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Molecular cloning and pharmacological characterization of bovine calcitonin receptor-like receptor from bovine aortic endothelial cells

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Abstract

A complementary DNA encoding calcitonin receptor-like receptor (CRLR) was isolated from a bovine aortic endothelial cell library. The bovine CRLR has 462 amino acids and 92% homology with the human CRLR. In a reverse transcriptase–polymerase chain reaction assay, bovine CRLR was found to be widely distributed, including in the heart and lungs. Stable transfection of bovine CRLR in human embryonic kidney cells (HEK-293) resulted in specific high-affinity [^{125}I] rat adrenomedulin (rADM)-binding (dissociation constant = 145 ± 15 pM). ADM-stimulated adenylyl cyclase activity with an EC_{50} value of 5.0 ± 1.2 nM. The human ADM receptor antagonist hADM(22-52) inhibited [^{125}I]rADM-binding and ADM-stimulated adenylyl cyclase activity. Interactions between bovine CRLR and individual receptor activity modifying proteins (RAMPs) were also investigated. Transient co-transfection of bovine CRLR cDNA with human receptor activity modifying protein 1 (hRAMP1) cDNA in HEK-293 cells resulted in the expression of a CRLR that displayed high-affinity binding to calcitonin gene-related peptide. Co-transfection of bovine CRLR with human RAMP2 or RAMP3 cDNAs in HEK-293 cells displayed high-affinity ADM receptors. These observations suggest that in the absence of exogenous RAMPs heterologous expression of bovine CRLR results in an ADM receptor phenotype.

Keywords: Adrenomedulin; Calcitonin gene-related peptide; Bovine calcitonin receptor-like receptor; Receptor activity modifying protein; Radioligand binding; Adenylyl cyclase

1. Introduction

ADM, a potent vasorelaxant/hypotensive peptide, shows partial structural homology to CGRP [1,2]. ADM is widely expressed in a variety of tissues, including vascular smooth muscle cells [3] and endothelial cells [4]. Both ADM and CGRP increase cAMP in various tissues including smooth muscle cells, which is thought to lead to subsequent vasorelaxation [5]. Both ADM and CGRP have been suggested to play an important role in the regulation of vascular tone. On the basis of sequence similarities observed among these peptides, several studies have focused on evaluating the binding characteristics of these molecules to receptors located on various tissues. There has been great difficulty in defining ADM receptor sites, mainly due to the lack of highly selective ADM receptor antagonists. In addition, it has been difficult to separate the effects of CGRP from those of ADM, since both peptides share many pharmacological effects and the CGRP receptor antagonist CGRP(8-37) blocks ADM binding as well as many of the ADM-mediated responses in various preparations [6–8].

The cloning of CGRP and ADM receptors has been the subject of intense research by a number of investigators. The initial effort to clone the CGRP receptor resulted in a receptor showing homology to the calcitonin receptor, and was named CRLR [9]. However, the endogenous ligand could not be identified following transient expression of either rat or human CRLR in COS-7 cells. On the other

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Abbreviations: ADM, adrenomedullin; cAMP, cyclic AMP; CGRP, calcitonin gene-related peptide; CRLR, calcitonin receptor-like receptor; RAMP, receptor activity modifying protein; RT–PCR, reverse transcriptase–polymerase chain reaction; sCT, salmon calcitonin.

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hand, when human CRLR was stably transfected in HEK-293 cells, the transfected cells were responsive to CGRP [10]. These studies confirmed that the human homologue of CRLR possessed the pharmacological properties of a CGRP type 1 receptor (CGRP1) [10]. Subsequently, CGRP1 receptors from rats [11] and pigs [12] were also cloned and characterized from the respective lung cDNA libraries. It is now widely accepted that the CRLR is, in fact, the CGRP1 receptor. The CGRP1 receptor was found to be sensitive to the antagonistic properties of the C-terminal fragment of CGRP, CGRP(8-37), but to have weak affinity for the linear analogue, [Cys(ACM^{2,7})] CGRP [13]. The cloning of human and rat ADM receptors was reported by Kapas *et al*. [14] and Hanze *et al*. [15]. The rat ADM receptor clone when expressed in COS-7 cells displayed specific high-affinity binding to [^{125}I]ADM. Exposure of these cells to ADM resulted in an increase in cAMP accumulation [15]. However, in subsequent studies by other investigators, it was difficult to successfully transfect and express this clone in a variety of cell lines [16].

