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# Calcitonin gene-related peptide receptor independently stimulates 3′,5′-cyclic adenosine monophosphate and $Ca^{2+}$ signaling pathways

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## Abstract

Calcitonin gene-related peptide (CGRP) is a neuropeptide with diverse biological properties including potent vasodilating activity. Recently, we reported the cloning of complementary DNAs (cDNAs) encoding the human and porcine CGRP receptors which share significant amino acid sequence homology with the human calcitonin receptor, a member of the recently described novel subfamily of G-protein-coupled 7TM receptors. Activation of this family of receptors has been shown to result in an increase in intracellular cAMP accumulation and calcium release. In this study, we demonstrate that HEK-293 cells expressing recombinant CGRP receptors (HEK-293HR or PR) respond to CGRP with increased intracellular calcium release ($EC_{50}$ = 1.6 nM) in addition to the activation of adenylyl cyclase ($EC_{50}$ = 1.4 nM). The effect of CGRP on adenylyl cyclase activation and calcium release was inhibited by CGRP (8–37), a CGRP receptor antagonist. Both effects were mediated by cholera toxin-sensitive G-proteins, but these two signal transduction pathways were independent of each other. While cholera toxin pretreatment of HEK-293PR cells resulted in permanent activation of adenylyl cyclase, the same pretreatment resulted in an inhibition of CGRP-mediated $[Ca^{2+}]_i$ release. Pertussis toxin was without effect on CGRP-mediated responses. In addition, CGRP-mediated calcium release appears to be due to release from a thapsigargin-sensitive intracellular calcium pool. These results show that the recombinant human as well as porcine CGRP receptor can independently increase both cAMP production and intracellular calcium release when stably expressed in the HEK-293 cell line. (Mol Cell Biochem **197**: 179–185, 1999)

*Key words*: CGRP-1 receptor, HEK-293 cells, calcium, cholera toxin

## Introduction

Calcitonin gene-related peptide (CGRP) is a 37-amino acid peptide which is synthesized from alternate processing of calcitonin mRNA [1]. CGRP is distributed throughout the central and peripheral nervous systems, and possesses diverse biological actions including regulatory effects on central sympathetic outflow, insulin secretion, neuronal differentiation and endothelial cell proliferation [2]. CGRP initiates its responses through an interaction with target organ receptors that are primarily coupled to the activation of adenylyl cyclase. CGRP receptors have been identified and characterized from several tissues, including those of neuronal and peripheral origin. Presently, two CGRP receptor subtypes (CGRP-1 and CGRP-2) have been defined based on pharmacological profiles [3]. The fragment CGRP (8–37) is a selective antagonist for CGRP-1 receptors. Reduction of the disulfide bond of CGRP, which destroys the N-terminal ring structure of the peptide, yields a linear analog, diacetoamidomethyl cysteine CGRP ([cys$(ACM)_{2,7}$]CGRP), that is a selective agonist for CGRP-2 receptors. In addition to stimulating adenylyl cyclase, CGRP increases inositol trisphosphate in skeletal muscle [4], increases $[Ca^{2+}]_i$ (the concentration of intracellular calcium) in bovine aortic endothelial cells [5] and activates protein kinase C in cardiomyocytes [6]. Recently, we reported the cloning and characterization of human and porcine CGRP receptors [7, 8]. Sequence analysis indicated that the porcine recombinant CGRP receptor shares

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93% identity with the human recombinant CGRP receptor. The amino acid sequence similarity suggests that they belong to a subgroup of G-protein-coupled receptors which also includes receptors for calcitonin, parathyroid hormone (PTH), secretin, glucagon, glucagon-like peptide, vasoactive intestinal peptide (VIP) and pituitary adenylyl cyclase-activating peptide (PACAP). This class of G-protein-coupled receptors (calcitonin (9), PTH [10], glucagon [11], glucagon-like peptide [12], secretin [13], vasoactive intestinal peptide and pituitary adenylate cyclase activating polypeptide [14]) generally have been shown to be coupled to multiple effector systems such as activation of adenylyl cyclase and phospholipase C [15]. The receptors in this group, including CGRP receptors, possess a large N-terminal extracellular domain, six conserved cysteine residues in the N-terminal extracellular domain, and multiple consensus N-glycosylation sites. Stable expression of CGRP receptor cDNA in human embryonic kidney 293 (HEK-293) cells produced specific, high affinity CGRP binding sites that displayed pharmacological and functional properties very similar to the native CGRP-1 receptors present in SKNMC cells [1]. Exposure of these cells to CGRP resulted in an increase in cAMP production, which was inhibited in a competitive manner by the CGRP-1 receptor antagonist, CGRP (8–37). In the present study, we investigated whether these recombinant CGRP receptors are also coupled to phospholipase C. Here, we report that the addition of CGRP to HEK-293 cells expressing recombinant human (HEK-293HR) or porcine (HEK-293PR) CGRP receptors resulted in the activation of phospholipase C, resulting in an increase in the formation of inositol phosphates and $[Ca^{2+}]_i$ release in addition to the activation of adenylyl cyclase.

