ltd, St Helens, Merseyside, UK). The mammalian expression vector pcDNA3.1(-) and Lipofectamine were obtained from Invitrogen (Invitrogen Ltd, Inchinnan Business Park, Paisley, UK). Dithiothreitol (DTT), GDP, forskolin, 2-[N-morpholino]ethane sulphonic acid (MES), 1,4-dioxane, triethylamine, 4-azidoanilide-HCl, 1-(3-dimethylamino propyl)-3-ethylenecarbodiimide hydrochloride (NDEC), 3-(aminopropyl)triethoxy silane (APES) and all other chemicals were purchased from Sigma-Aldrich Chemicals Company Ltd (Gillingham, Dorset, UK). Waters Sep-Pak C18 columns were obtained from Millipore (UK) Ltd (Watford, Herts, UK). Cyclic AMP assay kits were obtained from Dupont-NEN (Stevenage, Hertfordshire, UK). Phorbol 12-myristate 13-acetate (PMA), H-89 (N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide-2HCl), PKC inhibitors and the anti-Gs α polyclonal antibody, raised in rabbits immunized with synthetic peptides corresponding to the C-terminus of Gs α -protein, were obtained from Calbiochem (Merck Biosciences, Beeston, Nottingham, UK). Protein-A sepharose (CL-4B) was purchased from Amersham-Pharmacia Biotech AB (GE Healthcare, Chalfont St.Giles, Bucks, UK). [α -³²P]-GTP and reagents for enhanced chemiluminescence (ECL) were obtained from Amersham International (Amersham Place, Little Chalfont, Bucks, UK). CRH-R1 antibody (polyclonal antibody raised against a peptide mapping at the C-terminus of human CRH-R1) was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, San Diego, CA, USA). β -arrestin antibody (raised against a peptide mapping at amino acid residues 384-397 of human beta-arrestin2) and blocking peptide were from Abcam Plc (Cambridge Science Park, Cambridge, UK). The Alexa-Fluor® 594 and 488 antibodies were obtained from Molecular Probes-Invitrogen (Inchinnan Business Park, Paisley,

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UK). The DNA 3'-end labelling kit was purchased from Boehringer Mannheim (Lewes, East Sussex, UK). Synthetic oligo-nucleotide probes, PCR and cloning reagents, Dulbecco's modified Eagle's medium culture media and enzymes were purchased from Gibco-Invitrogen (Invitrogen Ltd, Inchinnan Business Park, Paisley, UK).

Transfection of CRH-R1s and HEK293 cell culture: Complementary DNAs for CRH-R1 α and R1 β cloned in pcDNA3.1(-) were transiently expressed in HEK 293 cells (293-R1 α or 293-R1 β cells) using the Lipofectamine method as previously described (Grammatopoulos et al., 1999). Using the same method CRH-R1 α and R1 β were transiently expressed in Chinese hamster ovary cells (CHO) stably expressing human oxytocin receptors (gift from Dr A. Jackson, University of Warwick).

For generation of HEK293 cell lines stably expressing CRH-R1 α or R1 β , each receptor variant cDNA cloned in pcDNA3.1(-), was transfected using Lipofectamine reagent (Life Technologies). The cells were grown in DMEM in the presence of G418 (500 μ g/ml) and those survived were subcultured. A number of these cell lines (st293-R1 α or st293-R1 β) were selected for characterisation of their binding and signalling properties.

Binding, cAMP assays and receptor desensitization studies: Binding affinity and maximum binding site concentrations (B_{max}) of CRH-R1 α and R1 β receptors was assessed in cell membrane preparations from 293-R1 α , 293-R1 β , st293-R1 α or st293-R1 β , by Scatchard analysis using ¹²⁵I-oCRH. Briefly, cells were re-suspended in ice-cold CRH binding buffer (50 mM Tris-HCl-pH 7.4, 5 mM EGTA, 10 mM MgCl₂, 1 mM PMSF, 1mM DTT, 100 IU/ml aprotinin and membrane-rich fractions were prepared as previously described (Hauger et al., 1997). The binding data were analyzed using the computer program EBDA (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 Ligand (Munson and Rodbard, 1980).

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Cyclic AMP stimulation assays of HEK293 cells transiently or stably expressing CRH-R1 α or R1 β receptors were carried out as previously described (Grammatopoulos et al., 1999). Cyclic AMP production was measured using a cAMP RIA kit. For desensitization studies, HEK293 cells transiently expressing CRH-R1 α or R1 β were plated in 12-well dishes, when up to 80% confluent the cells were pre-treated with h/r CRH (100 nM) or PMA (200 nM) in stimulation buffer (DMEM containing 1mg/ml 3-isobutyl-1-methylxanthine (IBMX) and 10mM MgCl₂) for 45 or 30 min, respectively. At the end of the incubation period, the medium was removed; the cells were rinsed with fresh DMEM, and then 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 10min (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 previously described (Grammatopoulos et al., 1999). In some experiments, results were calculated and expressed as % of maximum adelylyl cyclase (AC) stimulation (by forskolin) to correct for differences in the AC stimulation between various 293-R1 α or 293-R1 β cell preparations used.

In vitro phosphorylation of CRH-Rs: 293-R1 α or 293-R1 β cells (approx 5x10⁹) were incubated in phosphate-free DMEM containing 300 μ Ci/ml [³²P] orthophosphate for 3h 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 nM okadaic acid, followed by centrifugation at 40,000 x g for 1 h. The resulting pellet was resuspended in 1 ml PBS containing 1% Triton X-100, 0.05% SDS, 1 mM EGTA, 1

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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 nM okadaic acid; the samples were solubilized for 2 h on ice. Solubilized material was pre-incubated with pre-immune serum (1:200) for 1 h and CRH-Rs were immunoprecipitated with 25 μ l of CRH-R1 Ab and 100 μ l 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 HEK-293 cells were used as negative controls. The specificity of the primary antibody was shown by preabsorbtion of the primary antibody with a synthetic peptide (1 μ M).