McLatchie *et al*. [17] demonstrated that a seven-transmembrane receptor such as the CRLR can function as a CGRP type 1 or an ADM receptor depending upon the co-expression of one of the accessory proteins called RAMPs. RAMPs are integral membrane proteins with a large extracellular amino terminus, a single transmembrane spanning domain, and a C-terminal intracellular tail. The RAMPs are expressed widely in a number of tissues. Three different human RAMPs (RAMP1, RAMP2, and RAMP3) have been cloned. These proteins appear to be required for the glycosylation and transport of the CRLR to the plasma membrane, as well as for ligand binding. When RAMP1 associates with CRLR, the receptor responds to CGRP, whereas association with RAMP2 or RAMP3 results in a CRLR that is responsive to ADM. Recently, it was shown that the amylin receptor phenotype can be generated by co-expression of RAMP1 or RAMP3 with a calcitonin receptor [18].

Regulation of vascular tone has been shown to play a key role in controlling blood pressure [19]. Vascular tone is regulated by many factors released from the circulation and perivascular nerves [20]. The presence of specific ADM and CGRP receptors coupled to the activation of adenylyl cyclase has been demonstrated in endothelial and vascular smooth muscle cells [21–23]. To understand the role of ADM and CGRP in endothelial cell physiology, we initiated cloning of the CRLR from these cells. This report describes the cloning and characterization of a novel CRLR from bovine aortic endothelial cells. By screening the bovine aortic endothelial cell library, we identified a CRLR with 92% homology to human CRLR. In the present study, RT–PCR analysis of bovine CRLR indicated a wide distribution of this receptor. Stable expression of bovine CRLR cDNA in HEK-293 cells resulted in a receptor that displayed ADM receptor pharmacology. Interactions between bovine CRLR and individual RAMPs also were investigated.

2. Materials and methods

2.1. Materials

Human (h) ADM, rat (r) ADM, hαCGRP, hαCGRP(8-37), and sCT were purchased from Bachem Biochemicals. hADM(22-52) was purchased from Phoenix Pharmaceuticals. [^{125}I]rADM (specific activity 2000 Ci/mmol) and [^{125}I]hαCGRP (specific activity 2000 Ci/mmol) were obtained from Amersham. A bovine endothelial cell library was purchased from Stratagene. The bicinchoninic acid (BCA) protein assay kit was obtained from the Pierce Chemical Co. LipofectAMINE was obtained from Gibco BRL. All other reagents were obtained from the Sigma Chemical Co.

2.2. Methods

2.2.1. Screening of bovine endothelial cell cDNA libraries

A bovine endothelial cell cDNA library was constructed using the λ-Zap cDNA synthesis kit and following the standard protocol. The human CGRP receptor cDNA-coding sequence was used as a probe for the isolation of putative CRLR clones from the bovine endothelial cell library. The probe was radiolabled with [α-^{32}P]ATP by the random priming method and used to screen approximately 1×10^6 plaque-forming units of the endothelial cDNA library. Nylon filters (Hybond-N Amersham, Life Sciences) containing plaque lifts were hybridized at 42° in 5× SSC (150 mM NaCl, 15 mM sodium citrate), 5× Denhardt's solution, 0.1% SDS, and 20% formamide followed by autoradiography. Hybridizing clones were plaque purified, and the pBluescript-containing insert was rescued from the purified λ phage by *in vivo* excision using the Exassist/Solar system (Stratagene). cDNA was sequenced on both strands by the dideoxynucleotide chain termination reaction. Two oligonucleotide primers were designed to amplify the open reading frame of the bovine CRLR cDNA, and PCR amplification was carried out. A recombinant receptor of the bovine CRLR clone (BE-CRLR) was produced by transfection in human embryonic kidney cells (HEK-293) (BE-CRLR-293). Transfection was achieved by the LipofectAMINE procedure. Stable cell lines expressing this receptor were selected with G418 and screened for CGRP and ADM-dependent activation of adenylyl cyclase.

