ELSEVIER

European Journal of Pharmacology 403 (2000) 1–7

ejp

www.elsevier.nl/locate/ejphar

Involvement of G protein-coupled receptor kinase-6 in desensitization of CGRP receptors

Nambi Aiyar [a,*], Jyoti Disa [a], Khoa Dang [a], Alexey N. Pronin [b], Jeffrey L. Benovic [b], Ponnal Nambi [a,1]

[a] *Department of Cardiovascular Pharmacology, SmithKline Beecham Pharmaceuticals, 709 Swedeland Road, Box 1539, UW2510 King of Prussia, PA 19406-0939 USA*

[b] *Department of Microbiology and Immunology, Kimmel Cancer Center, Thomas Jefferson University, 233 South 10th Street, Philadelphia, PA 19107 USA*

Received 27 January 2000; received in revised form 5 June 2000; accepted 9 June 2000

Abstract

This investigation was undertaken to study the mechanisms of calcitonin gene-related peptide (CGRP)-mediated desensitization using recombinant porcine CGRP receptors stably expressed in human embryonic kidney (HEK-293) cells. Pretreatment of these cells with human αCGRP resulted in an ~60% decrease in CGRP-stimulated adenylyl cyclase activity and an ~10-fold rightward shift in the dose–response curve of CGRP. This effect was rapid ($t_{1/2}$ ~5 min) and was accompanied by a significant decrease in [^{125}I]CGRP binding to membrane preparations from CGRP-pretreated cells. In contrast, CGRP pretreatment had no effect on isoproterenol- or forskolin-stimulated adenylyl cyclase activity in these cells. The potential involvement of protein kinase A or protein kinase C in CGRP-mediated desensitization was studied using selective inhibitors or activators of these kinases. Pretreatment of the cells with forskolin (adenylyl cyclase activator) or phorbol dibutyrate (protein kinase C activator) had no effect on CGRP-mediated adenylyl cyclase activity and did not influence CGRP-mediated desensitization. However, pretreatment of the cells with 2-(8-[(dimethylamino)methyl]-6,7,8,9-tetrahydropyrido[1,2-a]indol-3-yl]-3-(1-methylindol-3-yl)maleimide hydrochloride (Ro 32-0432) (a potent inhibitor of protein kinase C) resulted in significant attenuation of CGRP-mediated desensitization with an IC_{50} ~3 μM. To establish whether this effect might be due to inhibition of other protein kinases by Ro 32-0432, its effect was tested against several G protein-coupled receptor kinases (GRKs). Ro 32-0432 was found to inhibit GRK2, GRK5, and GRK6 with IC_{50} values of 29, 3.6, and 16 μM, respectively, suggesting that its effect on CGRP-mediated desensitization might be a result of GRK inhibition. To further test this hypothesis, as well as the potential GRK specificity, the cells were treated with antisense oligonucleotides to GRK2, GRK5, and GRK6. While GRK2 and GRK5 antisense nucleotides had no effect on CGRP-mediated desensitization, the GRK6 antisense nucleotide treatment significantly reversed CGRP-mediated desensitization. These results suggest the involvement of GRK6 in CGRP-mediated desensitization in HEK-293 cells.

Keywords: CGRP receptor, porcine, cloned; G protein-coupled receptor kinase; Homologous desensitization; CGRP (calcitonin gene-related peptide); Ro 32-0432; Antisense oligonucleotide

1. Introduction

Seven transmembrane G protein-coupled receptors (GPCR) are subjected to a variety of regulatory mechanisms that can affect receptor function, localization and number. In general, chronic exposure of cells to an agonist results in a reduced ability of the cells to respond to a subsequent agonist challenge. This desensitization process is a general biological phenomenon that serves to protect cells from excessive stimulation. For GPCR, desensitization appears to be a multistep process that includes uncoupling of the receptor from the G protein followed by internalization and down-regulation (Hausdorf et al., 1990; Lohse, 1993; Freedman and Lefkowitz, 1996; Chuang et al., 1996; Krupnick and Benovic, 1998).

Two major patterns of desensitization have been characterized: agonist-dependent and agonist-independent. Ago-

* Corresponding author. Tel.: +1-610-270-5004; fax: +1-610-270-5080.

E-mail address: nambi_aiyar-1@sbphrd.com (N. Aiyar).

[1] Present address: Cardiovascular Sciences, E400/3255, Dupont Pharmaceuticals, Experimental Station, Wilmington, DE 19880-0400, USA.

0014-2999/00/$ - see front matter
PII: S0014-2999(00)00419-2

nist-dependent desensitization results in the response of a receptor to a particular agonist being attenuated by prior exposure to that agonist, while the response to other agonists or activators is unaffected. Phosphorylation of the agonist-occupied GPCR by G protein-coupled receptor kinases (GRKs) has been implicated in this process (Freedman and Lefkowitz, 1996; Krupnick and Benovic, 1998). In contrast, agonist-independent desensitization refers to a process where treatment of cells with one agonist results in the attenuation of cellular responses for numerous agonists as well as for non-receptor-mediated effectors such as forskolin and sodium fluoride. This process also often results in receptor phosphorylation, although this appears to be mediated primarily by second-messenger-dependent protein kinases such as protein kinase A and/or protein kinase C (Hausdorf et al., 1990; Freedman and Lefkowitz, 1996).

