0026-895X/04/6501-207–213$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 65:207–213, 2004

Vol. 65, No. 1
2852/1119547
Printed in U.S.A.

Characterization of the Human Calcitonin Gene-Related Peptide Receptor Subtypes Associated with Receptor Activity-Modifying Proteins

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Received July 29, 2003; accepted October 8, 2003

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Coexpression of receptor activity-modifying proteins (RAMPs) with calcitonin receptor 2 (CTR2) or calcitonin receptor-like receptor (CRLR) leads to the formation of four functional heterodimeric receptors for human calcitonin gene-related peptide (hCGRP). In this study, we transfected hCGRP receptors into human embryonic kidney 293 cells and examined their pharmacological profiles using three dominant-negative (DN) RAMP mutants and various hCGRPα analogs. Fluorescence-activated cell-sorting analysis revealed that their cotransfection with CTR2 induced cell surface expression of all three RAMPs, and the three CTR2/RAMP heterodimers mediated equivalent levels of cAMP production in response to hCGRPα that were approximately 50-fold greater than were seen with CTR2 alone. By contrast, [Tyr^0]hCGRPα binding and signaling were markedly weaker with CTR2/RAMP2 or -3 than with CTR2/RAMP1 or CRLR/RAMP1; likewise, ^{125}I-[His^{10}]hCGRPα bound most potently to CTR2/RAMP1. When CTR2 was coexpressed with DN RAMP1 or -2, hCGRPα-evoked responses were similar to those seen with CTR2 alone, despite the expression of both CTR2 and DN RAMP at the cell surface. But coexpression of DN RAMP3 with CTR2 significantly diminished hCGRPα signaling compared with that seen with CTR2 alone, indicating that DN RAMP3 is able to function as a negative regulator of CTR2 function. Competition experiments showed the relative agonist sensitivity of the four receptors to be hCGRPα > [Tyr^0]hCGRPα > [Cys(Et)^{2,7}]hCGRPα > [Cys(ACM)^{2,7}]hCGRPα. Of the linear analogs, [Cys(ACM)^{2,7}]hCGRPα (ACM, acetylmethoxy) enhanced cAMP formation only via CTR2/RAMP1, whereas [Cys(Et^{2,7})]hCGRPα acted via CRLR/RAMP1 and somewhat less potently via CTR2/RAMP1. Thus, among the three CGRP$_{8-37}$-insensitive receptors, CTR2/RAMP1 is most sensitive to the two linear analogs, suggesting that it could be classified as a CGRP2 receptor. Moreover, the combined use of iodinated CGRPα analogs may be useful for defining the CGRP1 receptor.

The α form of calcitonin gene-related peptide (CGRPα) is a 37-amino acid peptide generated from alternate tissue-specific splicing of the calcitonin gene (Amara et al., 1982), and, like adrenomedullin and amylin, it belongs to the calcitonin family of regulatory peptides (Wimalawansa, 1997). Notably, the β form of the peptide (CGRPβ) is not derived from the calcitonin gene, despite its high sequence homology with CGRPα (Amara et al., 1985). CGRPα and -β each contain a disulfide bridge between cysteine residues at positions 2 and 7 and a C-terminal phenylalanine amide, both of which are required for biological activity (Wimalawansa, 1997). Their binding sites are widely distributed among peripheral tissues and in the central nervous system, enabling CGRP to exert a wide variety of biological effects, including potent vasorelaxation (Van Rossum et al., 1997).

The existence of at least two CGRP receptor subtypes has been proposed from differential antagonist affinities and agonist potencies in a variety of in vivo and in vitro bioassays (Dennis et al., 1989, 1990; Dumont et al., 1997). The CGRP1 receptor subtype was found to be particularly sensitive to the antagonist fragment CGRP$_{8-37}$ (Chiba et al., 1989; Dennis et al., 1990; Mimeault et al., 1991). By contrast, the CGRP2 receptor was sensitive to the linear analogs [Cys(ACM)^{2,7}]- and [Cys(Et)^{2,7}]hCGRPα but was insensitive to CGRP$_{8-37}$ (Dennis et al., 1989, 1990; Dumont et al., 1997). In 1998, the

This study was supported in part by the grants-in-aid for Scientific Research on Priority Areas and for 21st Century Centers of Excellence Program (Life Science) from the Ministry of Education, Culture, Sports Science, and Technology, Japan, and by a grant-in-aid from the Uehara Memorial Foundation.

ABBREVIATIONS: CGRP, calcitonin gene-related peptide; CT, calcitonin; CTR, calcitonin receptor; RAMP, receptor activity-modifying protein; CRLR, calcitonin receptor-like receptor; ACM, acetylmethoxy; Et, ethylamide; h, human; DN, dominant-negative; FITC, fluorescein isothionate; HA, human hemagglutinin; FACS, fluorescence-activated cell-sorting; HEK, human embryonic kidney; PBS, phosphate-buffered saline; *D*, deletion; *Mock,* empty vector pCAGGS/Neo.

CGRP1 receptor was identified as a heterodimer composed of a novel single transmembrane domain accessory protein, receptor activity-modifying protein 1 (RAMP1), and an orphan receptor, calcitonin receptor-like receptor (CRLR) (McLatchie et al., 1998). RAMP2 and RAMP3 also transport CRLR to the cell surface, where the CRLR/RAMP complex exhibits a 1:1 stoichiometry and forms functional adrenomedullin receptors (McLatchie et al., 1998; Hilairet et al., 2001). The three RAMPs, which share only 30% sequence identity and differ in their tissue distributions, are all composed of ~160 amino acids, which make up a large extracellular N-terminal domain, a single membrane-spanning domain, and a very short cytoplasmic domain (Sexton et al., 2001). Upon binding their respective agonist, these receptors mediate mobilization of intracellular cAMP and Ca^{2+} (Kuwasako et al., 2000). We recently identified the individual RAMP domains responsible for agonist binding to CRLR/RAMP heterodimers and their deletion yielded a group of dominant-negative (DN) RAMP mutants (Kuwasako et al., 2001, 2002, 2003a).

