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The calcitonin receptor-like receptor/receptor activity-modifying protein 1 heterodimer can function as a calcitonin gene-related peptide-(8–37)-sensitive adrenomedullin receptor

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Abstract

The receptor activity-modifying protein (RAMP)/calcitonin receptor-like (CRL) receptor heterodimer is thought to function as a receptor for either a calcitonin gene-related peptide (CGRP) (CRL receptor/RAMP1) or adrenomedullin (CRL receptor/RAMP2 or -3), depending on the RAMP isoform present. We examined the receptor specificity of adrenomedullin-induced increases in cAMP in human embryonic kidney (HEK)293 cells coexpressing human CRL receptor and human RAMP1 or RAMP2. In cells expressing CRL receptor/RAMP1, adrenomedulin-induced increases in cAMP were comparable to those induced by α-CGRP, and the CGRP receptor antagonist α-CGRP-(8–37), but not the adrenomedullin receptor antagonist adrenomedullin-(22–52), blocked the adrenomedullin-evoked responses. Cells expressing CRL receptor/RAMP2 responded more selectively to adrenomedullin; in this case, the effect was blocked by adrenomedullin-(22–52) but not by α-CGRP-(8–37). Real-time quantitative polymerase chain reaction confirmed that cotransfection of CRL receptor and RAMP1 had no effect on the endogenous expression of RAMP2. Thus, CRL receptor/RAMP1 likely functions as an adrenomedullin receptor as well as a CGRP receptor, which may explain why many of the actions of adrenomedullin are potently antagonized by α-CGRP-(8–37).

Keywords: Adrenomedullin; CGRP (Calcitonin gene-related peptide); CRL (Calcitonin receptor-like) receptor; RAMP (Receptor activity-modifying protein); α-CGRP-(8–37); cAMP

1. Introduction

Calcitonin gene-related peptide (CGRP) and adrenomedullin belong to the calcitonin family of regulatory peptides and are both highly potent vasodilators (Brain et al., 1985; Kitamura et al., 1993). CGRP is a 37-amino acid neuropeptide present throughout the central and peripheral nervous systems (Wimalawansa, 1997). Adrenomedullin, by contrast, is comprised of 52 amino acids and acts as an autocrine/paracrine agent in a variety of tissues, though best understood is its role as a local regulator of cardiovascular function (Eto, 2001). Both peptides induce production of cAMP and Ca^{2+} mobilization in a variety of cell types (Shimekake et al., 1995; Drissi et al., 1998).

Since its discovery, adrenomedullin has been shown to share a number of pharmacological features with CGRP. For instance, many effects of adrenomedullin are blocked by α-CGRP-(8–37), a selective CGRP receptor antagonist (Chiba et al., 1989) state regarding the pharmacological features of α-CGRP-(8–37). Meanwhile Nuki et al. and the listed authors demonstrate the effects of adrenomedullin that are blocked by the α-CGRP-(8–37) (Nuki et al., 1993; Nishimura and Suzuki, 1997; Okamura et al., 1997; Yoshimoto et al., 1998). Moreover, α-CGRP-(8–37), but not adrenomedullin-(22–52), a selective adrenomedullin receptor antagonist (Eguchi et al., 1994), blocks adrenomedullin-induced cellular responses (Nishikimi et al., 1998; Hasbak et al., 2001; Tomoda et al., 2001). These findings suggest that adrenomedullin binds not only to its specific receptors, but also to receptors that bind both CGRP and adrenomedullin.