CGRP is a 37-amino-acid neuropeptide with potent cardiovascular effects that include positive inotropic and chronotropic actions, systemic vasodilation, and hypotension in animal and human studies (Poyner, 1992; Wimalawansa, 1997; Van Rossum et al., 1997). CGRP is produced in sensory neurons and is transported peripherally to terminal areas in visceral organs. Once released from the cell, the peptide initiates its biological responses by binding to specific cell-surface receptors that are predominantly coupled to the activation of adenylyl cyclase. CGRP receptors have been identified and pharmacologically characterized from a number of tissues and cell lines. For example, SK-N-MC is a well-characterized human neuroblastoma cell line that expresses high-affinity CGRP receptors (Van Valen et al., 1990). While the molecular mechanisms involved in CGRP receptor desensitization have not been elucidated, CGRP pretreatment of both SK-N-MC and rat mesangial cells results in desensitization of the CGRP-mediated stimulation of cAMP production (Van Valen et al., 1990; Aiyar et al., 1992).

Recently, we reported the cloning of complementary DNAs (cDNAs) encoding the human (Aiyar et al., 1996) and porcine (Elshourbagy et al., 1998) CGRP receptors. The CGRP receptor shares significant amino acid sequence homology with the human calcitonin receptor, a member of a recently described novel subfamily of GPCR. Stable expression of the porcine CGRP receptor cDNA in human embryonic kidney (HEK-293) cells produced a cell line (HEK-293-PR) that displayed specific, high affinity CGRP binding sites with pharmacological and functional properties very similar to the native $CGRP_1$ receptor. Exposure of these cells to CGRP resulted in increased cAMP production and intracellular calcium release (Aiyar et al., 1999). Moreover, both signal transduction pathways were inhibited in a competitive manner by human αCGRP-(8-37), a $CGRP_1$ receptor antagonist that lacks the seven N-terminal amino acids of CGRP (Dennis et al., 1989; Quirion et al., 1992). In the present study, we investigated the mechanisms involved in CGRP receptor desensitization using the HEK-293-PR cells as a model. Our results suggest that GRK6 may play the major role in CGRP-mediated desensitization in this system.

2. Materials and methods

2.1. Materials

(2-[^{125}I]Iodohistidyl10) human αCGRP [specific activity 2000 Ci/mmol] was obtained from Amersham (Chicago, IL). The hαCGRP and human αCGRP-(8-37) were purchased from Bachem Biochemicals (PA). Ro 32-0432 (a selective PKC inhibitor) was synthesized at SB Pharmaceuticals. Forskolin, phorbol dibutyrate and all other reagents were obtained from Sigma (St. Louis, MO). BCA protein assay kit was obtained from Pierce Chemicals (Rockford, IL).

2.2. Cell culture

HEK-293 cells, stably transfected with the porcine CGRP receptor cDNA (HEK-293-PR), were maintained in a minimum essential medium (MEM) supplemented with 10% fetal calf serum. Cells were treated in the absence or presence of test substances such as CGRP for the indicated times, after which they were washed extensively with phosphate-buffered saline (PBS) prior to membrane preparation for quantitative [^{125}I]CGRP binding and CGRP-mediated adenylyl cyclase activation.

2.3. Membrane preparation

Cells were scraped into ice-cold PBS and centrifuged for 10 min at $1000 \times g$ at 4°C. The pellet was resuspended in 10 mM Tris-HCl, pH 7.4, 10 mM Na-EDTA and homogenized using a Dounce ground glass homogenizer. The homogenate was centrifuged for 20 min at $12{,}000 \times g$ at 4°C, and the resultant membrane pellet was resuspended in 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$ and recentrifuged. The final pellet was resuspended in 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$ and assayed immediately.

2.4. Determination of adenylyl cyclase activity

Membrane-bound adenylyl cyclase activity was determined as the rate of conversion of [α^{32}P]ATP to [^{32}P]cAMP as previously described (Elshourbagy et al., 1998). Membranes (40–60 μg protein) were incubated in triplicate tubes in buffer containing 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 1.2 mM ATP, 1.0 μCi [α^{32}P]ATP, 0.1 mM cAMP, 2.8 mM phosphoenolpyruvate and 5.2 μg/ml myokinase in a final volume of 100 μl for 20 min at 30°C. The reactions were stopped with 1 ml of a solution containing 0.28 mM cAMP, 0.33 mM ATP and 22,000 dpm of

[^{3}H]cAMP. [^{32}P]cAMP was separated using sequential chromatography on Dowex and alumina columns (Salmon et al., 1974). Adenylyl cyclase activities were determined in the absence (basal) or presence of various concentrations of human αCGRP (1 pM to 1 μM). The effect of human αCGRP-(8-37), a CGRP receptor antagonist, on human αCGRP-mediated activation of adenylyl cyclase was also determined.