## Materials and methods

*Materials*

Human CGRPα (hα-CGRP), human α-CGRP (8–37) (hα-CGRP (8–37)), human adrenomedullin (hADM), salmon calcitonin (sCT), and endothelin (ET-1) were purchased from Bachem Biochemicals (King of Prussia, PA, USA). Fura-2 acetoxymethyl ester (fura-2/AM) was obtained from Calbiochem (La Jolla, CA, USA). Cholera toxin and pertussis toxin were from List Biologicals (Campbell, CA, USA), and the BCA protein assay kit was obtained from Pierce Chemicals Co. (Rockford, IL, USA). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

*Generation of stable cell line expressing recombinant human and porcine CGRP receptor clones*

Cloning of human and porcine CGRP receptors and generation of HEK-293HR and HEK-293PR cells have been described earlier [7, 8]. Briefly, the human CGRP type 1 receptor was cloned using an expressed sequence tag (EST) derived from a human synovial tissue cDNA library. This incomplete 800-bp cDNA clone was used as a probe to screen a human lung cDNA library to isolate a clone with a complete open reading frame [7]. A porcine lung cDNA library constructed in a λ ZAP expression vector was screened by hybridization using a $^{32}$ P-labeled human CGRP cDNA-coding sequence as a probe. A fragment containing the entire coding region of the human CGRP receptor (or porcine CGRP receptor) was subcloned into the mammalian expression vector pCDN [16] and transfected into HEK-293 cells using the LipofectAMINE (Life Technologies, Gaithersburg, MD, USA) protocol as suggested by manufacturer. Two days after transfection, G418 was added to the culture medium to enrich for cells that contained the plasmid. In 2–3 weeks, colonies of cells appeared from which single cells were isolated by dilution and further culture. The colonies were then tested for their ability to generate cAMP in response to the CGRP peptide and were subjected to radioligand binding assays using [$^{125}$I]CGRP [7, 8].

*Measurement of adenylyl cyclase activity*

Adenylyl cyclase activity was measured in triplicate as the rate of conversion of α[$^{32}$P]ATP to [$^{32}$P]cAMP as previously described [17]. Membranes (40–60 μg) 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 solution containing cAMP (0.28 mM), ATP (0.33 mM) and 22000 dpm of [$^3$H] cAMP. [$^{32}$P]cAMP was separated using sequential chromatography (Dowex and alumina columns) [18]. Adenylyl cyclase activities were determined in the absence (basal) or presence of various concentrations (1 pM to 1 μM) of hα-CGRP.

*Determination of $[Ca^{2+}]_i$ (intracellular free calcium concentration)*

For measurements of $[Ca^{2+}]_i$, HEK-293HR or HEK-293PR cells were loaded with 2 μM fura-2/AM in growth medium for 45 min after which fresh growth medium was added for 15 min to allow residual ester hydrolysis. The cells were then rinsed in Dulbecco's PBS, trypsinized, resuspended in growth medium, centrifuged, and resuspended into modified Kreb's Ringer's Henseleit (KRH) buffer, pH 7.4, containing 0.1% gelatin. The KRH is modified by replacing sodium bicarbonate with 25 mM HEPES. The cells were stored on ice at a con-

centration of $2 \times 10^6$ cells/ml and diluted to $1 \times 10^6$ cells/ml with fresh KRH buffer at 37°C just prior to use. Fluorescence of fura-2 in cells suspended in 2 ml of buffer was measured with a dual channel fluorometer (University of Pennsylvania Biomedical Instruments Group) [19]. Data were captured on-line as voltage recordings with the aid of a PC running the LabWindows application (National Instruments, Austin, TX, USA) under Microsoft® Windows (Microsoft Corp., Redmond, WA, USA) and transferred to a MacIntosh terminal for analysis by Igor version 1.28 software (WaveMetrics, Lake Oswego, OR, USA). CGRP was added from concentrated stock solutions in water at a volume of ≤ 1%. To establish the integrity of the transfected cells, we also measured $[Ca^{2+}]_i$ stimulated by endothelin-1 (ET-1) (endogenous receptors in HEK-293 cells).