Receptor activity-modifying proteins (RAMPs) are a recently identified group of accessory proteins that serve to transport calcitonin receptor-like (CRL) receptors to the cell surface, where they form functional CGRP and adrenomedullin receptors (McLatchie et al., 1998). The three

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RAMP isoforms (RAMP1, RAMP2 and RAMP3) are each comprised of approximately 160 amino acids, making up a large extracellular N-terminal domain, a single membrane-spanning domain and a short cytoplasmic domain. Coexpression of RAMP2 or RAMP3 with CRL receptor leads to both proteins being presented at the plasma membrane as a heterodimeric adrenomedullin receptor, while coexpression of CRL receptor with RAMP1 enables the resultant heterodimer to function as a CGRP receptor (McLatchie et al., 1998; Muff et al., 1998; Fraser et al., 1999; Kuwasako et al., 2000). Although α-CGRP has far less affinity than adrenomedullin for CRL receptor/RAMP2 and CRL receptor/RAMP3, the affinity of adrenomedullin for CRL receptor/RAMP1 is comparable to that of α-CGRP (Fraser et al., 1999; Kamitani et al., 1999; Kuwasako et al., 2000, 2001; Oliver et al., 2001). In addition, in cells expressing much higher levels of CRL receptor and RAMP1 (Hasbak et al., 2001; Tomoda et al., 2001) than RAMP2 and -3, the effects of adrenomedullin are blocked by α-CGRP-(8–37), but not by adrenomedullin-(22–52). Taken together, these results suggest that adrenomedullin-induced cellular responses are mediated via the CRL receptor/RAMP1 complex. To confirm this, we cotransfected human CRL receptor and human RAMP1 or RAMP2 into HEK293 cells, which otherwise express no functional CGRP or adrenomedullin receptors, and examined the effects of α-CGRP-(8–37) and adrenomedullin-(22–52) on adrenomedullin-evoked cAMP production in the transfected cells.

2. Materials and methods

2.1. Materials

A plasmid containing human CRL receptor cDNA (Fluhmann et al., 1995) was a kind gift from Dr. Kenji Kangawa (National Cardiovascular Research Institute, Osaka, Japan). Human α-CGRP, adrenomedullin, α-CGRP-(8–37) and adrenomedullin-(22–52) were purchased from the Peptide Institute (Osaka, Japan). All other chemicals were of reagent grade or higher and were obtained from various commercial suppliers.

2.2. Expression constructs

Human CRL receptor, RAMP1 and RAMP2 were modified to provide a consensus Kozak sequence as previously described (Aiyar et al., 1996). Expression vectors pCAGGS-human CRL receptor and pCAGGS-human RAMP1/2/3 were constructed by cloning human CRL receptor or human RAMP cDNA into the mammalian expression vector pCAGGS/Neo (Kuwasako et al., 2000) using the 5′ *Xho*I and 3′ *Not*I sites. The sequences of the resultant constructs were all verified using an Applied Biosystems 310 Genetic Analyzer.

2.3. Cell culture and cDNA transfection

HEK293 cells were maintained in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum, 100 U/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 culture plates; upon reaching 70% confluence, they were transiently transfected with human CRL receptor and human RAMP1 or -2 or using Lipofectamine transfection reagent (Invitrogen) according to the manufacturer's instructions. Briefly, the cells were incubated for 3 h in 250 μl Optimem 1 medium containing 200 ng/well plasmid DNA and 2 μl/well Lipofectamine. As a control, some cells were transfected with empty expression vector (pCAGGS/Neo). All experiments were performed 48 h after transfection.

2.4. cAMP measurements

To measure the evoked production of cAMP, transfected cells were incubated for 15 min at 37 °C in Hanks' buffer containing 20 mM HEPES, 0.1% bovine serum albumin, 0.5 mM 3-isobutyl-1-methylxanthine (Sigma Chemical, St. Louis, MO), and the indicated concentrations of human α-CGRP or adrenomedullin. In some cases, the cells were preincubated for 5 min at 37 °C with buffer containing the indicated concentration of human α-CGRP(8–37) or adrenomedullin-(22–52) before exposure to human α-CGRP or adrenomedullin. The reactions were terminated by addition of lysis buffer (Amersham), after which the lysates were centrifuged at $79 \times g$ for 10 min at 4 °C. The cAMP content was then determined in aliquots of the supernatant using a commercial enzyme immunoassay according to the manufacturer's (Amersham) instructions for a nonacetylation protocol.