Activated $G_{s\alpha}$ -protein labelling: 293-R1 α or 293-R1 β cells (approx 5×10^9) were pre-treated 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 - 32 P]-GTP- γ -azidoanilide followed by UV-cross-linking. Cell membranes were prepared as describe above and agonist-induced G_s -labelling was carried out as previously described (Grammatopoulos et al., 1999). Briefly, 32 P-GTP-AA-labelled G-proteins were precipitated by centrifugation and solubilized in 120 μ l of 2% SDS. Then 360 μ l 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, 10 μ g/ml aprotinin were added and insoluble material was removed by centrifugation. Aliquots of solubilized membranes (100 μ l) were incubated with 10 μ l of undiluted $G_{s\alpha}$ -protein antiserum at 4°C for 2 h under constant rotation. Then 50 μ l of protein A Sepharose beads (10% w/v in the above buffer) were added and the incubation was continued at 4°C 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% NP-40, 0.5% SDS, 600 mM NaCl and then were further washed twice with 1 ml of a 100 mM Tris-HCl buffer, pH 7.4, containing 300 mM NaCl, 10 mM EDTA

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and dried under vacuum in a Speed-Vac microconcentrator. The immune complexes were dissociated from protein A by reconstitution in Laemmli's buffer (100 μ l) 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 2–5 days for determination of the incorporation of [α - ^{32}P]-GTP- γ -azidoanilide into stimulated G_s proteins.

Receptor/ β -arrestin immunofluorescence and internalization studies: HEK293 cells transiently or stably expressing CRH-R1 α CRH-R1 β receptor variants, seeded on glass cover slips pre-treated with 3-(aminopropyl)triethoxy silane (APES), were grown in 6-well plates until 70-80% confluence. 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 previously described (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 (x63) using a Leica DMRE laser scanning confocal microscope with TCS SP2 scan head. Alexa-Fluor[®] 488 was excited with 488-nm Ar laser at 25% of power, and the fluorescent signal was collected with a 500-535 nm emission filter. For Alexa-Fluor[®] 594nm detection, the 543 nm Green HeNe laser at 50% of power was

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used with a 555-620 nm emission filter. Optical sections (0.5 μm) were taken and representative sections corresponding to the middle of the cells were presented. Images were collected in 1026x1026 pixels with a scan speed of 400 Hz. The images were manipulated with Leica (5x zoom) and Adobe Photoshop software.

For each treatment, between 20-30 individual cells in five random fields of view, randomly selected and examined. Fluorescence intensity profiles were generated along multiple line axes, analyzed and quantified using Image J 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 4-18 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, was also carried out in a blind fashion by an independent Biomedical Laboratory Officer of the Molecular Pathology Laboratory, Division of Pathology, University Hospitals Coventry and Warwickshire, NHS Trust.

Statistics: The results obtained are presented as the mean \pm SEM of each measurement. Data were tested for homogeneity and comparison between group means was performed by one or two-way analysis of variance (ANOVA) and probability values of $p < 0.05$ were considered to be significant.

RESULTS

Effect of PKC activation on CRH-induced cAMP production in CRH-R1 α and R1 β expressing cells

Although capable of activating multiple signalling pathways, the CRH-R1 couples preferentially to G_s to activate the adenylyl cyclase signalling cascade. Therefore, we focused on cAMP production following treatment of HEK293 cells transiently expressing CRH-R1 α or CRH-R1 β receptor variants, with various concentrations of h/rCRH (0.1-1000 nM). CRH-radioreceptor assays using ¹²⁵I-oCRH 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-R1 α isoform was able to bind CRH with 3-4 fold greater affinity (K_d for CRH-R1 α was 1.35±0.4nM, and for R1 β was 4.75±0.6nM, *p*<0.05). Furthermore, CRH-R1 α was significantly more potent than the R1 β isoform in stimulating adenylyl cyclase activity (maximal cAMP response 61±12.3 and 15±1.3 pmol/ml, respectively). To confirm that CRH-R1 α and R1 β signalling is susceptible to homologous desensitization under the experimental conditions used, we assessed the effect of CRH pre-treatment on subsequent CRH-induced accumulation of intracellular cAMP. Pre-treatment of 293-R1 α and 293-R1 β 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 R1 α and R1 β , 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-R1 α and 293-R1 β 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 signalling by the two CRH-R1 variants

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without affecting receptor-binding characteristics. The CRH-R1 α -mediated cAMP production was enhanced (170-240%), whereas R1 β -mediated cAMP production was significantly reduced (50-70%) (Fig.1). The phorbol ester 4 α -PDD (100-500 nM), which does not activate PKC, had no effect on either CRH-R1 α or CRH-R1 β dependent activation of adenylyl cyclase (*data not shown*). Similar experiments were carried out in HEK293 cells stably expressing CRH-R1 α or R1 β 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 response of CRH-R1 α or R1 β to PMA pretreatment were also evident in *st293*-R1 α or *st293*-R1 β cells (*data not shown*).

The effect of agonist-dependent PKC activation on CRH-R1 α or R1 β activity was tested by transient expression of each CRH-R1 variant in Chinese hamster ovary (CHO) cells stably expressing the oxytocin receptor (OTR) (OTR/CHO-R1 α or OTR/CHO-R1 β). Scatchard analysis using radiolabelled 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-R1 α or R1 β expression compared to the HEK293 expression system (Table 1).

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

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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. Pre-incubation of 293-R1 α and 293-R1 β cells with calphostin C (100 nM) or bisindolylmaleimide I (100 nM), but not with the PKA inhibitor H-89 (10 μ M), markedly inhibited the PMA or indolactam V (PKC activators) effects on CRH-R1 α and R1 β 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 *st*293-R1 α or *st*293-R1 β cells were used (*data not shown*).

Effect of PKC activation on CRH-R1 α and R1 β phosphorylation and receptor coupling to G_s α

PKC can modulate GPCR signalling by phosphorylation of specific isoforms of adenylyl cyclase (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-R1 α and 293-R1 β , PMA caused a modest (44 \pm 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-R1 α and R1 β , activation of G_s α -protein was determined by measurement of CRH-dependent binding of [α -³²P]-GTP- γ -azidoanilide to G_s α for each CRH-R1 variant. PMA treatment for 30 min increased CRH-induced G_s α activation by 180 \pm 10% in 293-R1 α cells, but decreased G_s α activation by 65 \pm 6% in 293-R1 β cells (Fig. 3b). Immunoprecipitation of CRH-R1 α or R1 β , 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

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might explain their contrasting behaviour. 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-R1 α and R1 β 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 employed using specific CRH-R1 and β -arrestin antibodies to monitor distribution of the transfected receptor and endogenous β -arrestin. In some experiments, antibodies were co-incubated 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-R1 α and R1 β 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 μ m, respectively). Treatment of HEK293 cells transiently expressing recombinant CRH-R1 α with 100 nM of 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 μ m). Identical results were shown in cells expressing CRH-R1 β (Fig.5b). In addition, in the absence of agonist activation, PMA treatment induced receptor internalization only in HEK293 cells transiently expressing recombinant CRH-R1 β receptor variant but not R1 α (Fig.5c). These observations were confirmed by quantification of intracellular fluorescence spectra of 20 individual cells which were

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randomly selected (Fig.6). These results were additionally confirmed by using a manual scoring of protein movement (0-no staining, 5-substantial cytoplasmic staining) by an independent observer (*data not shown*).