CRLR shares ~55% overall amino acid sequence identity with the calcitonin receptor (CTR), although the transmembrane domains are almost 80% identical (Poyner et al., 2002). The best characterized splice variants of hCTR differ depending on the presence (CTR1) or absence (CTR2) of 16 amino acids in the first intracellular loop (Poyner et al., 2002). In that regard, cotransfection of RAMPs with the most common variant, CTR2, leads to the formation of a 1:1 dimer at the cell surface (Christopoulos et al., 1999) and augmentation of responses evoked by amylin or CGRP (Christopoulos et al., 1999; Leuthauser et al., 2000; Tilakaratne et al., 2000). The CGRP-evoked responses mediated via CTR/RAMPs were blocked by the selective receptor antagonist $calcitonin_{8-32}$ but not by $CGRP_{8-37}$ (Kuwasako et al., 2003b; Leuthauser et al., 2000). Although $calcitonin_{8-32}$ should therefore be useful for defining the CGRP2 receptor subtype, whether the functional properties of this receptor are similar to those mediated by CTR2/RAMP heterodimers remains unclear. In addition, nothing is known about the effects of DN RAMPs on CTR2 function. We therefore cotransfected hCTR2 and individual hRAMPs into human embryonic kidney (HEK) 293 cells, which express no functional CGRP or amylin receptors, and examined their pharmacological features using the aforementioned DN RAMPs and various hCGRPα peptide analogs.

Materials and Methods

Chemicals. ^{125}I-[Tyr^0]hCGRPα (specific activity, 2 μCi/pmol), which contains an extra N-terminal tyrosine residue (Tyr^0), was produced in our laboratory using a modification of a method described previously (Kitamura et al., 1993; Kuwasako et al., 2003a). ^{125}I-[His^{10}]hCGRPα (specific activity, 2 μCi/pmol) was obtained from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK). Human CGRPα was purchased from Peptide Institute (Osaka, Japan). [Tyr^0]hCGRPα, [Cys(ACM)^{2,7}]hCGRPα (ACM, acetylmethoxy), [Cys(Et)^{2,7}]hCGRPα (Et, ethylamide), and rat amylin were from Phoenix Pharmaceuticals, Inc. (Belmont, CA). Rat FITC-conjugated monoclonal anti-HA antibody was from Roche Applied Science (Indianapolis, IN). All of the other reagents were of analytical grade and were obtained from various commercial suppliers.

Expression Constructs. Human CTR2 (Kuestner et al., 1994) and the three hRAMPs (McLatchie et al., 1998) were modified to provide a consensus Kozak sequence as described previously (Aiyar et al., 1996). An HA epitope tag (YPYDVPDYA) was ligated, in-frame, to the 5′ end of cDNAs encoding the intact and DN hRAMPs (DN hRAMP1, L94A/*D*101-103; DN hRAMP2, *D*86-92; DN hRAMP3, *D*59-65) used for CRLR/RAMP heterodimers (Kuwasako et al., 2001, 2003a), and the native signal sequences were removed and replaced with MKTILALSTYIFCLVFA (Guan et al., 1992), yielding HA hRAMPs and HA DN hRAMPs. Human CTR2 and individual HA hRAMPs and HA DN hRAMPs were cloned into the mammalian expression vector pCAGGS/Neo (Kuwasako et al., 2000) using the 5′-XhoI and 3′-NotI sites. The sequences of the resultant constructs were all verified using the 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). Each of the HA hRAMPs was compared with the native sequence in the assays and was found to behave identically (data not shown).

Cell Culture and DNA Transfection. HEK 293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B at 37°C under a humidified atmosphere of 95% air/5% CO_2. For experimentation, cells were seeded into 24-well plates and, upon reaching 70 to 80% confluence, were transiently cotransfected with hCTR2 plus HA hRAMP, HA DN hRAMP, or empty vector using LipofectAMINE transfection reagents (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The cells were incubated for 4 h in 250 μl of OptiMEM 1 medium containing 200 ng/well plasmid DNA, 2 μl/well Plus regent, and 2 μl/well LipofectAMINE. As a control, some cells were transfected with empty vector (pCAGGS/Neo) (*Mock*). All experiments were carried out 48 h after transfection.

FACS Analysis. Flow cytometry was carried out to assess the levels of whole-cell and cell-surface expression of each HA hRAMP or HA DN hRAMP mutant in HEK 293 cells. To evaluate cell-surface expression, cells were harvested after transient transfection, washed twice with PBS, resuspended in ice-cold FACS buffer (Kuwasako et al., 2000), and then incubated with anti-HA FITC antibody (1:50 dilution) for 60 min at 4°C in the dark. For the evaluation of whole-cell expression, cells were first permeabilized using IntraPrep reagents (Beckman Coulter, Fullerton, CA) according to the manufacturer's instructions and then incubated with anti-HA FITC antibody (1:50 dilution) for 15 min at room temperature in the dark. After two successive washes with FACS buffer, both groups of cells were subjected to flow cytometry in an EPICS XL flow cytometer (Beckman Coulter) and analyzed using EXPO 2 software (Beckman Coulter).