*Determination of inositol phosphates accumulation*

HEK-293PR cells were exposed to serum-free medium containing 1 µCi/ml myo [$^3$H] inositol for 24 h. On the day of the experiment, the medium was removed, the cells were washed twice with 2 ml Dulbecco's phosphate-buffered saline (DPBS) and then incubated in DPBS containing 10 mM LiCl for 10 min at 37°C. The experiment was initiated by the addition of indicated concentrations of hα-CGRP, and the incubation continued for an additional 5 min at 37°C after which the reaction was terminated by the addition of 10% (final concentration) trichloroacetic acid and centrifugation. The supernatants were extracted with ether saturated with water and the inositol phosphates were separated using ion-exchange chromatography [16].

## Results and discussion

Ligand binding studies using the HEK-293HR or HEK-293PR cells have shown high affinity specific binding sites for CGRP with a Kd of 19 and 38.5 pM, respectively [7, 8]. The binding data are consistent with the presence of a single class of high-affinity binding site. The pharmacological profile of ligands competing for [$^{125}$I]CGRP binding to the recombinant receptor was in accordance with that of the natural receptor. CGRP stimulated the accumulation of cAMP in HEK-293HR or HEK-293PR cells, with $EC_{50}$ values of 0.9 and 1.1 nM, respectively. We have also observed and presented here that in membranes isolated from HEK-293HR cells, CGRP stimulated adenylyl cyclase activity in a concentration-dependent manner, with an $EC_{50}$ value of 1.4 ± 0.3 nM (Fig. 1). In this study, we also examined CGRP-mediated $[Ca^{2+}]_i$ release using HEK-293HR cells. CGRP (100 nM) increased $[Ca^{2+}]_i$ in these cells from a resting value of 214 ± 17 nM to 312 ± 17 nM, (n = 4) followed by a gradual return to the prestimulatory level within 3–5 min, despite the continued presence of agonist (Fig. 2A). The increase in intracellular calcium release was dependent upon the concentration of CGRP, which gave an $EC_{50}$ value of 1.63 ± 0.37 nM (Fig. 2B). Similar observations were also made for HEK-293PR cells. The maximal response was observed in both cells at 100 nM of hCGRP. As controls, pCDN vector-transfected HEK-293 cells were used to study the effect of CGRP on $[Ca^{2+}]_i$ mobilization. Cells transfected with vector alone did not respond to CGRP or exhibit any specific binding of [$^{125}$I]CGRP (data not shown). While CGRP receptor antagonist, CGRP (8–37), by itself had no effect on $[Ca^{2+}]_i$ release, it inhibited the CGRP-stimulated $[Ca^{2+}]_i$ release (Fig. 2C). Stimulation of $[Ca^{2+}]_i$ release in HEK-293HR or PR cells was specific for CGRP, since CGRP-related peptides such as Cys (ACM)$_{2,7}$ CGRP, adrenomedullin and calcitonin did not cause any change (<10%) in $[Ca^{2+}]_i$ release in HEK-293HR or PR cells (Fig. 3). Also, exposure of these cells to forskolin did not cause $[Ca^{2+}]_i$ release (data not shown), suggesting that the stimulatory effect of CGRP on $[Ca^{2+}]_i$ release was not secondary to the activation of the adenylyl cyclase pathway. Furthermore, treatment of HEK-293HR or PR cells with CGRP also resulted in an increase of inositol phosphates accumulation as shown in Fig. 4 for HEK-293PR cells. Taken together, these results indicate that the recombinant human or porcine CGRP receptor expressed in HEK-293 cells is coupled to the activation of adenylyl cyclase as well as to phospholipase C. Our data also demonstrate that the concentration-response curves of CGRP for the stimulation of cAMP accumulation and intracellular calcium release are