2.5. Radioligand binding

Saturation binding experiments were carried out using membranes prepared from HEK-293-PR cells pretreated in the absence or presence of CGRP. The incubation mixture contained increasing concentrations of [^{125}I]CGRP (5–120 pM) and 40–60 μg of membrane protein in a final volume of 500 μl. Nonspecific binding was determined in the presence of 1 μM human αCGRP. After incubating for 30 min at 25°C, the reaction mixture was rapidly diluted with cold 0.9% NaCl; and bound and free ligands were separated by filtration through glass-fiber filters.

2.6. Antisense oligonucleotide treatment of HEK-293-PR cells

Antisense oligonucleotide treatment of HEK-293-PR cells was done following the protocol of Nakata et al. (1996). Briefly, HEK-293-PR cells were maintained in a MEM supplemented with 10% fetal calf serum until confluent. The culture medium was then changed to a MEM containing 1% fetal calf serum, and the synthetic sense or antisense oligonucleotides for human GRK2, GRK5 or GRK6 were added every 4 h for 24 h at a concentration of 10 μM. The sequences of the antisense oligonucleotides were 5′ CTCCAGGTCCGCCATCTT 3′; 5′ GTTTTCCAGCTCCATTGA 3′ and 5′ GTTCTCGAGCTCCATGGG 3′ for GRK2, GRK5, and GRK6, respectively. Following the 24-h treatment, the medium was removed, the cells were washed with PBS and then incubated with or without hαCGRP (30 nM) for 30 min (desensitization protocol). The cells were then washed with cold PBS and membranes were prepared to measure CGRP-stimulated adenylyl cyclase activity as described above.

3. Results

To determine whether CGRP-mediated adenylyl cyclase activation was modified after an initial exposure of the CGRP receptors to CGRP, HEK-293-PR cells were pretreated with increasing concentrations of CGRP for 30 min at 30°C. Following the treatment, the cells were washed extensively, and membranes were prepared to measure CGRP-stimulated adenylyl cyclase activity. Pretreatment of HEK-293-PR cells with increasing concentrations of CGRP led to desensitization of the CGRP-mediated activation of adenylyl cyclase as demonstrated by a progressive rightward shift in the concentration–response curve of CGRP and a progressive reduction in maximal adenylyl cyclase activity (Fig. 1). In contrast, CGRP pretreatment had no effect on forskolin-stimulated adenylyl cyclase activity (data not shown), suggesting that CGRP-mediated desensitization is agonist-specific or homologous.

CGRP-mediated desensitization was rapid as shown in Fig. 2, with half-maximal desensitization occurring at ~5 min after treatment with CGRP. In addition, this response is receptor-mediated because it was effectively blocked by inclusion of the CGRP receptor antagonist, CGRP-(8-37), during CGRP pretreatment (Fig. 3). The decrease in CGRP-mediated adenylyl cyclase activity was also accompanied by a decrease in [^{125}I]CGRP binding to membranes prepared from HEK-293-PR cells pretreated with CGRP. Scatchard analysis of saturation binding data obtained with membranes prepared from control HEK-293-PR cells indicated the presence of a single class of high-affinity binding sites with a dissociation constant of 39 pM and maximum binding (B_{max}) of 41 fmol/mg protein (Fig. 4). Membranes prepared from CGRP-pretreated cells displayed approximately 40% loss in CGRP binding capacity (B_{max} = 24 fmol/mg protein) with no significant change in affinity (Fig. 4).

To determine whether cAMP production and subsequent protein kinase A activation participate in CGRP-mediated desensitization, HEK-293-PR cells were pretreated with 10 μM forskolin for 30 min to directly activate adenylyl cyclase. Membrane preparations from these cells were then assayed for CGRP-mediated adenylyl cyclase activity. Treatment of the cells with forskolin had

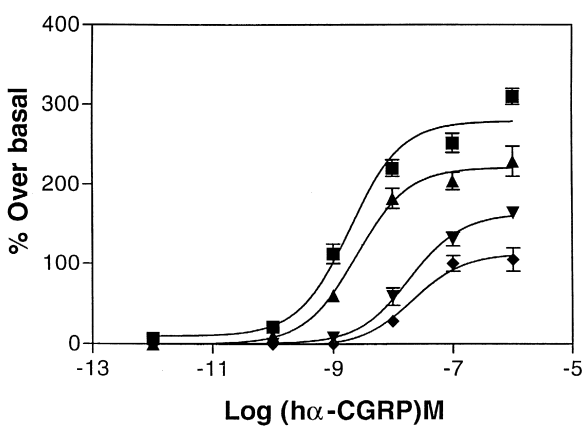

Fig. 1. Concentration–response curve of CGRP-mediated desensitization in HEK-293-PR cells. Monolayers of HEK-293-PR cells were pretreated for 30 min at 30°C with indicated concentrations of human αCGRP prior to extensive washing and membrane preparation. Adenylyl cyclase activity was then measured in the absence or presence of increasing concentrations of human αCGRP. Data are presented as mean ± S.E.M. of triplicate determinations from one experiment, which is representative of three separate experiments. Control cells (■), CGRP (1 nM) (▲), CGRP (10 nM) (▼), CGRP (100 nM) (◆).