2.5. mRNA expression measured by real-time quantitative polymerase chain reaction (PCR)

Total RNA was extracted from transfected HEK293 cells using Total RNA Isolation Reagent (Invitrogen) and then reverse-transcribed using superscript reverse transcriptase (Invitrogen), yielding the respective cDNAs. Expression of mRNAs encoding human CRL receptor and the three human RAMP isoforms was assessed using real-time quantitative PCR (Prism 7700 Sequence Detector, Applied Biosystems, Foster City, CA) as previously described (Mishima et al., 2001). Levels of CRL receptor and RAMP mRNA were normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, which served as an internal control. The oligonucleotide primers and fluorescently labeled probes used are listed in Table 1. DNA sequence analysis confirmed that the amplified products were identical to those of human CRL receptor and the three

Table 1
Oligonucleotide sequences used for quantitative PCR

Gene	Primer/probe	Sequence (5′–3′)
Human GAPDH	forward primer (504–524)	CAA-TGC-CTC-CTG-CAC-CAC-CAA
	reverse primer (668–689)	GAG-GCA-GGG-ATG-ATG-TTC-TGG-A
	labeled probe (583–612)	ATG-ACC-ACA-GTC-CAT-GCC-ATC-ACT-GCC-ACC
Human CRL receptor	forward primer (1458–1484)	CTG-TAC-ATG-AAA-GCT-GTG-AGA-GCT-ACT
	reverse primer (1571–1597)	TGG-AAG-TGC-ATA-AGG-ATG-TGC-ATG-ATG
	labeled probe (1526–1555)	TCC-ATG-GCG-ACC-TGA-AGG-AAA-GAT-TGC-AGA
Human RAMP1	forward primer (189–209)	GAG-ACG-CTG-TGG-TGT-GAC-TGG
	reverse primer (329–350)	GAT-GGG-GCA-GCT-CCT-GAA-GTA-G
	labeled probe (276–305)	TGC-TTC-TGG-CCC-AAT-GCA-GAG-GTG-GAC-AGG
Human RAMP2	forward primer (414–438)	GCA-GAG-AGG-ATC-ATC-TTT-GAG-ACT-C
	reverse primer (542–569)	CCT-CCA-TAC-TAC-AAG-AGT-GAT-GAG-GAA-G
	labeled probe (459–488)	TGC-TCC-CTG-GTG-CAG-CCC-ACC-TCT-TCT-GAC
Human RAMP3	forward primer (208–232)	CCG-AGT-TCA-TCG-TGT-ACT-ATG-AGA-G
	reverse primer (301–322)	CTG-TGG-ATG-CCG-GTG-ATG-AAG-C
	labeled probe (256–285)	AGG-CCA-ATG-TCG-TGG-GCT-ACT-GGC-CCA

human RAMP isoforms (Fluhmann et al., 1995; McLatchie et al., 1998).

2.6. Statistical analysis

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

3. Results

3.1. Ligand-induced cAMP production in HEK293 cells coexpressing human CRL receptor and human RAMP1 or -2

The EC_{50} for α-CGRP-and adrenomedullin-induced cAMP production in intact HEK293 cells was $> 2.0 \times 10^{-6}$ M for both agonists, which is indicative of the absence of functional CGRP and adrenomedullin receptors in this cell type (Fig. 1A). In HEK293 cells transfected with RAMP1, however, CGRP elicited eightfold increases in cAMP over the levels seen in untransfected cells (data not shown), suggesting that RAMP1 and endogenous calcitonin receptors are capable of forming a functional CGRP receptor (Christopoulos et al., 1999). In addition, α-CGRP and adrenomedullin elicited only small increases in the cAMP content of HEK293 cells expressing CRL receptor alone (Fig. 1B), and the effects of adrenomedullin were not different from those of α-CGRP. By contrast, we previously established stable CRL receptor transfectants using HEK293 cells and showed that adrenomedullin-induced cAMP production was about twofold higher than that obtained with α-CGRP (Kuwasako et al., 2000). This may be largely explained by differences in transfection efficacy in transient and stable transfectants. Far more robust responses to α-CGRP or adrenomedullin were elicited in cells expressing both CRL receptor and RAMP1 (Fig. 1C). α-CGRP and adrenomedullin each elicited concentration-dependent increases in cAMP that reached levels nearly 50-fold greater than in