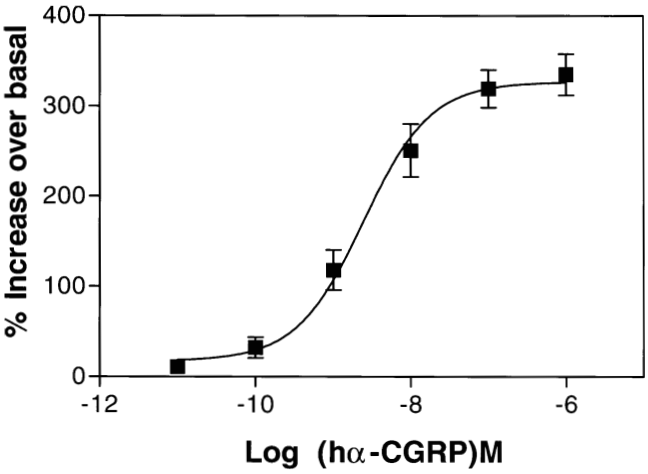


*Fig. 1.* Stimulation of adenylyl cyclase activity in the membranes from HEK-293HR cells by hα-CGRP. The membranes (40–60 µg protein) were incubated in the presence of 10 µM GTP and increasing concentrations of hα-CGRP at 30°C for 20 min and processed as explained in Materials and methods. Data are mean from 5 separate experiments performed in triplicates.