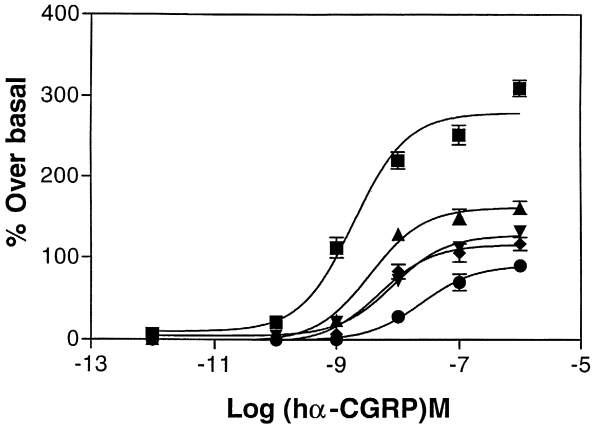

Fig. 2. Time course of CGRP-mediated desensitization in HEK-293-PR cells. Monolayers of HEK-293-PR cells were pretreated with or without 30 nM human αCGRP at 30°C for the indicated times before extensive washing and membrane preparation. Adenylyl cyclase activity was measured in the absence or presence of increasing concentrations of human αCGRP as described in Section 2. Data are presented as mean ± S.E.M. of triplicate determinations from one experiment, which is representative of three separate experiments: Control (■), 5 min (▲), 15 min (▼), 30 min (♦) and 60 min (●).

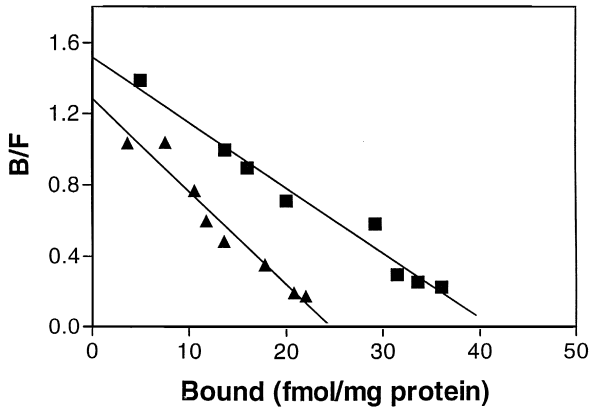

Fig. 4. Scatchard analysis of [^{125}I]CGRP binding to membranes prepared from control (■) and CGRP-pretreated (▲) HEK-293-PR cells. HEK-293-PR cells were pretreated in the absence or presence of human αCGRP (30 nM) for 30 min at 30°C before membrane preparation for [^{125}I]CGRP saturation binding experiments. Data are presented from a single experiment, which is representative of two separate experiments.

no effect on CGRP-mediated adenylyl cyclase activation, suggesting that protein kinase A activation does not directly promote CGRP receptor desensitization, and that agonist occupancy of the receptor is a prerequisite for the onset of desensitization (Fig. 5). Experiments were also designed to evaluate the potential role of protein kinase C in CGRP-mediated desensitization. This was performed using activators as well as inhibitors of protein kinase C. Pretreatment of the cells with a protein kinase C activator such as phorbol dibutyrate had no effect on CGRP receptor responsiveness, suggesting that protein kinase C is not involved in this process (Fig. 5). Pretreatment of cells with staurosporin, a nonselective protein kinase C inhibitor, resulted in loss of viability of the cells. However, pretreatment of HEK-293-PR cells with 3 μM Ro 32-0432, a potent and specific inhibitor of protein kinase C, resulted in partial attenuation of CGRP-mediated desensitization (Fig. 6). While this effect was dependent on the concentration of Ro 32-0432, the IC_{50} value obtained for inhibiting CGRP-mediated desensitization (IC_{50} ~ 3 μM) was much higher than that reported for inhibition of protein kinase C (IC_{50} 3–15 nM; Birchall et al., 1994). Ro 32-0432 had no effect on basal adenylyl cyclase activity. The lack of effect of phorbol dibutyrate on desensitization and the relatively high IC_{50} value obtained for Ro 32-0432 suggested that the effect of this compound on CGRP receptor desensitiza-