The involvement of endogenous β -arrestin in receptor desensitization/internalization was also investigated. In both 293-R1 α and R1 β unstimulated cells β -arrestin immunofluorescence (green) was widely distributed in the cytoplasm, whereas CRH-R1 immunofluorescence (red) was confined to the plasma membrane (Fig.7a and 8a). In both CRH-R1 α and R1 β 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 co-localised with CRH-R1 α or R1 β , as demonstrated by a significant increase in plasma membrane immunostaining of β -arrestin signal and the appearance of yellow signal in the overlap image (Fig.7b and 8b, *top panel*). 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 μ m, respectively). Within 30min a significant pool of CRH-R1 α and R1 β receptors were internalised. Interestingly a fraction of receptors (both CRH-R1 α and R1 β) appeared to be co-localized with cytosolic β -arrestin (Fig.6b and 7b, *bottom panel*). 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-R1 α and R1 β cellular models, PMA treatment for 2-30 min did not affect β -arrestin cellular distribution (Fig.7c and 8c). As expected, PMA treatment did not alter CRH-R1 α cellular distribution. In contrast, PMA induced CRH-R1 β internalization demonstrated by a significant redistribution of cellular immunostaining, indicative of receptor internalization. These observations were again confirmed by quantification of

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intracellular fluorescence spectra of 20 individual cells which were randomly selected (Fig.7 and 8 *insets*).

Collectively, these results suggest that CRH-R1 β internalization occurs without β -arrestin involvement. Identical results were obtained when *st293*-R1 α and *st293*-R1 β cells were used (*data not shown*).

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DISCUSSION

The human specific CRH-R1 β receptor splice variant is identical to the CRH-R1 α except for a 29 amino acid insert in the first intracellular loop (IC1) of the R1 β which results in impaired agonist binding and G-protein coupling (Xiong et al., 1995). Indeed, our data suggest that the CRH-R1 β receptor can only weakly activate the Gs-protein/adenylyl cyclase pathway, in agreement with published data (Xiong et al., 1995), and shows no significant coupling to Gi-, Gq- or Go-proteins (*unpublished* data). Lack of CRH-R1 β receptor isoform-specific antibodies have prevented the conclusive demonstration of CRH-R1 β protein expression in native tissues. Regardless of whether this receptor transcript is significantly expressed as protein product, possible increased expression of the CRH-R1 β 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-R1 β variant mRNA is exclusively expressed in the absence of CRH-R1 α mRNA, suggesting the presence of splicing mechanisms controlling the balance of CRH-R1 α and CRH-R1 β mRNA expression levels. CRH-R1 β 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-R1 β in certain tissues would render these 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 β activation. At present, there are no data about the ratio of expression of the R1 α and R1 β receptor proteins in tissues, probably reflecting the methodological difficulties mentioned above, however, preliminary data from our laboratory have identified a

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specific mechanism involving progesterone that regulates the ratio of CRH-R1 α /R1 β 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-R1 α and R1 β . This study provides novel evidence that although both R1 α and R1 β , 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-R1 β susceptible to signalling desensitization and internalization. In contrast, CRH-R1 α ability to stimulate the G_s α -AC pathway and cAMP production is enhanced in response to PKC activation. Evidence from rat cells natively expressing only the CRH-R1 isoform (equivalent to the human CRH-R1 α), such as rat anterior pituitary, supporting the physiological significance of this signalling amplification mechanism, as CRH actions in the rat anterior pituitary are positively modulated by PKC (Bilezikjian et al., 1987; Carvallo and Aguilera, 1989); AVP-induced PKC activation augments CRH effects by increasing cAMP formation and proopiomelanocortin gene transcription. This different response of the two CRH-R1 variants is also in agreement with our previous observations showing that some, but not all, CRH-R variants detected by isoelectric focusing are sensitive to oxytocin-induced PKC activation in human term myometrium (Grammatopoulos and Hillhouse, 1999).

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The effects of PMA on isoforms of AC are well-described (Yoshimasa et al., 1987), and were confirmed in our experimental model. However, alterations in AC activity cannot explain the disparate responses of the two CRH-R1 variants to PKC activation, and a more likely explanation is that PKC-induced phosphorylation, results in differential functional effects on the two CRH-R1 variants affecting the receptors coupling to G_sα-protein. Indeed, this is supported by our G-protein activation experiments, which demonstrated that phorbol esters treatment resulted in enhanced G_sα-protein coupling of CRH-R1α and diminished G_sα-protein coupling of CRH-R1β. These alterations paralleled changes in CRH stimulation of AC after PKC activation. The CRH-R1 itself or other signalling proteins involved in receptor G-protein interactions and signalling might be targeted by PKC actions. The CRH-R1 amino acid sequence contains several Ser/Thr residues, identical in both R1α and R1β variants, located in the IC1 and IC2 as well as the proximal and distal portion of the cytoplasmic tail, that are putative targets for PKC phosphorylation. Our results presented here suggest that both CRH-R1 variants can be phosphorylated following PKC activation, in agreement with previous reports (Hauger et al., 2003). Although the *in vitro* phosphorylation assays suggest that the degree of each receptor phosphorylation is similar, it is possible that subtle differences are undetectable with the methodology used and distinct phosphoacceptor residues in each of the CRH-R1 variants are targeted by PKC due to potential differences in the tertiary structure of the receptor; this requires further investigations. Interestingly, both CRH-R1 variants exhibited measurable basal phosphorylation levels, indicative of either basal PKC activity targeting CRH-R1 or alternative effects of other kinases that are activated as a result of some degree of constitutive activity of the CRH-R1 and regulate CRH-R1 function in the absence of agonist-induced receptor activation.

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The latter is possible since the basal phosphorylation state of the CRH-R1 α was higher than that of the signalling impaired R1 β variant.

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 GRK isoforms, facilitating GRK translocation to the membrane or enhancing GRK 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 and 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-R1 α homologous desensitization involves multiple GRK isoforms (Teli et al., 2005), thus potential PKC-GRK interactions might also modulate heterologous CRH-R1 β desensitization.