Radioligand Binding. To assess whole-cell radioligand binding, transfected HEK 293 cells in 24-well plates were washed twice with ice-cold PBS and then incubated with 100 pM ^{125}I-[Tyr^0]hCGRPα or ^{125}I-[His^{10}]hCGRPα in binding buffer (modified Krebs-Ringers-HEPES medium) (Kuwasako et al., 2000) in the absence (total binding) or presence of increasing concentrations of unlabeled hCGRPα. The total incubation volume was 300 μl. Nonspecific binding was defined as binding in the presence of 1 μM unlabeled hCGRPα. After incubation for 4 h at 4°C, the cells were washed twice with ice-cold PBS and solubilized with 0.5 M NaOH, after which the associated cellular radioactivity was measured in a γ-counter.

cAMP Assay. Analysis of intracellular cAMP accumulation was carried out after transfection of the indicated cDNAs into HEK 293 cells. In Hanks' buffer solution containing 20 mM HEPES and 0.1% bovine serum albumin, the cells were exposed to the indicated concentrations of hCGRPα, [Tyr^0]hCGRPα, [Cys(ACM)^{2,7}]hCGRPα, [Cys(Et)^{2,7}]hCGRPα, or rat amylin for 15 min at 37°C in the presence of 0.5 mM 3-isobutyl-1-methylxanthine (Sigma Chemical, St. Louis, MO). The reactions were terminated by the addition of lysis buffer (Amersham Biosciences). The resultant lysates were centrifuged at 2000 rpm for 10 min at 4°C, after which aliquots of the supernatants were collected, and the cAMP contents were determined using a commercial enzyme immunoassay kit according to the manufacturer's instructions for a nonacetylation protocol (Amersham Biosciences).

Statistical Analysis. Results are expressed as means ± S.E. of at least three independent experiments. Differences between two groups were evaluated with the use of Student's *t* tests. The differences among multiple groups were evaluated with one-way analysis of variance followed by Scheffé's tests. Values of $P < 0.05$ were considered significant.

Results

We used HEK 293 cells in this study because they lack functional CGRP receptors (Fig. 3A). Figure 1A shows the effect of cotransfection of each of the epitope-tagged hRAMP isoforms with hCTR2 on cell surface expression in nonpermeabilized cells (Fig. 1A). Increasing concentrations of hCTR2 induced corresponding increases in cell surface hRAMP delivery compared with that obtained with each hRAMP alone. The EC_{50} value for hCTR2 was approximately 50 ng for all three hRAMPs. Although hRAMPs appeared at the cell surface, even when transfected alone, specific ^{125}I-[Tyr^0]hCGRPα binding was not increased (Fig. 1B). Cotransfection of hRAMP1 with increasing concentrations of hCTR2 markedly increased specific ^{125}I-[Tyr^0]hCGRPα binding, whereas hCTR2/hRAMP2 or -3 showed only small increases in the specific binding. In all cases, ^{125}I-[Tyr^0]hCGRPα binding was approximately maximal when 100 ng of hCTR2 DNA was transfected (EC_{50} = 50 ng).

The four hCGRP receptors generated by coexpression of each of the hRAMPs with hCTR2 or hCRLR were characterized using the two ^{125}I-labeled CGRPα analogs ^{125}I-[Tyr^0]hCGRPα and ^{125}I-[His^{10}]hCGRPα (Fig. 2). There was little specific binding of either iodinated analog to HEK 293 cells transfected with empty vector (*Mock*) or hCTR2 alone. The specific binding of [Tyr^0]hCGRPα to cells coexpressing hCTR2 with hRAMP1 was 26,000 cpm/well (nonspecific/total binding ratio = 0.11) (Fig. 2A). Similar levels of specific binding were obtained in cells coexpressing hCRLR with hRAMP1 (17,500 cpm/well), whereas the specific binding to cells coexpressing hCTR2 with hRAMP2 or -3 was somewhat lower (2800 and 4500 cpm/well, respectively) (Fig. 2A). On the other hand, hRAMP1 markedly increased levels of specific ^{125}I-[His^{10}]hCGRPα binding when coexpressed with hCTR2 (39,800 cpm) but failed to significantly affect binding when coexpressed with hCRLR (13,100 cpm) (Fig. 2B). Much lower levels of specific binding were observed when hCTR2 was coexpressed with hRAMP2 or -3 (9200 and 6000 cpm, respectively) (Fig. 2B).