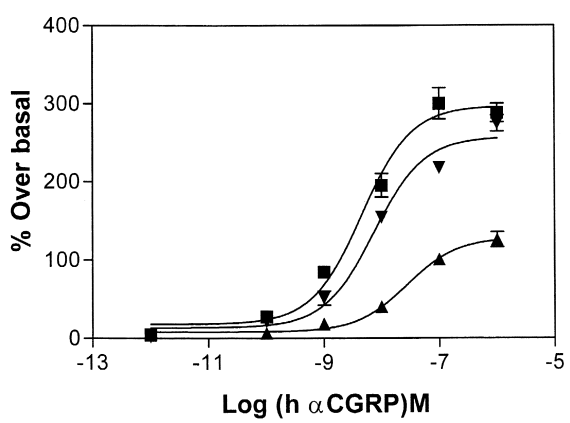

Fig. 3. Effect of human CGRP-(8-37) on CGRP-mediated desensitization in HEK-293-PR cells. HEK-293-PR cells were pretreated in the absence (control (■)) or presence (▲) of human αCGRP (30 nM) or human αCGRP plus human CGRP-(8-37) (100 nM) (▼) for 30 min at 30°C and adenylyl cyclase activity was measured in the membranes as described in Section 2. Data are presented as mean ± S.E.M. of triplicate determinations from one experiment, which is representative of at least two separate experiments.

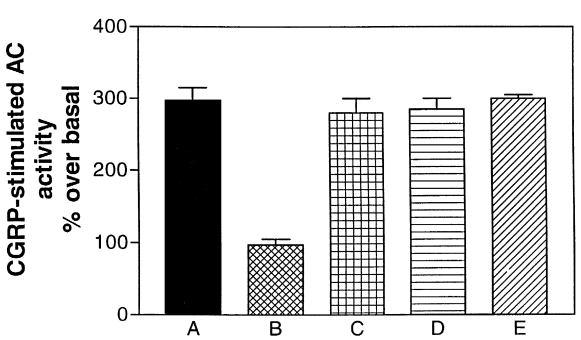

Fig. 5. Involvement of protein kinase A and protein kinase C in human αCGRP-mediated desensitization in HEK-293-PR cells. HEK-293-PR cells were pretreated with PBS (A), 3 nM CGRP (B), 10 μM forskolin (C), 1 μM phorbol(12-myristate-13)dibutyrate (D) or 1 μM α PDD (E) for 30 min at room temperature prior to measuring hαCGRP (100 nM)-stimulated adenylyl cyclase activity. Membranes were then prepared to measure adenylyl cyclase activity in the absence or presence of 0.1 μM CGRP. Data are presented as mean ± S.E.M. of three determinations.

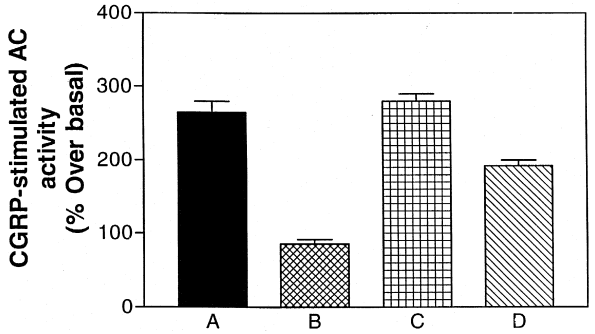

Fig. 6. Effect of Ro 32-0432 on CGRP-mediated desensitization in HEK-293-PR cells. HEK-293-PR cells were pretreated with or without Ro 32-0432 (3 μM) for 10 min prior to CGRP desensitization protocol (30 min treatment at 30°C with or without hαCGRP (30 nM)). Membranes were then prepared for measurement of adenylyl cyclase activity in the absence or presence of 0.1 μM CGRP. Data are presented as mean ± S.E.M. of three determinations. Control (A), CGRP desensitization (B), pretreatment with Ro 32-0432 (C), pretreatment with Ro 32-0432 prior to CGRP desensitization (D).

tion may be due to inhibition of kinases other than protein kinase C. Studies have shown that GRKs mediate the agonist-dependent phosphorylation and uncoupling of many GPCR. To assess the possible effect of Ro 32-0432 on GRK activity, Ro 32-0432 was tested for its ability to inhibit GRK-promoted phosphorylation of rhodopsin. Ro 32-0432 was found to inhibit the activity of GRK2, GRK5, and GRK6 with IC_{50} values of 29.2 ± 3.6, 3.6 ± 0.3, and 15.8 ± 1.4 μM, respectively. These values reveal a good correlation between the IC_{50} for Ro 32-0432 inhibition of CGRP-mediated desensitization (~3 μM) and inhibition of GRK activity (4–29 μM).