Confocal microscopy studies in 293-R1 α and 293-R1 β cells revealed that both CRH-R1 variants were susceptible to homologous desensitization and internalization, demonstrating for the first time that the CRH-R1 β 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 co-localization with the CRH-R1, in agreement with previous studies (Teli et al., 2005; Rasmussen et al., 2004; 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-R1 α and R1 β receptors co-localise with β -arrestin raising the possibility of distinct pathways (β -arrestin-dependent

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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-R1 β , but not CRH-R1 α , receptors available for agonist binding by inducing heterologous receptor endocytosis. This might have a significant contribution to the diminished functional response of CRH-R1 β following PKC activation. In addition, the finding that PMA-induced CRH-R1 β desensitization and internalization was not associated with recruitment of β -arrestin to the plasma membrane points towards 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 signalling activity utilizing the ability of the CRH-R1 gene to generate receptor variants with distinct responses to PKC-induced phosphorylation. It appears 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-R1 α 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-R1 α -Gs α -protein interactions. In contrast, increased expression of CRH-R1 β (e.g. in pregnant myometrium at term which is associated with inhibition of progesterone activity), will reduce tissue sensitivity to CRH actions due to the presence of signalling-impaired CRH-Rs that are susceptible to PKC induced desensitization and internalization.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1

Effects of protein kinase activation on CRH-induced cAMP production in 293-R1 α and 293-R1 β cells. HEK293 cells transiently expressing CRH-R1 α or R1 β receptors were pre-treated with CRH (100 nM) for 45min, PMA (200 nM) or indolactam V (100 nM) for 30 min, prior to 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 \pm SEM of three estimations and are representative from four individual transfections.

Figure 2

CRH-induced cAMP production from OTR/CRH-R1 α -CHO and OTR/CRH-R1 β -CHO cells pre-treated with oxytocin before addition of CRH. CHO cells stably expressing oxytocin receptors (OTR) were transiently transfected with CRH-R1 α or R1 β receptor cDNA (cloned in pcDNA3.1) using the Lipofectamine method. Following PKC activation by pre-treatment with various concentrations of OT for 30 min, cells were stimulated with CRH (100 nM) for 15 min and cAMP production was determined by RIA. Results are expressed as the mean \pm SEM of three estimations and are representative from four individual transfections. *, $P < 0.05$ compared to basal values; †, $P < 0.05$ compared to OT-untreated values.

Figure 3

Effect of PKC activators on forskolin-induced cAMP production, CRH-induced G $_s$ -protein activation and CRH-Rs *in vitro* phosphorylation, in HEK 293 cells expressing CRH-R1 receptor variants. (a) Treatment of 293-R1 α or R1 β cells for 30 min in the presence or absence of PMA (200 nM), or indolactam V (100 nM) or 4 α -PDD

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(500 nM) was followed by addition of forskolin (10 μ M) or CRH (100 nM) for 15 min and estimation of cAMP production, by RIA. (b) Treatment of 293-R1 α or 293-R1 β cells with or without PMA (200 nM) for 30 min was followed by addition of CRH (100 nM) in the presence of [α -³²P]-GTP- γ -azidoanilide followed by UV cross-linking, immunoprecipitation, fractionation on SDS-PAGE and autoradiography for determination of the incorporation of the [α -³²P]-GTP- γ -azidoanilide into the stimulated Gs-protein. (c) PMA-treated cells (200 nM for 30 min) pre-labelled with 300 μ Ci/ml [³²P] orthophosphate were solubilized, and the CRH-Rs were immunoprecipitated, fractionated on SDS-PAGE, and subjected to autoradiography (-70°C, 10-14 days) using intensifying screens as described in *Materials and Methods*. Results are expressed as the mean \pm SEM of three estimations and are representative from three individual transfections. *, $P < 0.05$ compared to basal values; +, $P < 0.05$ compared to CRH-untreated values.

Figure 4

Detection of CRH-R1 and β -arrestin distribution in 293-R1 α cells by fluorescent confocal microscopy studies. 293-R1 α cells were grown on coverslips and CRH-R1 and β -arrestin distribution was monitored by indirect immunofluorescence using specific primary antibodies in the presence or absence of corresponding blocking peptides (10 fold molar excess) and Alexa-Fluor® 594 secondary antibody for CRH-R1 (red) and Alexa-Fluor® 488 secondary antibody for β -arrestin (green). Cell nuclei (blue) were also stained using the DNA-specific dye DAPI. Identical results were obtained from 4 independent experiments.

Figure 5

Effects of CRH and PMA on internalization characteristics of CRH-R1 α and CRH-R1 β receptor variants transiently expressed in HEK293 cells: visualization by

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fluorescent confocal microscopy. 293-R1 α or 293-R1 β cells were grown on coverslips and following exposure to CRH (100 nM) for 45min or PMA (200 nM) for 30 min, CRH-R distribution was monitored over the ensuing time period by indirect immunofluorescence using CRH-R1 (red) specific antiserum and Alexa-Fluor® 594 secondary antibody. Cell nuclei (blue) were also stained using the DNA-specific dye DAPI. Identical results were obtained from 4 independent experiments. Scale bar 10 μ m. Representative profiles of fluorescence intensity are also shown, generated along the lines depicted in the overlap images, by using Image J software.

Figure 6

Relative quantification of CRH-R1 α and CRH-R1 β 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 μ m).

Figure 7

CRH-R1 α and β -arrestin subcellular distribution following CRH or PMA (200 nM): visualization by fluorescent confocal microscopy.

HEK293 cells transiently expressing CRH-R1 α were stimulated with CRH (100 nM) or PMA (200 nM) for 2-30 min. CRH-R1 α 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). Co localization appears as yellow in the overlap image. Identical results were obtained from 4 independent experiments. Scale bar 10 μ m. Also shown, representative profiles of fluorescence intensity, generated along the lines depicted in the overlap images, by using Image J software.

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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 μ m) or plasma membrane (1-3 and 19-21 μ m) fluorescence was measured. Results are expressed as the mean \pm SEM of 3 estimations from 20 individual cells. *, $P < 0.05$ compared to untreated values.

Figure 8

CRH-R1 β and β -arrestin subcellular distribution following CRH or PMA (200 nM): visualization by fluorescent confocal microscopy.

HEK293 cells transiently expressing CRH-R1 α or R1 β receptor variants were stimulated with CRH (100 nM) or PMA (200 nM) for 2-30 min. CRH R1 β 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). Co localization appears 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 4 independent experiments. Scale bar 10 μ m. In some experiments, profiles of fluorescence intensity were generated along the lines depicted in the overlap images, by using Image J 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 μ m) or plasma membrane (1-3 and 19-21 μ m) fluorescence was measured. Results are expressed as the mean \pm SEM of 3 estimations from 20 individual cells. *, $P < 0.05$ compared to untreated values.

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Table 1 CRH binding characteristics of different cell lines expressing CRH-R1 variants. Data are expressed as the mean \pm SEM (n=3), * $p < 0.05$ compared to CRH-R1 β expressed in HEK293 or OTR/CHO cells .