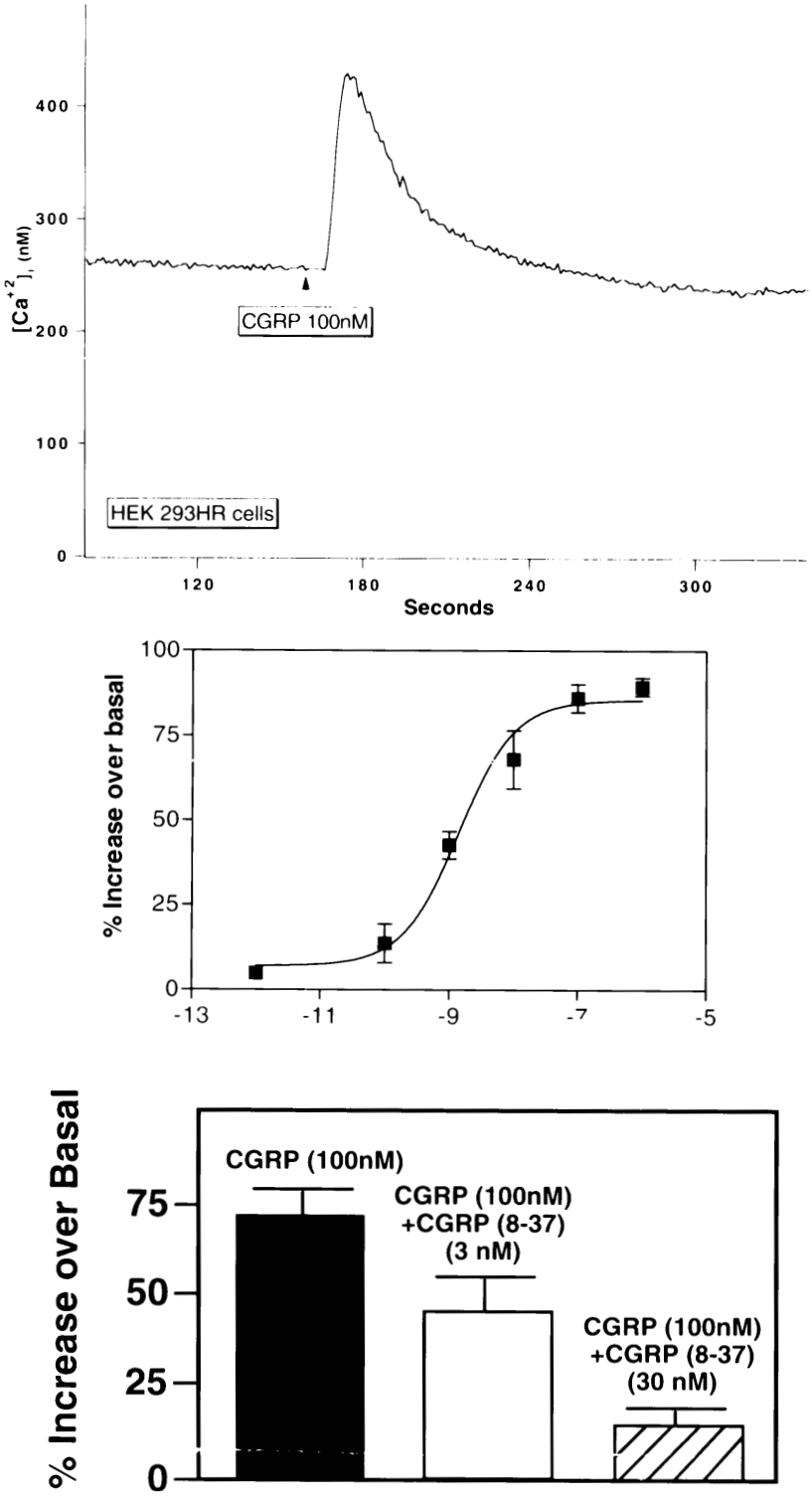


*Fig. 2.* (Top panel): Human α-CGRP-stimulated $[Ca^{2+}]_i$ transients in HEK-293HR cells. Addition of 100 nM hα-CGRP is indicated by the arrow. The results presented are representative of four different experiments performed in duplicates. Cells were loaded with fura-2 AM, and $[Ca^{2+}]_i$ release was determined using a fluorescence spectrophotometer as described in Materials and methods. (Middle panel): Concentration-response curve for hα-CGRP-mediated $[Ca^{2+}]_i$ increase in HEK-293HR cells. Each point represents the mean ± S.E.M. from 3 separate experiments performed in duplicates. (Bottom panel): Effect of hα-CGRP (8–37) on CGRP (100 nM)-mediated $[Ca^{2+}]_i$ responses in HEK-293HR cells. Data are the mean ± S.E.M. from 4 different experiments performed in duplicates.

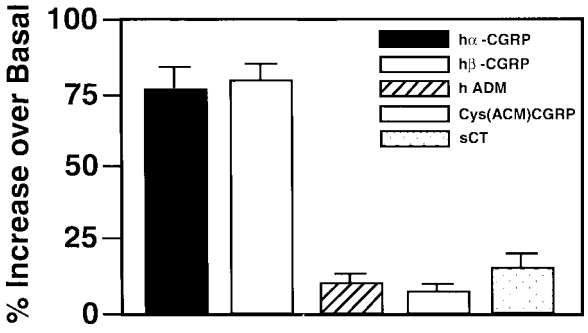


*Fig. 3.* Effect of 100 nM hα-CGRP, hβ-CGRP, hADM, Cys $(ACM)_{2,7}$ CGRP and sCT on $[Ca^{2+}]_i$ release in HEK-293HR cells. Data are the mean ± S.E.M. from 3 different experiments performed in duplicates.

very similar, indicating that the same receptor efficiently couples to both effector systems. As observed with other seven transmembrane G-protein-coupled receptors, the CGRP receptor also may activate adenylyl cyclase and phospholipase C pathways via G proteins. It is not clear whether a single G protein mediates both effects.

To determine the potential role of Gs-protein in CGRP-mediated $[Ca^{2+}]_i$ release, cholera toxin (CTX) was used to ADP ribosylate and permanently activate the Gs protein. CTX pretreatment (0.2 µg/ml for 2 h) of HEK-293PR cells resulted in a 7.5 fold increase in basal adenylyl cyclase activity but had no effect on $[Ca^{2+}]_i$ release. This is very similar to what was observed with forskolin which also stimulated adenylyl cyclase 6.4 fold over basal without increasing $[Ca^{2+}]_i$ release, further emphasizing that the activation of adenylyl cyclase may not be a prerequisite for the stimulation of $[Ca^{2+}]_i$ release.