To further test the potential role of GRKs in CGRP-mediated desensitization in HEK-293-PR cells, we used an antisense approach. Eighteen base-pair antisense and sense oligonucleotides specific for human GRK2, GRK5, and GRK6 were initially synthesized (Nakata et al., 1996). HEK-293-PR cells were pretreated with these oligonucleotides for 24 h (with fresh oligo added every 4 h) and CGRP-mediated desensitization was then studied. While pretreatment of the cells with antisense oligonucleotides to GRK2 and GRK5 had no effect on CGRP-mediated desensitization, pretreatment of the cells with the GRK6-specific antisense oligonucleotide markedly attenuated CGRP-mediated desensitization (Fig. 7). In the same experiment, there was no effect on CGRP-mediated desensitization when the cells were treated with sense oligonucleotides. These results suggest that GRK6 plays an important role in agonist-specific desensitization of CGRP receptors in HEK-293-PR cells. This observation was further strengthened by performing additional experiments in which CGRP binding and response were quantitated in cells transiently overexpressing GRK2, GRK5 or GRK6. The hypothesis behind this set of experiment was to test if GRK6 might attenuate CGRP binding and response. As shown in Fig. 8, overexpression of GRK6 decreased CGRP binding (30%)

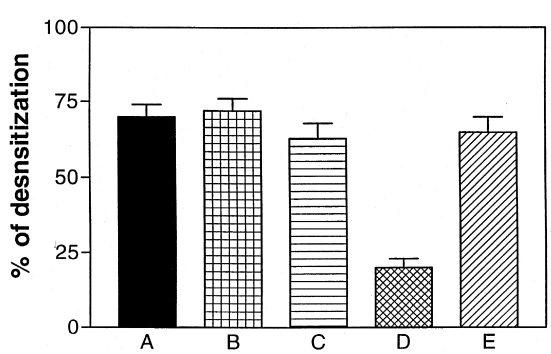

Fig. 7. Effect of GRK2, GRK5 and GRK6 sense or antisense oligonucleotide pretreatment on CGRP-mediated desensitization in HEK-293-PR cells. HEK-293-PR cells were pretreated with or without sense or antisense oligonucleotides to GRK2, GRK5 and GRK6 for 24 h prior to desensitization with CGRP (30 nM for 30 min). The membranes were then prepared to quantitate CGRP (100 nM)-mediated adenylyl cyclase activity. Control (A), GRK2 antisense (B), GRK5 antisense (C), GRK6 antisense (D), and GRK6 sense (E). The values are means ± S.E.M. from three separate experiments done in duplicate.

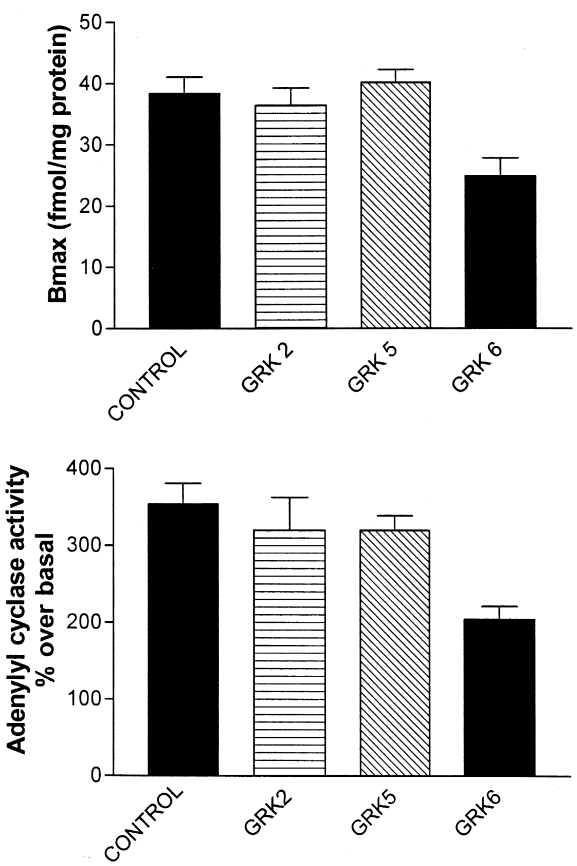

Fig. 8. [^{125}I]CGRP binding and CGRP-mediated adenylyl cyclase activity in membranes prepared from HEK-293 cells with and without the overexpression of GRK2, GRK5, and GRK6. Transient transfection of HEK-293-PR cells with expression plasmids encoding vector, GRK2, GRK5, or GRK6 was performed following the standard protocol. Membranes were prepared from these cells to quantitate [^{125}I]CGRP binding (top panel) and CGRP-mediated adenylyl cyclase activity (bottom panel). Data are presented as mean ± S.E.M. from three separate experiments, each done in duplicate.

as well as function (43%). In the same experiment, overexpression of GRK2 or GRK5 had no effect on CGRP binding or function. These data further confirm the observation that GRK6 is involved in CGRP-mediated desensitization of CGRP receptors expressed in HEK-293 cells.

4. Discussion

CGRP receptors belong to seven transmembrane GPCR of the secretin family (Group II) of GPCR. In the present study, we demonstrated that CGRP receptors undergo agonist-induced homologous desensitization. Pretreatment of HEK-293-PR cells with CGRP results in a decrease in adenylyl cyclase activation in response to a second challenge with CGRP, with no change in forskolin-stimulated adenylyl cyclase activity. The attenuated response was accompanied by a loss of CGRP receptors. The results suggest that the desensitization and downregulation caused by CGRP is homologous in nature. The desensitization and downregulation caused by CGRP was dose-dependent and rapid, with half-maximal desensitization occurring at ~ 5 min after treatment with CGRP. The desensitization was effectively blocked by inclusion of the antagonist, CGRP-(8-37), during CGRP-pretreatment.