Type of HEK293 cells expressing CRH-R1 variants	<i>B</i>_{max} (nmol/mg protein)	K_D (nM)
293-R1 α (trans)	8.8 \pm 2.7	1.35 \pm 0.4*
293-R1 β (trans)	11.1 \pm 2.2	4.75 \pm 0.6
OTR/CHO-R1 α (trans)	0.4 \pm 1.5	2.7 \pm 0.5*
OTR/CHO-R1 β (trans)	1.2 \pm 2.1	7.6 \pm 0.9
st293-R1 α (stable)	430 \pm 87	2.1 \pm 0.5*
st293-R1 β (stable)	520 \pm 112	5.1 \pm 0.8

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Table 2. The effects of PKC and PKA inhibitors on CRH-induced cAMP production. The 293-R1 α and 293-R1 β cells were treated with various PKC (calphostin C -100nM or bisindolylmaleimide I-100nM) or PKA (H-89-10 μ M) inhibitors to investigate the effect on CRH-induced cAMP production in the presence or absence of PKC activators (PMA or indolactam V) as described in *Material and Methods*. Results are expressed as the mean \pm SEM of five estimations from four individual transfections

Treatment	293-R1 α		293-R1 β	
	EC50(nM)	Max AC stimulation (100nM CRH) (% of forskolin)	EC50(nM)	Max AC stimulation (100nM CRH) (% of forskolin)
Control cells				
+CRH	3 \pm 2.2	61 \pm 12.3	19 \pm 5.0	15 \pm 1.3
+PMA/CRH	4.8 \pm 0.2	145 \pm 21.4*	30 \pm 4.7	4 \pm 1.3*
+indoV/CRH	6 \pm 1.2	132 \pm 32.3*	23 \pm 6.8	4.7 \pm 1.6*
H-89 treated cells				
+CRH	4 \pm 0.2	57 \pm 7.6	14 \pm 2.2	13 \pm 2.1
+PMA/CRH	3.1 \pm 1.5	146 \pm 12.1*	25 \pm 6.0	3.7 \pm 0.6*
+indoV/CRH	3.8 \pm 0.9	139 \pm 25.1*	22 \pm 2.5	4.2 \pm 0.7*
Calphostin C treated cells				
+CRH	5 \pm 1.2	50 \pm 8.9	17 \pm 3.2	11 \pm 1.0
+PMA/CRH	4 \pm 0.2	65 \pm 12.9	19 \pm 2.4	8.5 \pm 2.4
+indoV/CRH	4.7 \pm 0.8	59 \pm 7.2	23 \pm 1.4	9.5 \pm 2.1
Bisindolylmaleimide I treated cells				
+CRH	3.2 \pm 1.4	53 \pm 9.9	13 \pm 2.2	14 \pm 3.2
+PMA/CRH	6 \pm 0.7	55 \pm 7.6	15 \pm 4.5	9 \pm 1.1

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+indoV/CRH	6.5±0.8	60±5.9	16±2.6	9.5±2.4
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p<0.05 compared to cAMP response of cells without any pretreatment