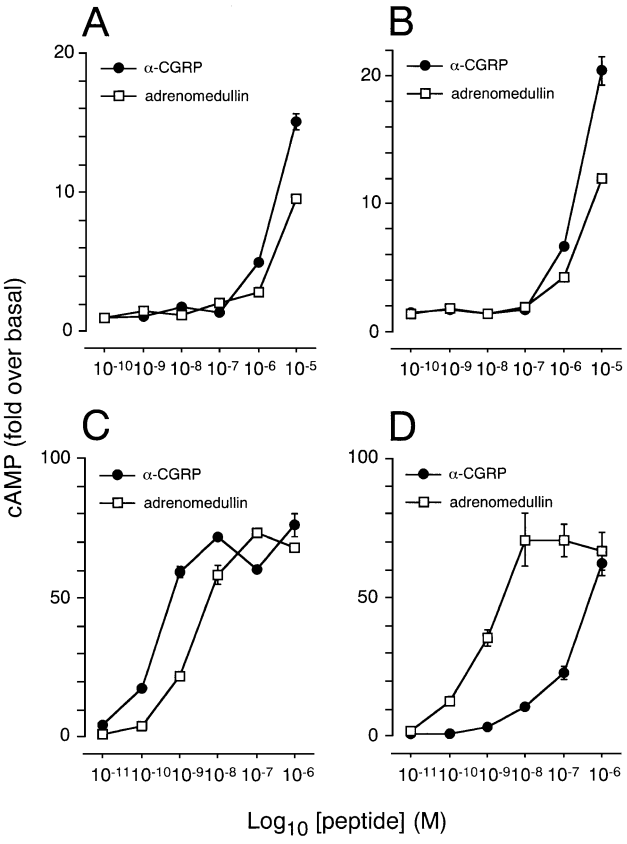

Fig. 1. Agonist-evoked cAMP production in HEK293 cells coexpressing human CRL receptor and human RAMP1 or RAMP2. Following transient transfection of empty vector (A), human CRL receptor (B), human CRL receptor/human RAMP1 (C) or human CRL receptor/human RAMP2 (D), cells were incubated in the presence of 0.5 mM 3-isobutyl-1-methylxanthine for 15 min at 37 °C with the indicated concentrations of α-CGRP or adrenomedullin and then lysed. Cell lysates were then analyzed for cAMP content. Symbols represent means ± S.E.M. from three separate identical experiments.

cells expressing empty vector. Moreover, there was no dramatic difference in their potencies: the EC_{50} for α-CGRP was 3.0×10^{-10} M, while that for adrenomedullin was 2.6×10^{-9} M. On the other hand, HEK293 cells expressing CRL receptor and RAMP2 responded much more selectively to adrenomedullin: the EC_{50} for adrenomedullin was 9.4×10^{-10} M, while that for α-CGRP was $>1.2 \times 10^{-7}$ M (Fig. 1D).

3.2. Examination of endogenous RAMP mRNA levels after transfection

To examine whether the response to adrenomedullin by cells expressing CRL receptor/RAMP1 could be attributed to increases in the level of endogenous RAMP2 or -3, we used real-time quantitative PCR with the appropriate primers and probes to selectively evaluate endogenous expression of human CRL receptor and human RAMP1/2/3 mRNA in transfected and untransfected HEK293 cells. Untransfected cells were found to express low levels of RAMP2 mRNA (5.1 ± 0.19) (Fig. 2A), which were unchanged 48 h after transfection of CRL receptor and/or RAMP1 (Fig. 2B) and remained constant for at least an additional 24 h thereafter (data not shown). Endogenous levels of CRL receptor mRNA (0.21 ± 0.04) were extremely low compared to those of RAMP2 mRNA (Fig. 2A), which may explain why HEK293 cells lack functional adrenomedullin receptors. Untransfected cells expressed little or no RAMP1 or -3 (less than 1/500 of the amount of RAMP2), and the transfection of CRL receptor and/or RAMP1 had no effect on RAMP3 gene expression (data not