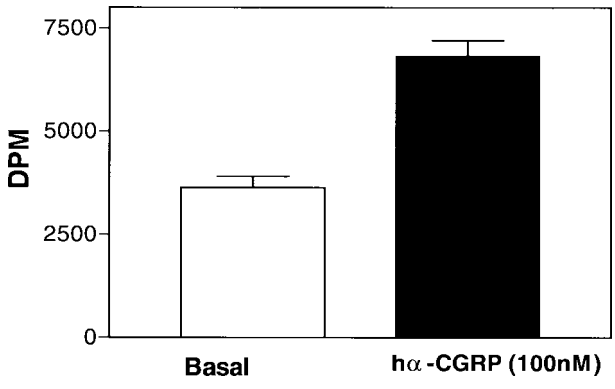


*Fig. 4.* Inositol phosphates accumulation in response to hα-CGRP (100 nM) in HEK-293PR cells. HEK-293PR cells were treated with myo-[$^3$H]inositol (1.0 µCi/ml) overnight and then washed to remove excess myo-[$^3$H]inositol. The cells were challenged with 100 nM hα-CGRP for 5 min, and the total inositol phosphates were separated from free inositol using ion-exchange chromatography. Data are the mean ± S.E.M. values from 3 different experiments performed in duplicates.

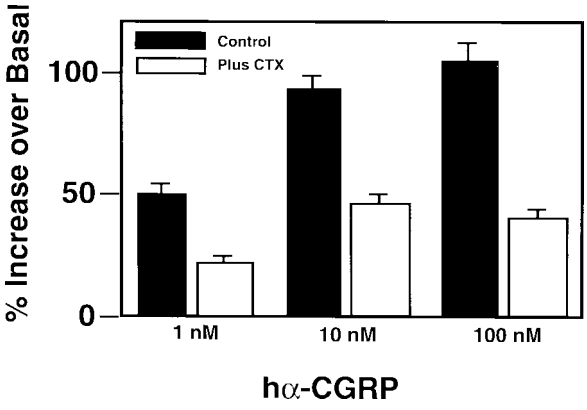


*Fig. 5.* Effects of cholera toxin pretreatment on hα-CGRP- mediated $[Ca^{2+}]_i$ release. HEK-293PR cells were pretreated with 0.2 µg/ml CTX for 2 h and then challenged with 100 nM of hα-CGRP. The results are expressed as percentage increase over basal. Data are the mean ± S.E.M. from 4 different experiments performed in duplicates.

In fact, pretreatment of HEK-293HR or PR cells with CTX inhibited CGRP-mediated $[Ca^{2+}]_i$ release by up to 70% (Fig. 5). Higher concentrations of CTX (2.0 µg/ml for 2 h) did not significantly change the inhibition of CGRP-mediated $[Ca^{2+}]_i$ release or the increase in basal adenylyl cyclase activity (data not shown). These data clearly indicate that activation of adenylyl cyclase alone is not enough to cause an increase in $[Ca^{2+}]_i$ release because both CTX and forskolin stimulated adenylyl cyclase activity without changing the intracellular $[Ca^{2+}]_i$ release. In addition, the data also indicate that activation of adenylyl cyclase does not result in inhibition of $[Ca^{2+}]_i$ release because pretreatment of the cells with forskolin resulted in a significant increase in adenylyl cyclase activity with no inhibition of $[Ca^{2+}]_i$ release. Thus, these data suggest that these two pathways (activation of adenylyl cyclase and intracellular $[Ca^{2+}]_i$ release) mediated by CGRP are independent pathways. While one of these pathways (activation of adenylyl cyclase) is stimulated by CTX pretreatment, the other pathway (stimulation of $[Ca^{2+}]_i$ release) is inhibited by CTX pretreatment. We then determined whether the inhibitory effect of CTX on CGRP-mediated $[Ca^{2+}]_i$ release was unique to the CGRP receptor or was shared by other receptors such as endothelin in HEK-293 cells. We found that CTX pretreatment did not affect endothelin-1-mediated $[Ca^{2+}]_i$ increase in these cells (262.2 ± 40.8 nM (control) and 259.87 ± 25.4 nM (CTX-Pretreated) (n = 4)). So, the CTX-mediated inhibition appears to be selective for CGRP receptor. Similarly, pertussis toxin (PTX) was used to study the involvement of the Gi family of G-proteins in CGRP-mediated responses. Neither CGRP-mediated $[Ca^{2+}]_i$ release nor activation of adenylyl cyclase in HEK-293PR was affected by PTX (100 ng/ml, 18 h) pretreatment (data not shown), ruling out a role for Gi. These results suggest that

CTX-sensitive G-protein(s) is(are) involved in CGRP-mediated $[Ca^{2+}]_i$ release as well as in CGRP-mediated activation of adenylyl cyclase. This type of activation of intracellular $[Ca^{2+}]_i$ increase through CTX-sensitive G-proteins has been previously reported for a small group of peptides such as adrenomedullin [5], secretin [13] and calcitonin [20].