Figure 1

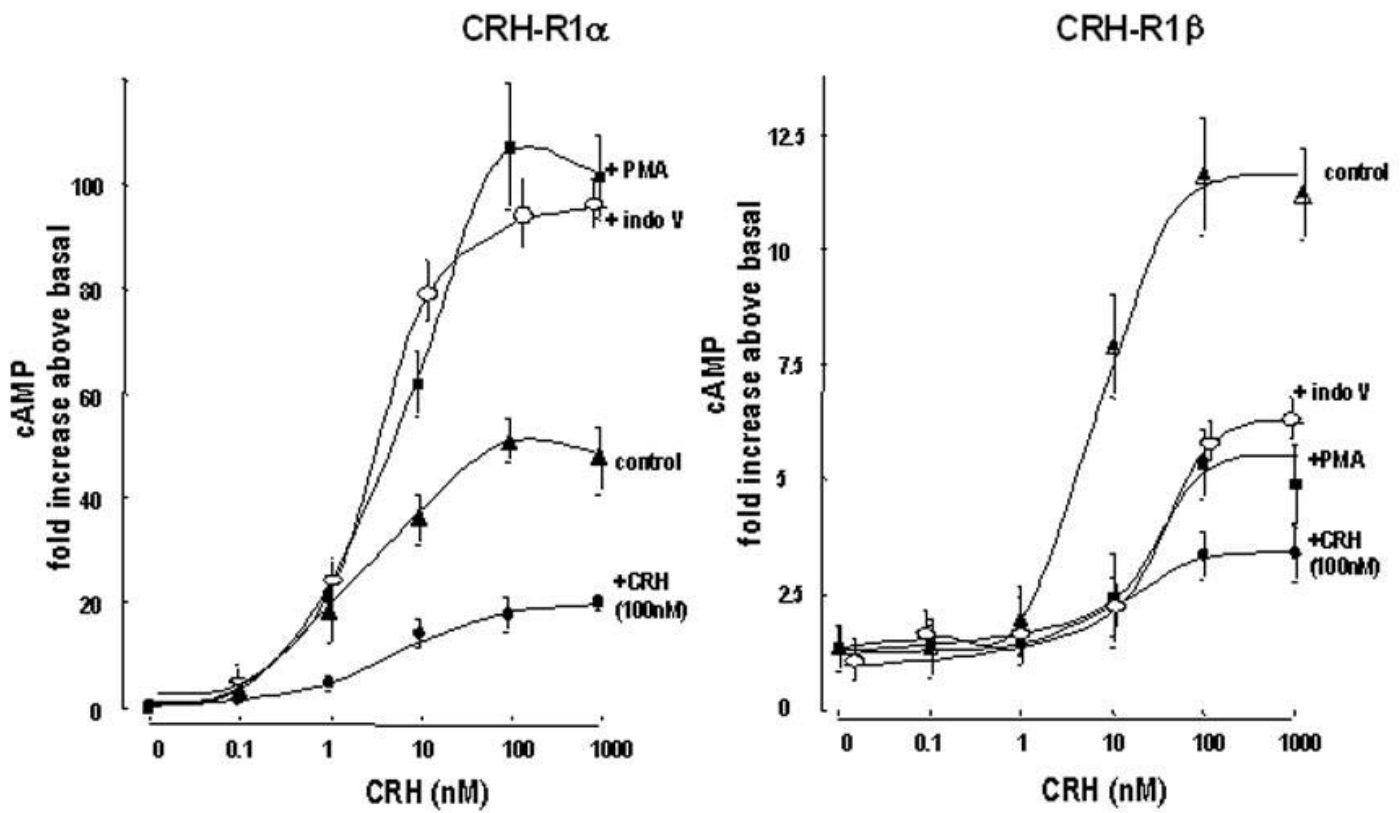


Figure 2

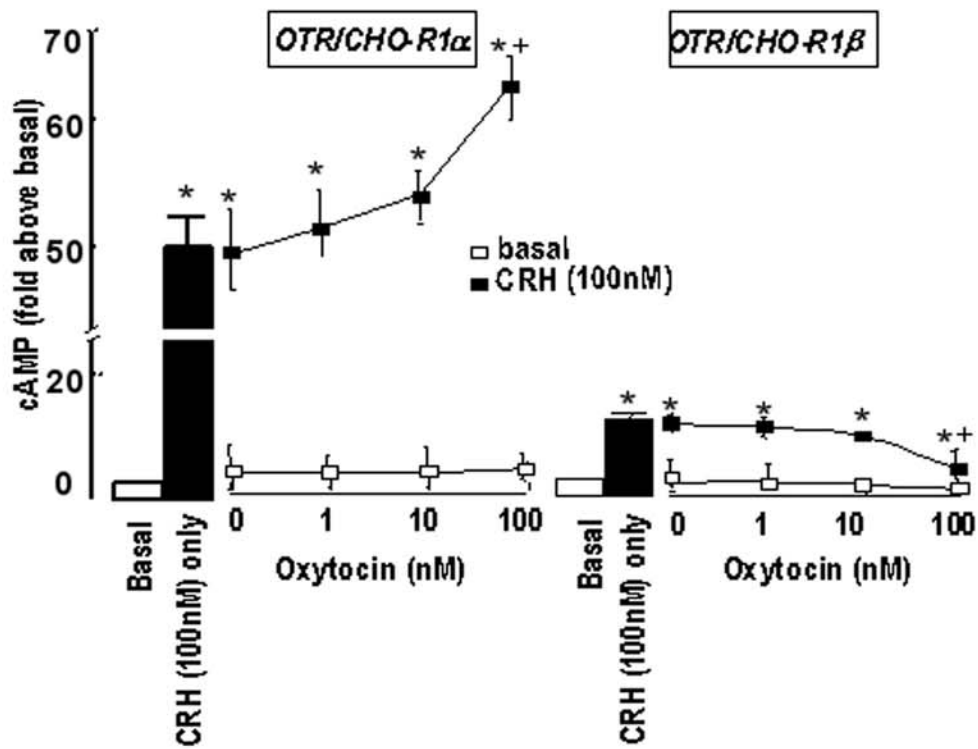


Figure 3