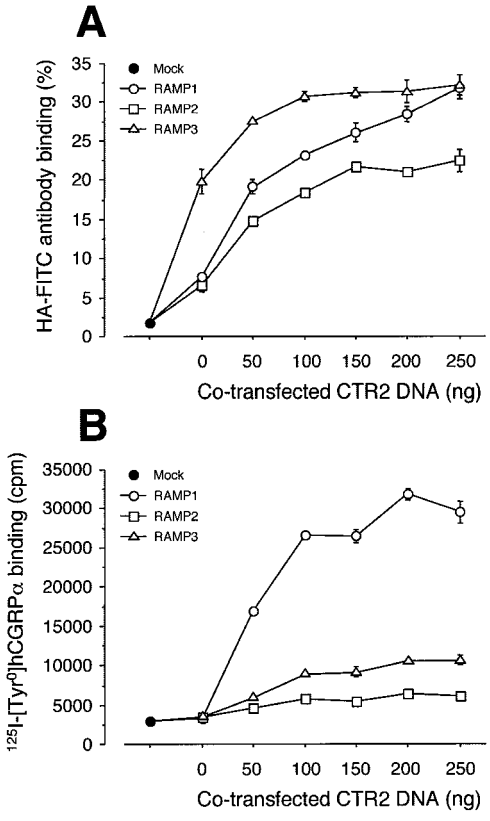

Fig. 1. Effect of hCTR2 DNA concentration on cell-surface hRAMP delivery (A) and ^{125}I-[Tyr^0]hCGRPα binding (B) in HEK 293 cells transfected with 100 ng of hRAMP1, -2, or -3. *A*, FACS analysis of nonpermeabilized HEK 293 cells expressing HA hRAMPs in the absence and presence of hCTR2. Forty-eight hours after transfection, cells were incubated with monoclonal anti-HA FITC antibody for 1 h at 4°C. *Mock* incubation with the antibody served as the control. B, ^{125}I-[Tyr^0]hCGRPα binding to HEK 293 cells coexpressing one of the hRAMPs with hCTR2 or empty vector (*Mock*). Cells were transiently transfected with the indicated plasmids and then incubated with 100 pM ^{125}I-[Tyr^0]hCGRPα for 4 h at 4°C. Total transfected DNA concentrations were all adjusted to 350 ng using empty vector. Bars represent means ± S.E. of three experiments.

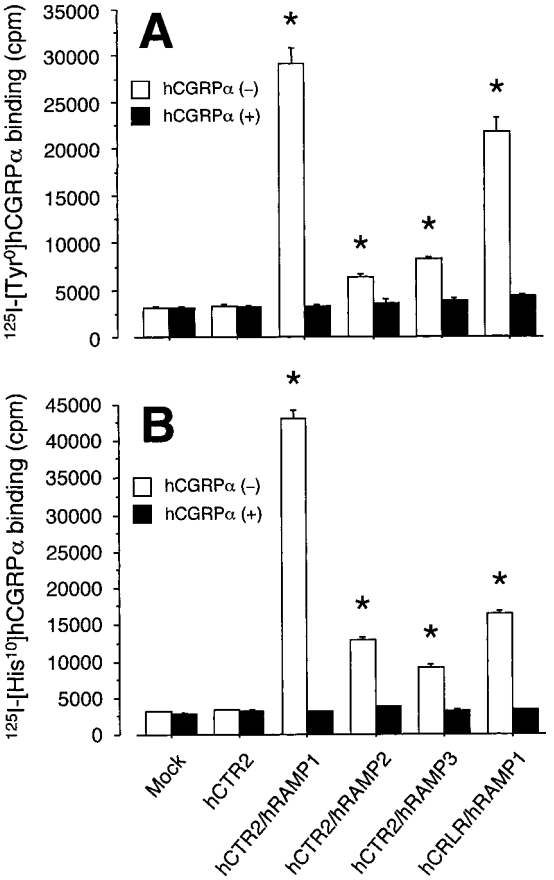

Fig. 2. Binding of ^{125}I-[Tyr^0]hCGRPα (A) and ^{125}I-[His^{10}]hCGRPα (B) to HEK 293 cells cotransfected with 100 ng of each hRAMP and 100 ng of hCTR2 or hCRLR. Shown is the total (□) and nonspecific (■) binding. Cells were transiently transfected with the indicated plasmids and then incubated for 4 h at 4°C with 100 pM ^{125}I-[Tyr^0]hCGRPα or ^{125}I-[His^{10}]hCGRPα in the presence (for nonspecific binding) or absence (for total binding) of 1 μM unlabeled hCGRPα. Bars represent means ± S.E. of three experiments (⋆, $P < 0.01$ versus corresponding nonspecific binding).

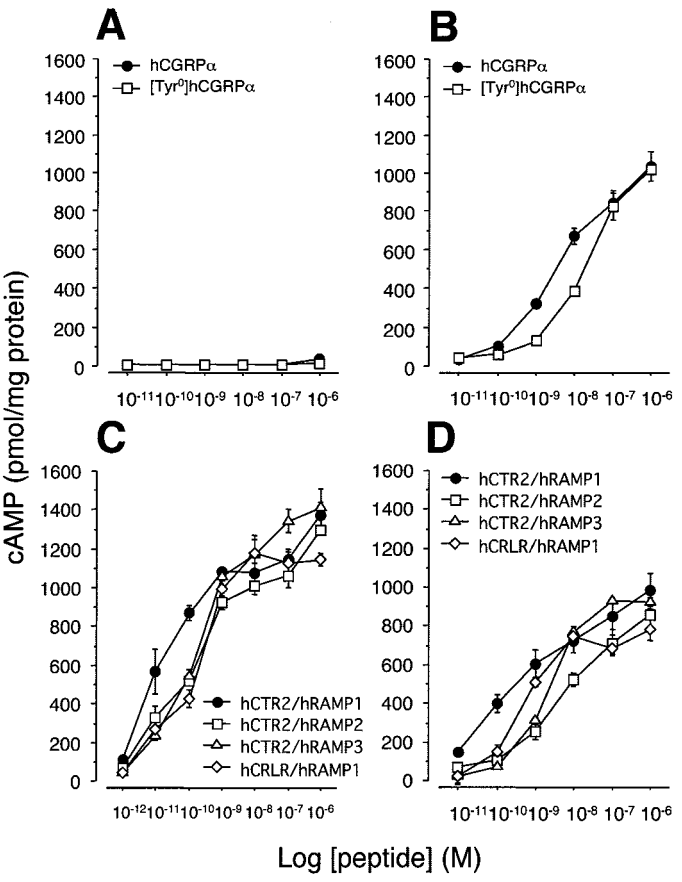

Fig. 3. Agonist-evoked cAMP production in HEK 293 cells transfected with 200 ng of vector (A), 100 ng of vector plus 100 ng of hCTR2 (B), or 100 ng of the indicated hRAMP plus 100 ng of hCTR2 or hCRLR (C and D). A and B, hCGRPα or [Tyr^0]hCGRPα responses. C, hCGRPα responses. D, [Tyr^0]hCGRPα responses. Cells were transiently transfected with the indicated plasmids, after which they were incubated for 15 min at 37°C with the indicated concentrations of hCGRPα or [Tyr^0]hCGRPα and then lysed. The resultant lysates were analyzed for cAMP content. Bars represent means ± S.E. of three experiments.