Agonist-induced desensitization is a common feature of G-coupled receptors, and often results from phosphorylation of specific serine or threonine residues. CGRP receptors are primarily coupled to the activation of adenylyl cyclase, and cyclic AMP-mediated phosphorylation of the CGRP receptor could occur by direct phosphorylation of the CGRP receptor by protein kinase A. Although protein kinase A is activated as a result of agonist binding, our evidence indicates that this kinase does not phosphorylate the receptor since forskolin failed to cause CGRP-mediated desensitization. In addition, activation of the CGRP receptor has been shown to activate protein kinase C because this receptor has been shown to generate two second messengers (inositol phosphates and diacylglycerol) (Aiyar et al., 1999). In this study, activation of protein kinase C has no effect on CGRP receptor responsiveness, suggesting that protein kinase C is not involved in CGRP-mediated desensitization. However, Ro 32-0432, a potent and specific inhibitor of protein kinase C, resulted in partial attenuation of CGRP-mediated desensitization. This data suggest that the effect of this compound on CGRP receptor desensitization may be due to inhibition of kinases other than PKC. The inability of protein kinase A and protein kinase C activation to mimic CGRP desensitization suggested that receptor-specific kinases of the GRK family may be responsible for CGRP-mediated desensitization. GRKs are a subfamily of protein kinases (currently containing six mammalian members) that have been implicated in agonist-specific phosphorylation and desensitization of multiple GPCR (Freedman and Lefkowitz, 1996; Chuang et al., 1996). HEK-293 cells have served as a useful model for studying GPCR regulation by GRKs. These cells have been shown to contain primarily GRK2, GRK5, and GRK6 (Freedman et al., 1995; Lazari et al., 1998). In this study, we observed that Ro 32-0432 inhibited the activity of GRK2, GRK5, and GRK6 at micromolar concentration.

To investigate further the potential role of GRKs in CGRP-mediated desensitization in HEK-293-PR cells, we used an antisense approach. This strategy has been used successfully by several groups to address the role of GRKs in the regulation of the β_2-adrenergic (Shih and Malbon, 1994), H_2 histamine (Nakata et al., 1996), and thyrotropin (Nagayama et al., 1996) receptors. Pretreatment of the cells with the GRK6-specific antisense oligonucleotide markedly attenuated CGRP-mediated desensitization. The specificity of the antisense approach was shown by demonstrating that the GRK3 and GRK5 antisense had no effect on CGRP receptor desensitization. Furthermore, there was no effect on CGRP-mediated desensitization when the cells were treated with sense oligonucleotides. This observation was further strengthened by performing additional experiments in which CGRP binding and response were quantitated in cells transiently overexpressing GRK6. The hypothesis behind this set of experiments was that if GRK6 was responsible for CGRP-mediated desensitization, then the overexpression of GRK6 should result in a decrease of CGRP binding as well as function. Overexpression of GRK6 in HEK-293 cells resulted in $\sim 40\%$ loss in CGRP binding and $\sim 30\%$ loss of CGRP-mediated activation of adenylyl cyclase. These data confirm the observation that GRK6 is involved in CGRP-mediated desensitization of CGRP receptors expressed in HEK-293 cells.

In summary, the results of the present study demonstrate that CGRP-induced homologous desensitization of recombinant CGRP receptors stably transfected in HEK-293 cells involves the activation of GRK6. The present results do not rule out the possibility that, in addition to GRK6, a specific yet unidentified kinase may also be involved in homologous desensitization of CGRP receptors in HEK-293-PR cells, but several lines of evidence point to a central role for GRK6 under the experimental conditions used in this study. It will be of interest to localize the sites of phosphorylation on the receptor for agonist-induced desensitization to understand the regulatory mechanism of CGRP receptors in a number of pathological conditions.

Acknowledgements

The authors would like to thank Sue Tirri for her excellent secretarial assistance.

References

Aiyar, N., Griffin, E., Albrigtson-Winslow, C., Feuerstein, G., Nambi, P., 1992. Homologous desensitization of calcitonin gene-related peptide response in rat glomerular mesangial cells in culture. Mol. Cell. Biochem. 113, 17–23.

Aiyar, N., Rand, K., Elshourbagy, N.A., Zeng, Z., Adamou, J.E., Bergsma, D.J., Li, Y., 1996. cDNA encoding the calcitonin gene-related peptide type 1 receptor. J. Biol. Chem. 271, 11325–11329.

Aiyar, N., Disa, J., Stadel, J., Lysko, P., 1999. Recombinant calcitonin gene-related peptide (CGRP) human receptor independently stimulates 3′,5′-cyclic adenosine monophosphate and Ca^{2+} signaling pathways. Mol. Cell. Biochem. 197, 179–185.