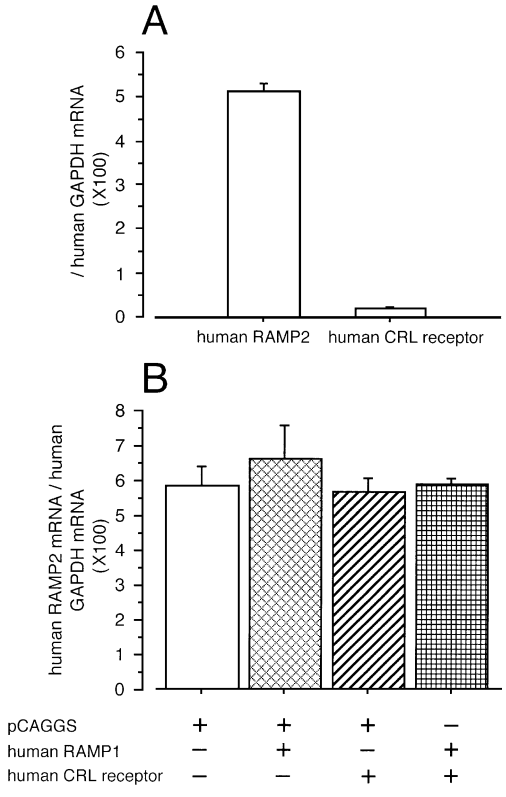

Fig. 2. Effects of transfection of human CRLR and/or human RAMP1 on endogenous expression of human RAMP2 in HEK293 cells. (A) Endogenous human RAMP2 and human CRL receptor mRNA levels in HEK293 cells. (B) HEK293 cells were transfected with the indicated combinations of equal amounts of control vector and vectors encoding human CRL receptor and human RAMP1. Forty-eight hours after cell transfections, human RAMP2 mRNA levels were measured as described in Materials and methods. Bars represent the means ± S.E.M. of three independent experiments.