The functionality of these receptors was assessed by measuring agonist-induced intracellular cAMP accumulation (Fig. 3). Although they endogenously express functional hCTR2 (Kuwasako et al., 2003b), high concentrations of hCGRPα or [Tyr^0]hCGRPα elicited no significant increases in cAMP in HEK 293 cells expressing empty vector (*Mock*) (Fig. 3A). This may be caused by the low level of hCTR2 protein expression. In HEK 293 cells transfected with hRAMP1, however, hCGRPα increased the cAMP content slightly; maximal cAMP levels (92 pmol/mg protein) reached ~8-fold over baseline (data not shown). This suggests that hRAMP1 and endogenous hCTR2 are capable of forming a functional CGRP receptor (Christopoulos et al., 1999). Transfection with hCTR2 enabled hCGRPα (EC_{50} = 3.8 nM) and [Tyr^0]hCGRPα (EC_{50} = 19 nM) to elicit significant increases in cAMP (Fig. 3B), and cotransfection of hCTR2 and hRAMP1 markedly increased the potency of hCGRPα (EC_{50} = 0.014 nM) (Fig. 3C). hCGRPα acted somewhat less potently in cells expressing hCTR2/hRAMP2 or -3 or hCRLR/hRAMP1 (EC_{50} = 0.20, 0.21, and 0.18 nM, respectively) (Fig. 3C). In cells expressing hCTR2/hRAMP, [Tyr^0]hCGRPα was approximately 10 to 20-fold less potent than hCGRPα (EC_{50} = 0.30 nM for hCTR2/hRAMP1 versus 4.8 nM for hCTR2/hRAMP2 versus 2.3 nM for hCTR2/hRAMP3), but the two analogs were equipotent in cells expressing hCRLR/hRAMP1 (EC_{50} = 0.48 nM for [Tyr^0]hCGRPα) (Fig. 3D). Notably, the responses to [Tyr^0]hCGRPα closely paralleled the profile of ^{125}I-[Tyr^0]hCGRPα binding (Fig. 2A). Rat amylin was highly potent in cells expressing hCTR2/hRAMP1, -2, or -3 (EC_{50} = 0.02, 0.35, and 0.15 nM, respectively) but not hCRLR/hRAMP1 (EC_{50} > 180 nM) (data not shown). Thus, coexpressed hCTR2 with hRAMP seems to form high-affinity receptors for both CGRPα and amylin.

To explore further the role of hRAMP in hCTR2 function, the effects of expressing three hRAMP deletion mutants (DN hRAMPs) on the whole-cell and cell-surface expression of epitope-tagged hRAMPs were analyzed. After transient transfection, all of the mutants were expressed in HEK 293 cells at levels similar to those seen with intact hRAMPs (Table 1). Moreover, the cell-surface expression of all three mutants was significantly increased by cotransfection with hCTR2, again reaching levels similar to those seen in intact hRAMPs. This may reflect the fact that the mutants encode all of the cysteine residues and *N*-glycosylation sites required for cell-surface expression of RAMPs (McLatchie et al., 1998; Flahaut et al., 2002; Kuwasako et al., 2003c). Total [Tyr^0]hCGRPα binding obtained with mutant receptor heterodimers composed of hCTR2 complexed with each of the DN hRAMPs was not significantly different from that obtained with hCTR2 alone (Fig. 4A). The levels of specific binding of [Tyr^0]hCGRPα to cells coexpressing hCTR2 with hRAMP1, -2, or -3 (250–630 cpm/well) were also similar to those seen with hCTR2 alone (140 cpm/well) (Fig. 4A).

The functionality of the receptors composed of hCRLR and

TABLE 1
HA FITC antibody binding to HEK 293 cells expressing 100 ng of HA hRAMPs or HA DN hRAMP mutants with or without 100 ng of CTR2
Data are presented as mean ± S.E. (n = 3).

	Permeabilized Cells		Nonpermeabilized Cells	
	hCTR2 (−)	hCTR2 (+)	hCTR2 (−)	hCTR2 (+)
	%			
Control	1.88 ± 0.03	1.98 ± 0.17	1.47 ± 0.11	1.85 ± 0.04
HA hRAMP1	53.6 ± 0.75*	55.1 ± 0.92#	3.30 ± 0.23*	26.2 ± 1.79#†
HA hRAMP2	57.5 ± 0.85*	58.6 ± 1.71#	9.31 ± 0.36*	16.7 ± 1.12#†
HA hRAMP3	53.8 ± 1.01*	54.5 ± 0.37#	22.0 ± 0.48*	32.5 ± 0.38#†
HA DN hRAMP1	51.5 ± 1.34*	57.9 ± 1.44#†	4.64 ± 0.73*	24.2 ± 0.43#†
HA DN hRAMP2	60.9 ± 2.06*	62.6 ± 0.71#	12.0 ± 0.37*	17.4 ± 0.52#†
HA DN hRAMP3	56.5 ± 0.72*	59.2 ± 0.41#†	23.2 ± 1.02*	32.5 ± 2.97#†