Birchall, A.M., Bishop, J., Bradshaw, A., Cline, J., Coffey, L.H., Elliot, V.M., Bibson, A., Greenham, T.J., Hallam, W., Harris, C.H., Hill, A., Hutchings, A.G., Lamont, G., Lawton, E.J., Lewis, A., Maw, J.S., Nixon, D., Pole, D., Wadsworth, J., Wilkinson, S.E., 1994. Ro 32-0432, a selective and orally active inhibitor of protein kinase C prevents T-cell activation. J. Pharmacol. Exp. Ther. 268, 922–929.

Chuang, T.T., Sallese, M., De Blasi, A., 1996. G-protein-coupled receptors: heterologous regulation of homologous desensitization and its implications. TIPS 17, 416–421.

Dennis, T.B., Fournier, A., St. Pierre, S., Quirion, R., 1989. Structure-activity profile of calcitonin gene-related peptide in peripheral and brain tissues: evidence for receptor multiplicity. J. Pharmacol. Exp. Ther. 251, 718–725.

Elshourbagy, N.A., Adamou, J.E., Swift, A.M., Disa, J., Mao, J., Ganguly, S., Bergsma, D.J., Aiyar, N., 1998. Molecular cloning and characterization of the porcine calcitonin gene-related peptide receptor. Endocrinology 139, 1678–1683.

Freedman, N.J., Lefkowitz, R.J., 1996. Desensitization of G protein-coupled receptors. Recent Prog. Horm. Res. 51, 319–351.

Freedman, N.J., Liggett, S.B., Drachman, D.E., Pei, G., Caron, M.G., Lefkowitz, R.J., 1995. Phosphorylation and desensitization of the human beta 1-adrenergic receptor: involvement of G protein-coupled receptor kinases and cAMP-dependent protein kinase. J. Biol. Chem. 270, 17953–17961.

Hausdorf, W.P., Caron, M.G., Lefkowitz, R.J., 1990. Turning off the signal: desensitization of β-adrenergic receptor function. FASEB J. 4, 2881–2889.

Krupnick, J.G., Benovic, J.L., 1998. The role of receptor kinases and arrestins in G protein-coupled receptor regulation. Annu. Rev. Pharmacol. Toxicol. 38, 289–319.

Lazari, M.F., Bertrand, J.E., Nakamura, K., Liu, X., Krupnick, J.G., Benovic, J.L., Ascoli, M., 1998. Mutation of individual serine residues in the C-terminal tail of the lutropin/choriogonadotropin receptor reveals distinct structural requirements for agonist-induced uncoupling and agonist-induced internalization. J. Biol. Chem. 273, 18316–18324.

Lohse, M.J., 1993. Molecular mechanisms of membrane receptor desensitization. Biochim. Biophys. Acta. 1179, 171–188.

Nagayama, Y., Tanaka, K., Hara, T., Namba, H., Yamashita, S., Taniyama, K., Niwa, M., 1996. Involvement of G protein-coupled receptor kinase 5 in homologous desensitization of the thyrotropin receptor. J. Biol. Chem. 271, 10143–10148.

Nakata, H., Kinoshita, Y., Kishi, K., Fukuda, H., Kawanami, C., Matsushima, Y., Asahara, M., Okada, A., Maekawa, T., Chiba, T., 1996. Involvement of β-adrenergic receptor kinase-1 in homologous desensitization of histamine H_2 receptors in human gastric carcinoma cell line MKN-45. Digestion 57, 406–410.

Poyner, D.R., 1992. Calcitonin gene-related peptide: multiple actions, multiple receptors. Pharmacol. Ther. 56, 23–51.

Quirion, R., Van Rossum, D., Domont, Y., St. Pierre, S., Fournier, A., 1992. Characterization of CGRP1 and CGRP2 receptor subtypes. Ann. N. Y. Acad. Sci. 657, 88–105.

Salmon, Y., Londos, C., Rodbell, M., 1974. A highly selective adenylate cyclase assay. Anal. Biochem. 58, 541–548.

Shih, M., Malbon, C.C., 1994. Oligonucleotides antisense to mRNA encoding protein kinase A, protein kinase C, and β-adrenergic receptor kinase reveals distinctive cell-type-specific roles in agonist-induced desensitization. Proc. Natl. Acad. Sci. U.S.A. 91, 12193–12197.

Van Rossum, D., Hanisch, U., Qurion, R., 1997. Neuroanatomical localization, pharmacological characterization and functions of CGRP-related peptides and their receptors. Neurosci. Behav. Rev. 21, 649–678.

Van Valen, F., Piechot, G., Jurgens, H., 1990. Calcitonin gene-related peptide (CGRP) receptors are linked to cyclic adenosine monophosphate production in SK-N-MC human neuroblastoma cells. Neurosci. Lett. 119, 195–198.

Wimalawansa, S.J., 1997. Calcitonin gene-related peptide and its receptors: molecular genetics, physiology, pathophysiology, and therapeutic potentials. Endocr. Rev. 17, 533–585.