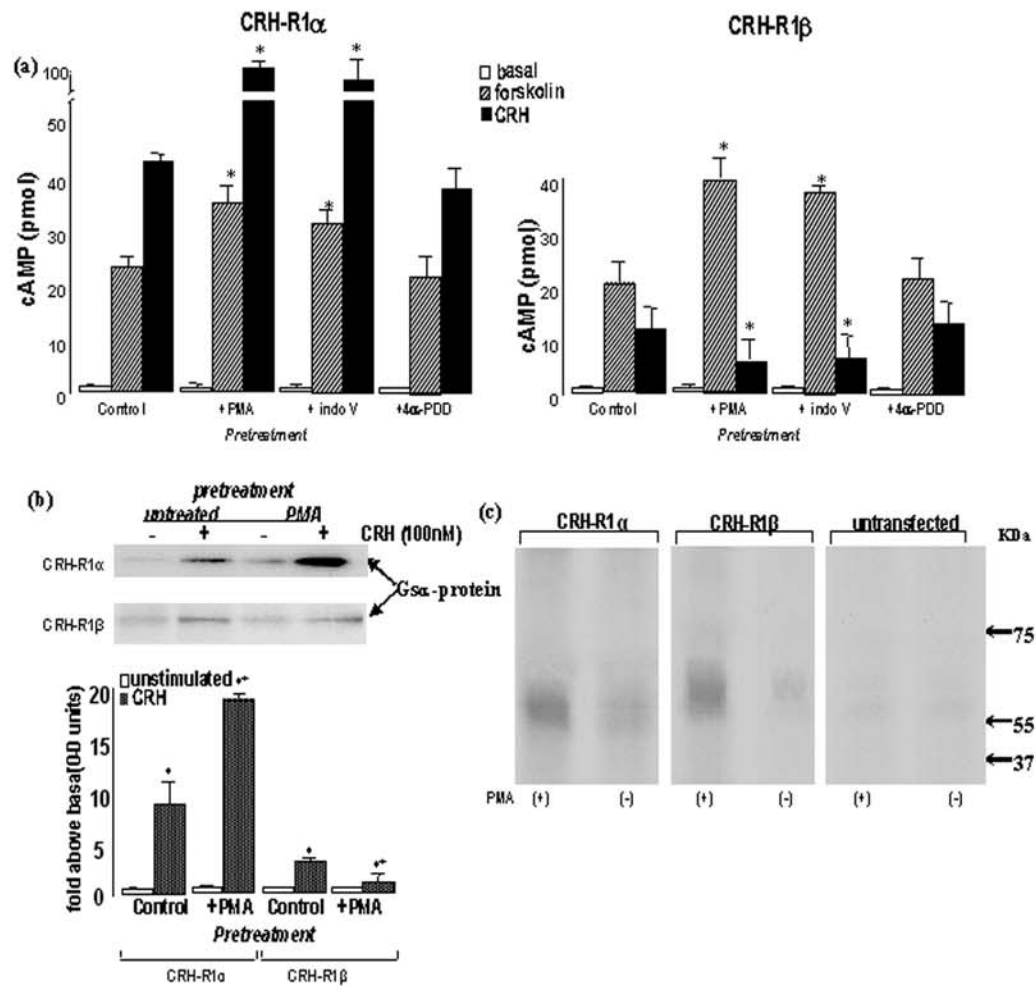


Figure 4

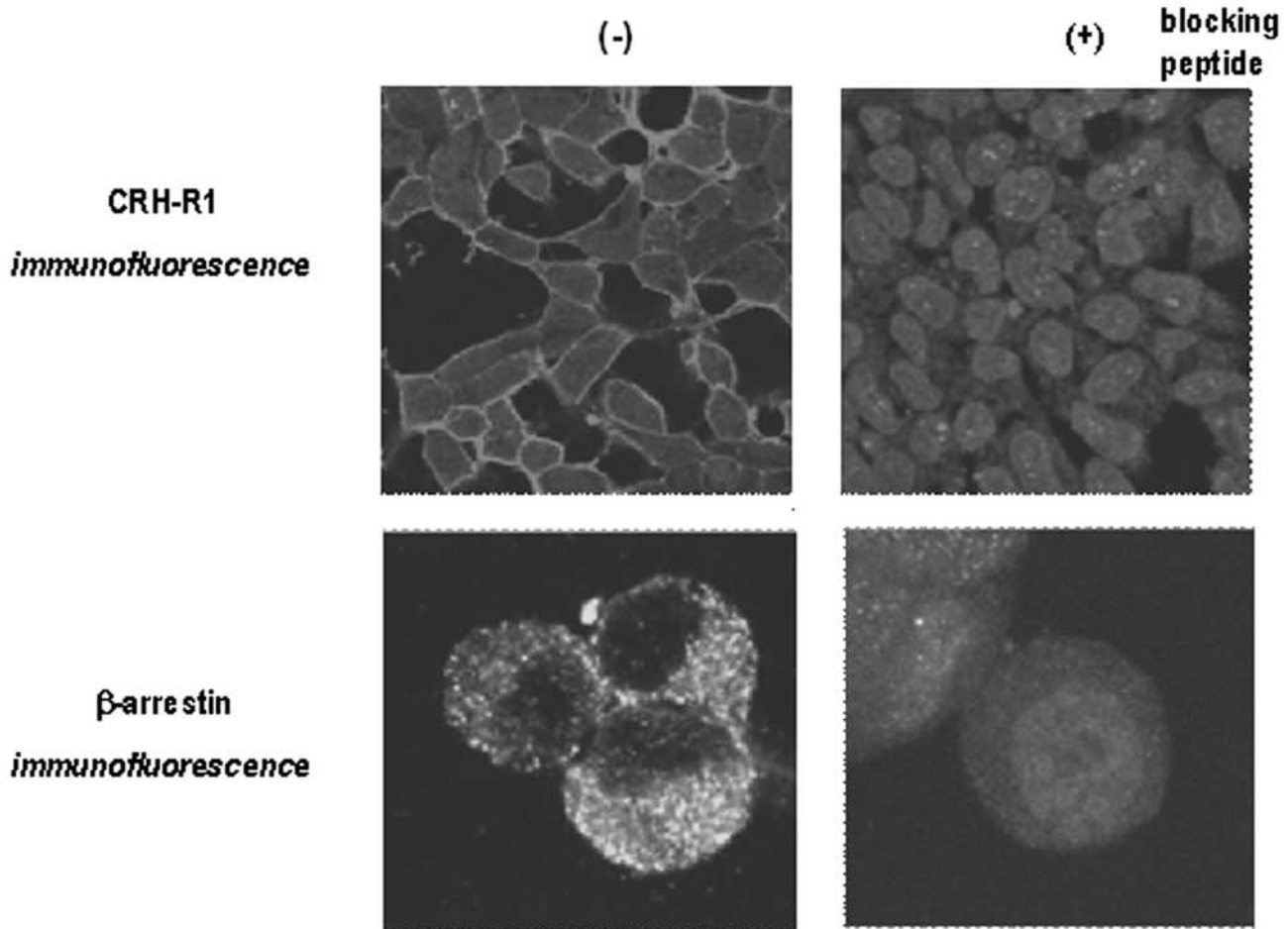


Figure 5

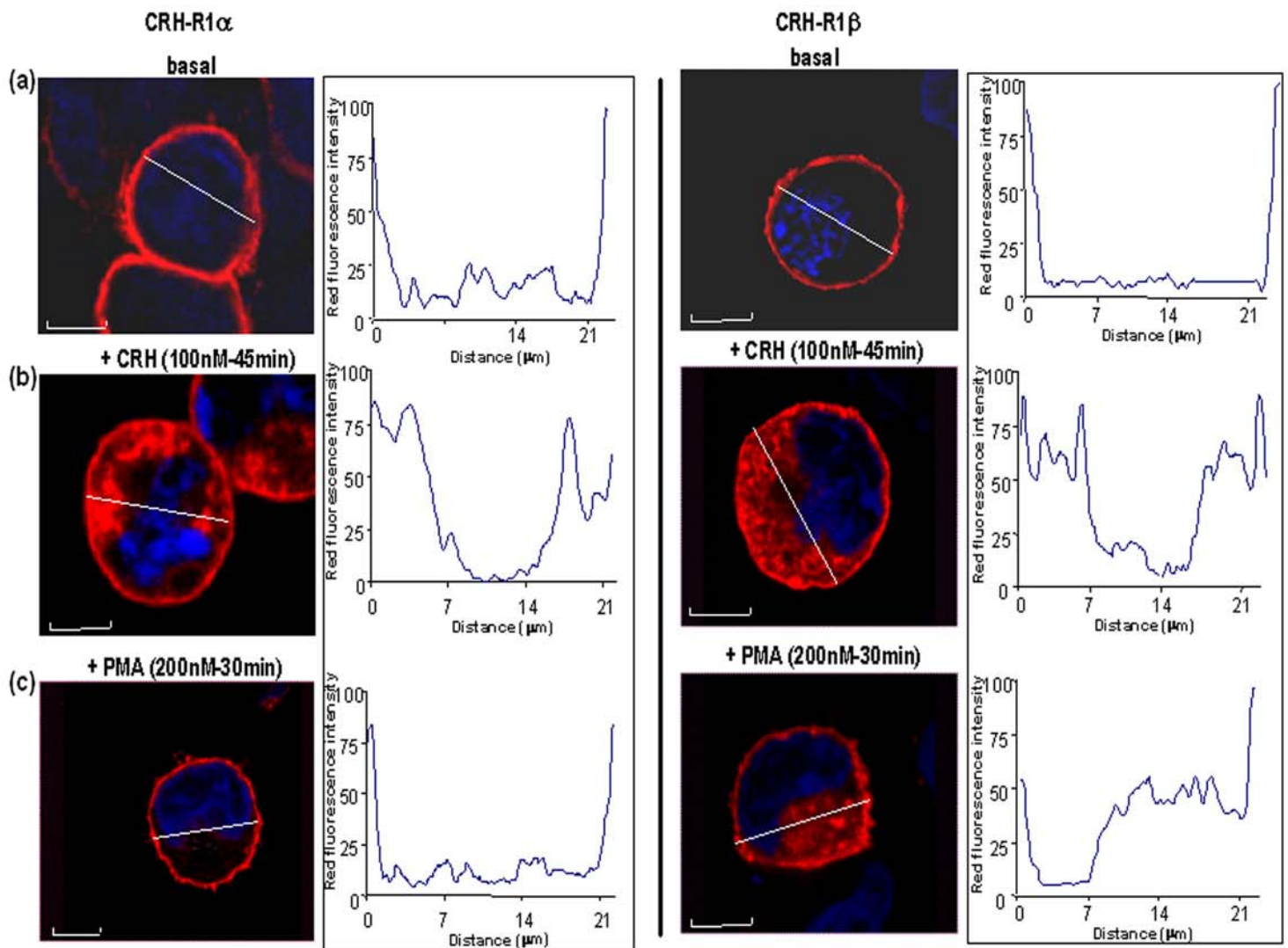