We also studied the effects of nifedipine (L-type calcium channel blocker) and thapsigargin ($Ca^{2+}$-ATPase inhibitor) on CGRP-mediated $[Ca^{2+}]_i$ release in HEK-293HR or HEK-293PR cells. Nifedipine did not affect CGRP-mediated calcium release. (Fig. 6, top panel). On the other hand, thapsigargin alone (3 µM) produced a rapid increase in $[Ca^{2+}]_i$ from 226–405 nM. Addition of CGRP (100 nM) to the cell suspension after exposure to thapsigargin resulted in significantly diminished $[Ca^{2+}]_i$ release (90% inhibition, Fig. 6, bottom panel). These results suggest that by depleting intracellular $Ca^{2+}$ stores, thapsigargin blocked CGRP-mediated $[Ca^{2+}]_i$ release. It has been reported [21, 22] that thapsigargin acts by irreversibly inhibiting the reuptake of intracellular $Ca^{2+}$ and thus preventing further agonist-stimulated $[Ca^{2+}]_i$ release.

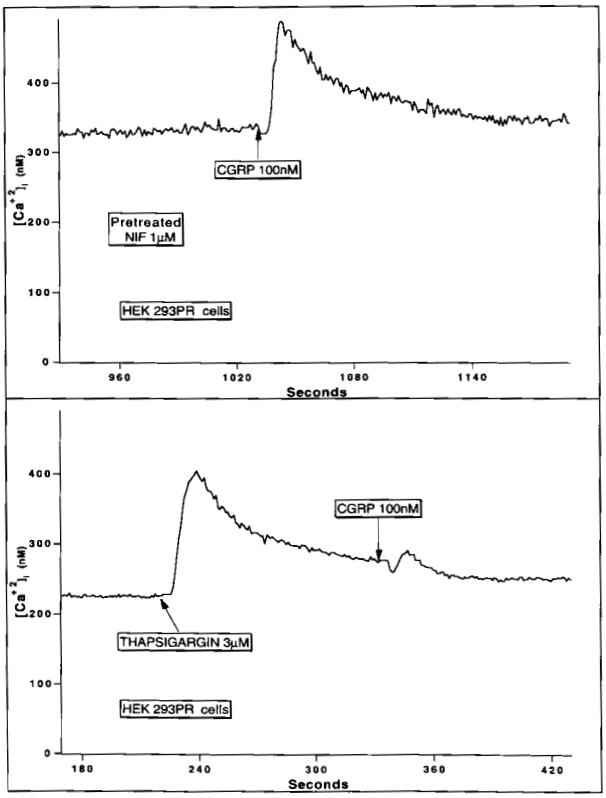


*Fig. 6.* (Top panel): Effects of nifedipine (l µM) on hα-CGRP-mediated $[Ca^{2+}]_i$ release in HEK-293PR cells. Data are from 1 experiment which is representative of 2 separate experiments performed in duplicates. (Bottom panel): Effects of thapsigargin (3 µM) on hα-CGRP-mediated $[Ca^{2+}]_i$ increase in HEK-293PR cells. Data are from 1 experiment which is representative of 2 separate experiments performed in duplicates.

Thus, the data presented here clearly demonstrate that the recombinant human or porcine CGRP receptors can simultaneously activate two distinct intracellular signaling pathways independently of each other. In this respect, these findings show some analogy to those reported for the calcitonin-secretin family of G protein-coupled receptors. Furthermore, there is no evidence of interaction between these two signal transduction pathways. The concept that a single ligand can activate more than one signal transduction pathway has been demonstrated for a small number of recombinant G-protein-coupled receptors such as the adrenomedullin [5], secretin [13] and calcitonin receptors [18]. Since HEK-293 cells appear to be the only mammalian cell that is capable of expressing recombinant CGRP receptors, it is possible that the presence of a diverse population of G proteins in this cell has facilitated this coupling event.

In summary, we report a novel intracellular signaling pathway for CGRP receptors. Activation of CGRP receptors in HEK-293HR or HEK-293PR cells results in parallel, independent, increases in cAMP and intracellular calcium levels via CTX-sensitive G-proteins.

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