* $P < 0.001$ versus empty vector.
\# $P < 0.001$ versus hCTR2 alone.
† $P < 0.05$ versus corresponding CRLR (−).

each of the DN RAMPs was then evaluated by measuring hCGRPα-evoked cAMP production (Fig. 4B). The EC_{50} values obtained in cells coexpressing hCTR2 and DN hRAMP1 or -2 (2.0 and 1.3 nM, respectively) did not differ from that obtained with hCTR2 alone (1.6 nM). Surprisingly, coexpression of hCTR2 with DN hRAMP3 reduced hCGRPα-evoked cAMP accumulation by 20 to 88% at concentrations ranging from 10^{-11} M to 10^{-7} M (EC_{50} = 14 nM), suggesting that DN hRAMP3 is able to act as a negative regulator of hCTR2 function.

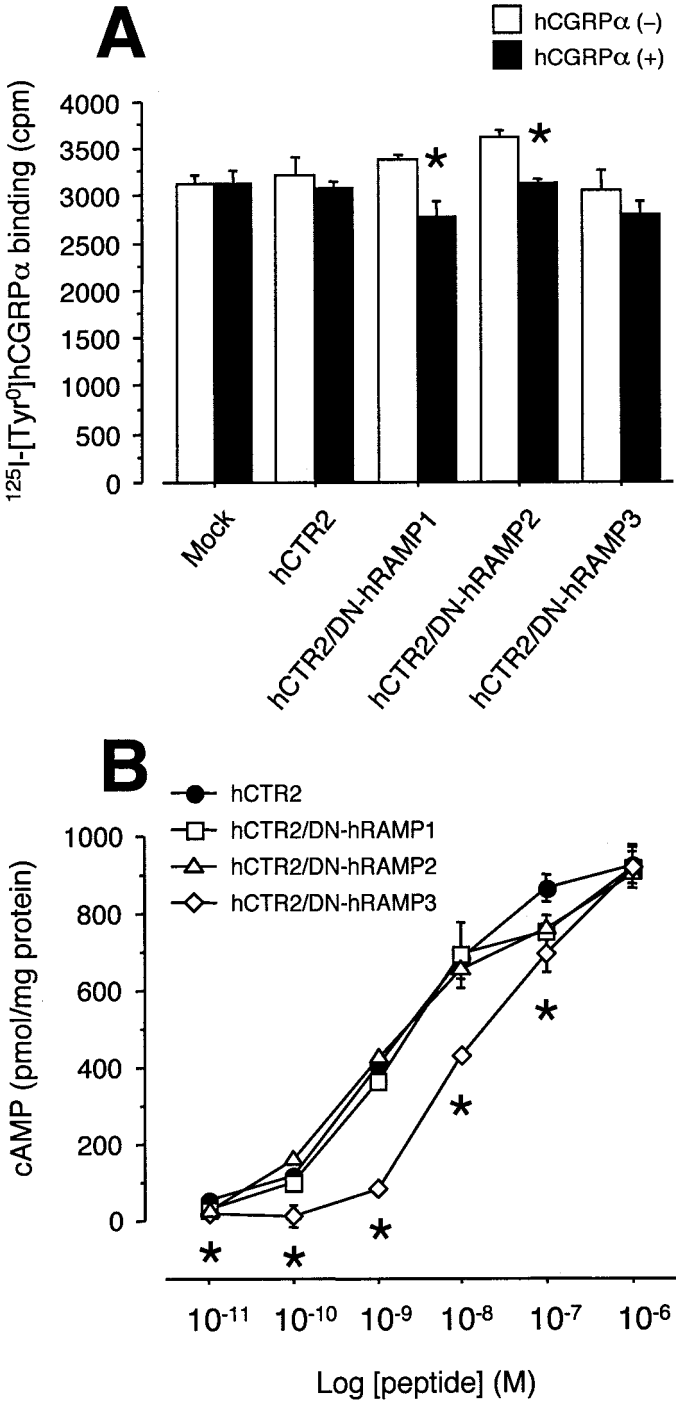

Fig. 4. Effects of DN hRAMPs on hCTR2 function. A, ^{125}I-[Tyr^0]hCGRPα (100 pM) binding to HEK 293 cells transfected with hCTR2 alone (100 ng) or cotransfected with hCTR2 (100 ng) plus one of three DN hRAMP mutants (100 ng). Shown is total (□) and nonspecific (■) binding. Cells were transiently transfected with the indicated plasmids and analyzed as in Fig. 3. Bars represent the means ± S.E. of three experiments (⋆, $P <$ 0.05 versus corresponding nonspecific binding). B, HEK 293 T cells expressing hCTR2 alone or hCTR2 plus one of three DN hRAMP mutants were treated with the indicated concentrations of hCGRPα for 15 min at 37°C. Bars represent means ± S.E. of three experiments (⋆, $P < 0.05$ versus hCTR2-expressing cells).

Using transfectants expressing each of the four CGRP receptors (Table 2), the affinities of various hCGRPα analogs were evaluated in ^{125}I-[Tyr^0]hCGRPα radioreceptor assays. With the exception of rat amylin, the IC_{50} values for the various analogs were similar with both hCTR2/hRAMP1 and hCRLR/hRAMP1, and the relative sensitivities of the two receptors were the following: hCGRPα > [Tyr^0]hCGRPα > [Cys(Et)^{2,7}]hCGRPα > [Cys(ACM)^{2,7}]hCGRPα. The profiles of the competition curves obtained with hCTR2/hRAMP2 and hCTR2/hRAMP3 were similar for each of the analogs tested, although the IC_{50} values were much lower than were seen with hCTR2/hRAMP1 and hCRLR/hRAMP1.