Figure 6

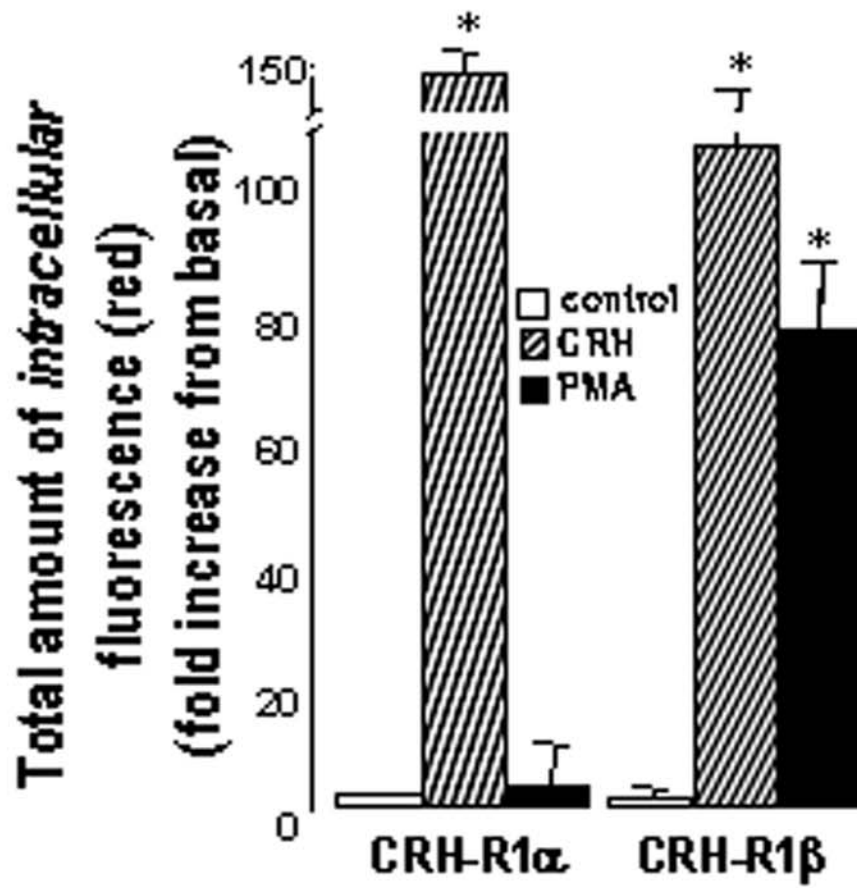


Figure 7

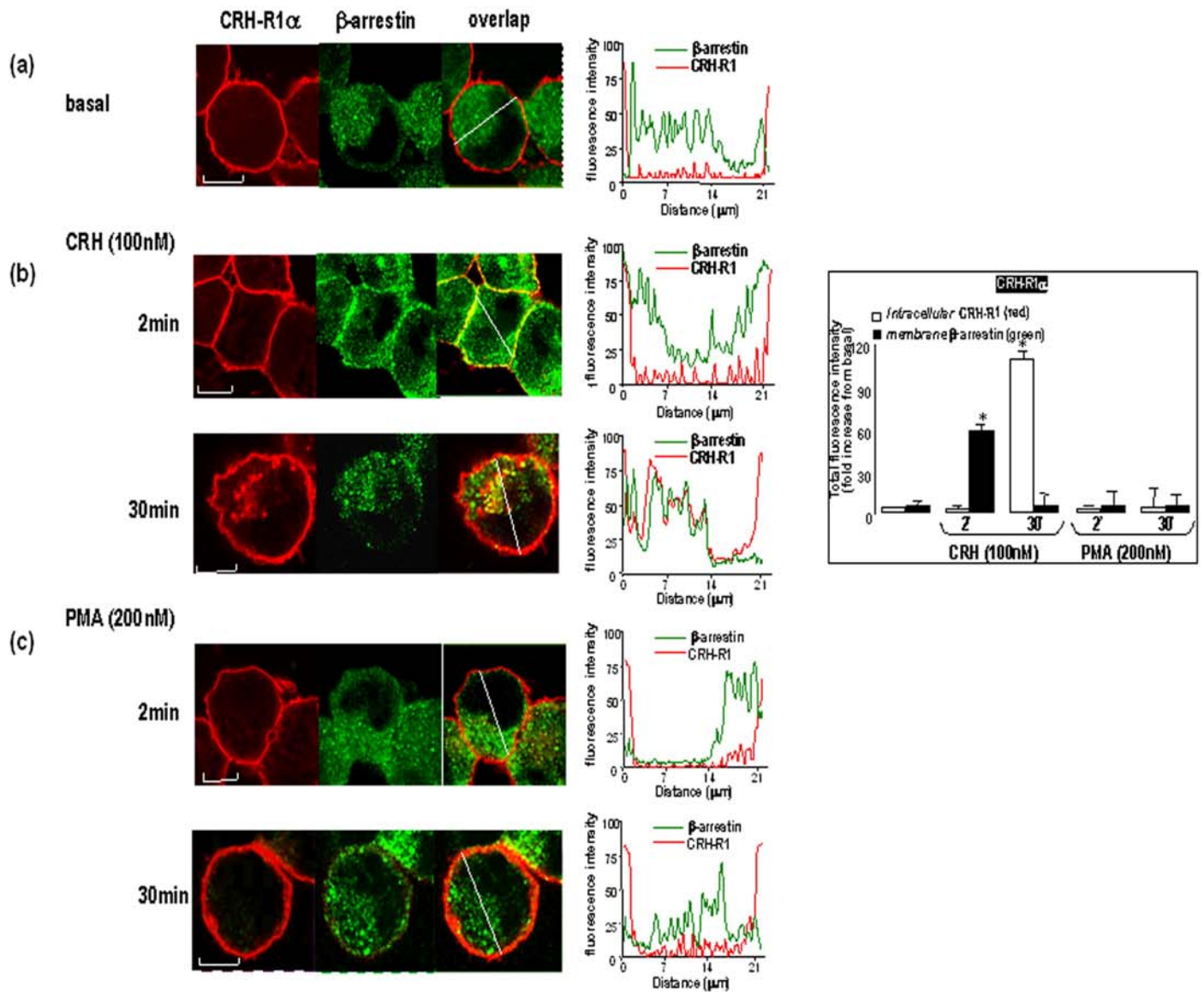


Figure 8