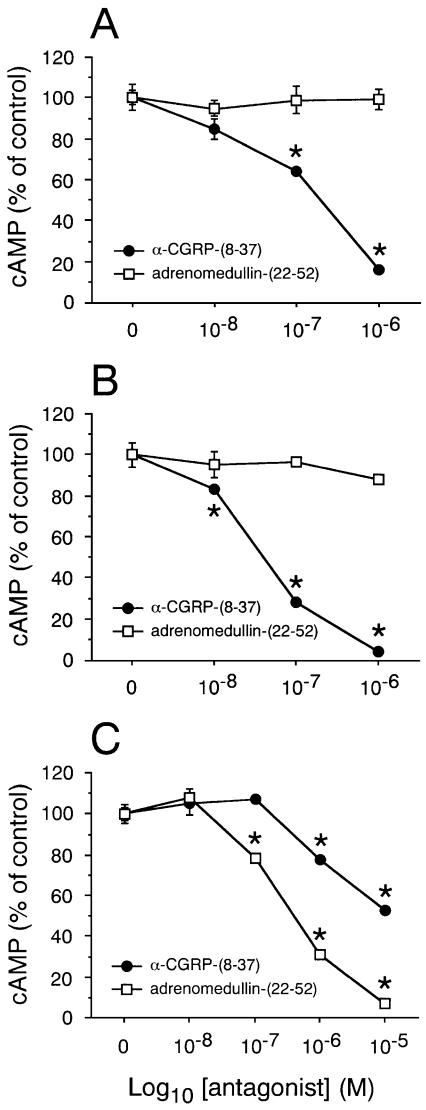

Fig. 3. Effects of α-CGRP-(8–37) or adrenomedullin-(22–52) on cAMP production induced by adrenomedullin or α-CGRP in HEK293 cells expressing CRL receptor/RAMP1 or CRL receptor/RAMP2. Following transient transfection of human CRL receptor/human RAMP1 (A and B) or human CRL receptor/human RAMP2 (C), cells were preincubated for 5 min at 37 °C with the indicated concentrations of human α-CGRP-(8–37) or adrenomedullin-(22–52), after which human α-CGRP (1 nM for (A)) or adrenomedullin (10 nM for (B) and 1 nM for (C)) were added, and the incubation was continued for an additional 15 min. The cells were then lysed, and the cAMP content of the lysates determined. Bars represent the means ± S.E.M. of three independent experiments. * $P < 0.05$ vs. α-CGRP or adrenomedullin without α-CGRP-(8–37) or adrenomedullin-(22–52).

shown). Thus, the robust responses to adrenomedullin by cells expressing CRL receptor/RAMP1 cannot be explained by changes in the expression of either RAMP2 or -3.

3.3. Effects of α-CGRP-(8–37) and adrenomedullin-(22–52) on agonist-induced cAMP production

We next examined the effects of the antagonists α-CGRP-(8–37) and adrenomedullin-(22–52) on intracellular cAMP production induced by adrenomedullin or CGRP in HEK293 cells expressing human CRL receptor/human RAMP1 or -2 (Fig. 3). In cells expressing empty vector or CRL receptor, 10 μM α-CGRP-(8–37) had no apparent effect on cAMP production evoked by 5×10^{-7} M adrenomedullin (data not shown). This response to adrenomedullin could not have been mediated via the RAMP2/CRL receptor complex because HEK293 cells express no functional adrenomedullin receptors. In cells expressing CRL receptor/RAMP1, α-CGRP-(8–37) concentration dependently inhibited the increase in cAMP evoked by 10^{-8} M adrenomedullin, almost completely abolishing the effect at a concentration of 10^{-6} M (Fig. 3B). α-CGRP-(8–37) had a similar effect on cAMP production evoked by 10^{-9} M α-CGRP (Fig. 3A). Adrenomedullin-(22–52), by contrast, had no effect on cAMP production induced by either adrenomedullin or α-CGRP (Fig. 3A,B). In cells expressing CRL receptor/RAMP2, however, adrenomedullin-(22–52) concentration dependently inhibited cAMP production evoked by 10^{-8} M adrenomedullin, almost totally blocking the effect at a concentration of 10^{-5} M (Fig. 3C). α-CGRP-(8–37) had a weaker effect (Fig. 3C).

4. Discussion

We demonstrated that in HEK293 cells coexpressing human CRL receptor and human RAMP1, adrenomedullin elicited concentration-dependent increases in the levels of intracellular cAMP that were comparable to those elicited by α-CGRP and that were blocked by α-CGRP-(8–37) but not by adrenomedullin-(22–52). Consistent with these findings, there was no remarkable difference in the EC_{50} values for adrenomedullin-evoked cAMP production mediated via CRL receptor/RAMP1 and CRL receptor/RAMP2. By contrast, HEK293 cells coexpressing human CRL receptor and human RAMP2 responded more selectively to adrenomedullin, and the responses were blocked by adrenomedullin-(22–52) but not by α-CGRP-(8–37), which is consistent with earlier findings obtained when CRL receptor and RAMP2 were cotransfected into mammalian cells (McLatchie et al., 1998; Aiyar et al., 2001). Taken together, these results suggest that the CRL receptor/RAMP1 heterodimer is a fully functional adrenomedullin receptor, distinct from CRL receptor/RAMP2, and that the α-CGRP-(8–37)-sensitive effects of adrenomedullin are, at least in part, mediated via CRL receptor/RAMP1.