As shown in Fig. 5, the linear analogs [Cys(ACM)^{2,7}]hCGRPα and [Cys(Et)^{2,7}]hCGRPα were virtually inactive at micromolar concentrations in cAMP assays, even in cells expressing hCTR2 or hCRLR. Both linear analogs had a moderate effect on the cAMP content of HEK 293 cells expressing hCTR2/hRAMP1 (EC_{50} = 54 and 4.1 nM, respectively), which was ~10-fold greater than was seen in cells expressing hCTR2/hRAMP2 or hCTR2/hRAMP3. The most potent responses were elicited by [Cys(Et)^{2,7}]hCGRPα in cells expressing hCRLR/hRAMP1 (EC_{50} = 0.56 nM). In this case, however, there was not a comparable increase in potency with [Cys(ACM)^{2,7}]hCGRPα.

Discussion

We have shown that coexpression of CTR2 with RAMP1, -2, or -3 led to significant increases in the potency of both CGRP and amylin; the RAMP1-mediated responses exhibited ~10-fold greater potency than those mediated by RAMP2 or -3. These results differ somewhat from earlier findings indicating that in monkey COS-7 cells, CGRP-evoked responses were augmented only by RAMP1, whereas amylin-evoked responses were augmented by RAMP1 and -3 (Christopoulos et al., 1999; Leuthauser et al., 2000; Tilakaratne et al., 2000). We suggest that although both COS-7 and HEK 293 cells are derived from kidney, this discrepancy mainly reflects differences in cell background. In contrast to CRLR, a large number of CTR2 molecules appeared at the surface of HEK 293 cells transfected with 100 ng of CTR2 but not those cotransfected with RAMP, and the cell-surface expression levels were unaffected by RAMP transfection (100 ng) (Kuwasako et al., 2003b). Conversely, CTR2, like CRLR, increased the appearance of epitope-tagged RAMP molecules at the cell surface in this and earlier studies (McLatchie et al., 1998; Kuwasako et al., 2002, 2003a). Taken together, these results suggest that although RAMP accessory proteins do not chaperone CTR2 to the cell surface as they do CRLR, they nevertheless interact with CTR2 effectively modifying the binding of both CGRP and amylin.

In addition to the cell-surface RAMP expression, ^{125}I-[Tyr^0]hCGRPα binding was found to reach approximately maximum levels with transfection of 100 ng of hCTR2 DNA (Fig. 1). Christopoulos et al. (1999) previously investigated concentration-dependent effects of each hRAMP on ^{125}I-la-

TABLE 2

IC_{50} values for analogs in competition for ^{125}I-[Tyr^0]hCGRPα binding to HEK 293 cells cotransfected with 100 ng of hRAMP and 100 ng of hCTR2 or hCRLR

Data are presented as mean ± S.E. (n = 3).

Competing Analogs	CTR2/RAMP1	CTR2/RAMP2	CTR2/RAMP3	CRLR/RAMP1
		nM		
hCGRPα	2.3 ± 0.2	>1000	91.0 ± 24.8	4.0 ± 0.9
[Tyr^0]hCGRPα	5.5 ± 0.4	>1000	760 ± 379	5.4 ± 0.2
[Cys(ACM)^{2,7}]hCGRPα	277 ± 29.6	>1000	>1000	124 ± 33.1
[Cys(Et)^{2,7}]hCGRPα	59.3 ± 4.4	>1000	573 ± 75.1	27.3 ± 4.4
Rat amylin	3.3 ± 0.4	52.0 ± 6.9	17.5 ± 4.8	347 ± 53.7

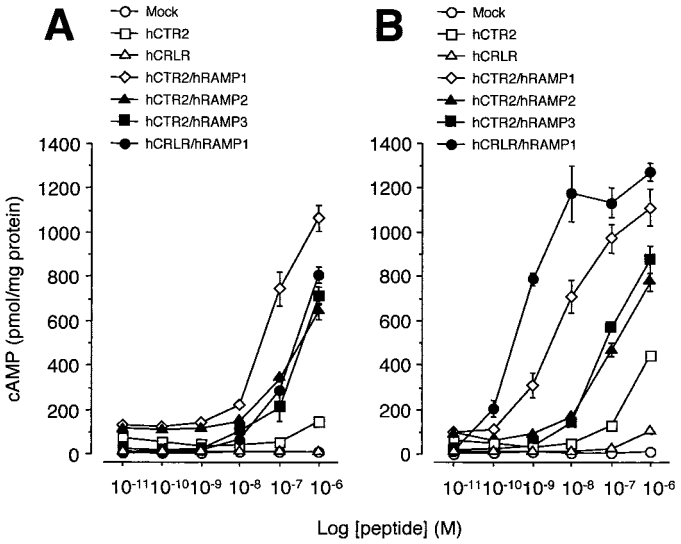

Fig. 5. Agonist-evoked cAMP production in HEK 293 cells transfected with 100 ng of vector (*Mock*), hCTR2 or hCRLR, or one of the hRAMPs plus 100 ng of hCTR2 or hCRLR. Transfected cells were incubated for 15 min at 37°C with the indicated concentrations of [Cys(ACM)^{2,7}]hCGRPα (A) or [Cys(Et)^{2,7}]hCGRPα (B) and then lysed. The resultant lysates were analyzed for cAMP content. Bars represent means ± S.E. of three experiments.

beled amylin binding in HEK 293 cells transiently transfected with 100 ng of hCTR2. In that study, too, specific ^{125}I-amylin binding was almost maximal with cotransfection of 100 ng of hRAMP1 or hRAMP3. Moreover, using covalent cross-linking analysis, those investigators were able to show that hRAMPs form a 1:1 dimer at the cell surface. Collectively, those results, along with the findings presented herein, suggest that the CTR2/RAMP complex exhibits 1:1 stoichiometry, although we have no data directly measuring the molar ratio of individually expressed proteins.

We have iodized the tyrosines of various vasoactive peptides, including adrenomedullin (Kitamura et al., 1993), and in the present study, we iodized a tyrosine residue added to the N terminus of hCGRPα. In cells transfected with hCTR2 alone, both hCGRPα and [Tyr^0]hCGRPα elicited moderate increases in cAMP, albeit that [Tyr^0]hCGRPα responses were ~10-fold weaker than those evoked by hCGRPα. Nevertheless, there was little specific binding of ^{125}I-[Tyr^0]hCGRPα to hCTR2. This is probably because the structures added at the N terminus of CGRP interfere with the nearest disulfide bond required for both binding to the receptor and biological activity. Interestingly, cotransfection of hRAMP1, but not hRAMP2 or -3, led to significant increases in the specific binding of ^{125}I-[Tyr^0]hCGRPα to hCTR2 and in [Tyr^0]hCGRPα-evoked responses, although [Tyr^0]hCGRPα exhibited 10 to 20 times less potency for the three hCTR2/hRAMP heterodimers than for hCGRPα. In contrast, hCRLR/hRAMP1 responded equally to both analogs, which suggests that [Tyr^0]hCGRPα could be used to define the non-CGRP1 receptor subtype. Interestingly, ^{125}I-[His^{10}]CGRPα bound most potently to cells expressing hCTR2/hRAMP1, and binding to hCRLR/hRAMP1 was much lower than the levels seen with ^{125}I-[Tyr^0]CGRPα. This suggests that the combined use of the two iodinated CGRPα analogs may enable the definition of the CGRP1 receptor.

Recent cross-linking and mutagenesis studies suggest that RAMPs determine ligand specificity by contributing to the structure of the ligand-binding pocket or by allosteric modulation of the conformation of CRLR (Hilairet et al., 2001; Kuwasako et al., 2001, 2003a). The responsible domains were found to be a seven-residue segment situated between three residues (Trp to Cys and Tyr) in human and rat RAMP2 and RAMP3 (Kuwasako et al., 2001, 2002). Moreover, their deletion (*D*) mutants were able to serve as negative regulators of endogenous adrenomedullin receptor function (Kuwasako et al., 2001, 2002). Very recently, expression of an hRAMP1 L94A/*D*101-103 double mutant was found to markedly attenuate the activity of endogenous hCGRP receptor (Kuwasako et al., 2003a). Although there have been several studies of the relative affinities of various RAMPs for CRLR (Muff et al., 1998; Buhlmann et al., 1999; Husmann et al., 2000), it remains unclear whether the inhibition of receptor function by DN RAMP mutants was caused by competitive inhibition, formation of heterodimeric complexes, or both. In the present study, hCGRPα-evoked responses in cells coexpressing hCTR2 with hRAMP1 L94A/*D*101-103 or hRAMP2 *D*86-92 were not enhanced, despite hCTR2-induced cell-surface expression of the RAMP mutants. This raises the possibility that both mutants might also inhibit the function of endogenous hCTR2/hRAMP receptors. It is of particular interest to us that coexpression of hRAMP3 *D*59-65 with hCTR2 led to a significant reduction in hCGRPα signaling via hCTR2 because it suggests that *D*59-65 is able to function as a negative regulator for hCTR2 function.

It has been reported that the linear CGRPα analogs [Cys(ACM)^{2,7}]- and [Cys(Et)^{2,7}]hCGRPα are reportedly more potent agonists at CGRP2 than at CGRP1 receptors (Dennis et al., 1989; Dumont et al., 1997; Moreno et al., 2002). Our competition experiments showed the relative agonist sensitivity of the four CGRP receptors tested to be hCGRPα > [Cys(Et)^{2,7}]hCGRPα > [Cys(ACM)^{2,7}]hCGRPα. We also showed that [Cys(ACM)^{2,7}]hCGRPα weakly stimulated cAMP formation only via CTR2/RAMP1 (EC_{50} = 54 nM), whereas [Cys(Et^{2,7})]hCGRPα acted via both hCRLR/hRAMP1 (EC_{50} = 0.56 nM) and hCTR2/hRAMP1 (EC_{50} = 4.1

nM). The EC_{50} values reflecting the affinity of [Cys(ACM)^{2,7}]- and [Cys(Et^{2,7})]hCGRPα for hCTR2/hRAMP1 were very similar to those obtained in the rat vas deferens, which is believed to express typical CGRP2 receptors (Dennis et al., 1989; Dumont et al., 1997; Juaneda et al., 2000). Collectively, these results suggest that among the three $CGRP_{8-37}$–insensitive receptors, CTR2/RAMP1 could be classified as a CGRP2 receptor, although a single use of [Cys(Et^{2,7})]hCGRPα could not draw a sharp distinction between the CGRP1 and CGRP2 receptor subtypes. Because RAMPs, CTR, and CRLR commonly coexist in native tissues and cells (Sexton et al., 2001; Poyner et al., 2002), additional studies will be needed to clarify the precise relationships among the endogenously expressed agonists, receptors, and receptor functions.

Acknowledgments

We are grateful to Dr. Patrick M. Sexton for his helpful discussion.

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