Our finding that the EC_{50} for adrenomedullin is about 10-fold higher than for α-CGRP in HEK293 cells expressing human CRLR/human RAMP1 is consistent with an earlier report in which HEK293T cells were used (Fraser et al., 1999). However, our findings are not consistent with those of McLatchie et al. (1998), who reported that the IC_{50} for inhibition by [^{125}I]α-CGRP of adrenomedullin binding to mouse Swiss 3T3 cells expressing human CRL receptor/human RAMP1 was 90-fold greater than that for α-CGRP binding (IC_{50} = 9.0 vs. 0.1). Furthermore, they differ from those of Buhlmann et al. (1999), who found adrenomedullin to be substantially less potent and less efficacious when acting via CRL receptor/RAMP1 than we did. It is notable, however, that those investigators examined the effects of rat agonists on monkey COS-7 cells expressing rat CRLR and human RAMPs. We suggest that in both of the aforementioned studies, changes in the potency and efficacy of adrenomedullin may reflect the combined use of agonists, receptors and host cells from different species. Supporting this notion is the recent report from Aiyar et al. (2001), who, like us, used materials exclusively from humans and found that the maximal responses elicited by human adrenomedullin via CRL receptor/RAMP1 were similar to those reported here. The fact that they calculated the EC_{50} for adrenomedullin to be about 100-fold higher than for α-CGRP, whereas we calculated only a 10-fold increase, may reflect differences in the plasmids used—for example, differences in promoter, transfection efficacy, etc.

Adrenomedullin, but not α-CGRP, evokes significant increases in cAMP in human and rabbit aortic endothelial cells, human aortic smooth muscle cells and rat cerebral microvessels, all of which are known to endogenously express only CRL receptor/RAMP2 (Muff et al., 1998; Kamitani et al., 1999; Kobayashi et al., 2000). Moreover, Kobayashi et al. (2000) clearly demonstrated that, in rat cerebral microvessels, adrenomedullin-(22–52) blocked the actions of adrenomedullin more effectively than α-CGRP-(8–37) did, which is consistent with earlier studies (Osajima et al., 1996; Fujioka et al., 1999; Osajima et al., 1999; Takao et al., 1999; Yousufzai et al., 1999; Ashton et al., 2000; Clementi et al., 2000; Santiemma et al., 2001). By contrast, with the exception of a single study of rat cardiac fibroblasts (Tomoda et al., 2001), there is little information available for cells that express only functional CRL receptor/RAMP1. Still, in porcine coronary arteries, where both RAMP1 and RAMP2 are expressed, α-CGRP-(8–37) inhibited adrenomedullin-induced vasodilation, while adrenomedullin-(22–52) had little effect (Hasbak et al., 2001). This may be explained by the finding that the interaction between CRL receptor and RAMP1 predominates over that between CRL receptor and RAMP2 (Buhlmann et al., 1999; Husmann et al., 2000). In isolated rat uterus, where all three RAMPs were highly expressed, 1 μM α-CGRP-(8–37) or adrenomedullin-(22–52) completely blocked the actions of adrenomedullin, suggesting that adrenomedullin binds to both CGRP and adrenomedullin receptors (Yanagita et al., 2000).

Consistent with this interpretation, the cAMP response to α-CGRP was 47% smaller in rabbit aortic endothelial cells co-transfected with RAMP1 and -3, but not RAMP2, than in cells transfected with RAMP1 alone (Muff et al., 1998). Additional studies will be necessary to clarify the precise relationships among endogenously expressed RAMPs, agonist binding sites, receptor functions, and the effects of receptor antagonists such as α-CGRP-(8–37) and adrenomedullin-(22–52).

In conclusion, coexpression of human CRLR and human RAMP1 produces fully functional AM receptors that are distinct from human CRLR/human RAMP2, which is often referred to as the adrenomedullin-specific receptor. This likely explains why many of the actions of adrenomedullin are potently antagonized by α-CGRP-(8–37).

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