2.2.2. RT–PCR

Total RNA was prepared from different bovine tissues using Trizol Reagent (Gibco BRL), and 2-μg aliquots were then subjected to reverse transcription using a Superscript

II cDNA synthesis kit (Gibco BRL). CRLR was detected by nested PCR using a set of specific primers. The sense primer was 5′-CAT CTC CTC TAC ATT ATC CAT GG-3′, and the anti-sense primer was 5′-GAA CCTCTC CAT TAA AGA AGC-3′. All PCRs were carried out on a GeneAmp PCR system 9600 (Perkin Elmer) using the following protocol: an initial denaturation step at 94° for 4 min was followed by 30 cycles at 94° for 30 sec, 58° for 30 sec, and 72° for 30 sec. Resulting PCR products were analyzed on a 1.5% agarose gel. A set of GAPDH primers was used as a control for the RT–PCR (Fig. 2). Amplification fidelity was confirmed by the observation that no amplicon was detected if either (a) *Taq* polymerase or (b) reverse transcriptase was omitted from the RT–PCR reaction. Nested PCRs were then performed using the same protocol and 1 μL of the first PCR product as template. The expression of RAMPs in HEK-293 cells (vector- and CRLR-transfected) was studied by using a similar RT–PCR protocol. The primers, as described by Kamitani *et al.* [24], were made and used for the reaction.

HEK-293 cells were grown in Eagle's MEM supplemented with 10% fetal bovine serum, 2 mM glutamine and maintained at 37° in a humidified atmosphere containing 95% air and 5% CO_2. For the experiment, HEK-293 cells were seeded at a density of 12×10^6 in T-150 flasks. After 24 hr, cells were co-transfected in serum-free Eagle's MEM with 10 μg of bovine CRLR cDNA and/or RAMP cDNA subcloned in pCDN vector using Lipofectamine Plus reagent.

2.2.3. Membrane preparation

Cells from BE-CRLR-293 were scraped into ice-cold PBS and centrifuged for 10 min at 1000 *g* at 4°. 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 *g* at 4°, and the resultant membrane pellet was resuspended in 50 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$ and recentrifuged at 12,000 *g* for 20 min at 4°. The final pellet was resuspended in 50 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$ and assayed immediately.

2.2.4. Radioligand binding assays

Saturation binding experiments were carried out using membranes prepared from BE-CRLR-293 cells. The incubation mixture contained various concentrations of [^{125}I]rADM (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 rADM. After incubating for 30 min at 25°, the reaction mixture was diluted rapidly with cold 0.9% NaCl, and bound and free ligands were separated by filtration on glass fiber filters. In competition binding studies, the membranes were incubated with increasing concentrations of rADM or hαCGRP (1 pM to 1 μM) and 150 pM [^{125}I]rADM for 30 min at 25°.

2.2.5. Determination of adenylyl cyclase activity

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 of myokinase in a final volume of 100 μL for 20 min at 30°. The reactions were stopped with a 1-mL 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 [25]. Adenylyl cyclase activities were determined in the absence (basal) or presence of various concentrations of hADM or hαCGRP (1 pM to 1 μM). The effects of hADM(22-52) and of hαCGRP(8-37) on ADM-mediated activation of adenylyl cyclase were also determined.

2.3. Data analysis

The apparent equilibrium dissociation constants (K_d) and the maximum binding sites (B_{max}) from saturation binding experiments and the K_i values from competition binding experiments were calculated using the interactive nonlinear curve-fitting program of GraphPad Prism.

3. Results

The human CGRP-R cDNA, previously cloned in our laboratory [10], was used to probe the bovine endothelial cell cDNA library. Several positive clones were identified. Nucleotide sequence analysis revealed that several of these positive clones encoded for bovine CRLR. Sequence analysis of the bovine CRLR clones revealed an open reading frame of 1389 nucleotides. The deduced polypeptide consisted of 462 amino acid residues with a calculated molecular mass of approximately 50.8 kDa (Fig. 1). The deduced amino acid sequence of the bovine CRLR was 92, 88, and 94% identical to the human, rat, and porcine CRLR, respectively. This high degree of homology among the CRLR amino acid sequences of different species suggests that the plasmid encodes the bovine CRLR homologue.

As originally observed with human CRLR (using the conventional northern blot approach), RT–PCR analysis (as described in Section 2) of bovine CRLR distribution revealed that the most abundant tissue source of bovine CRLR expression was the lungs and heart (Fig. 2). A significant level of expression was also observed in bovine bladder and pancreas (data not shown). Trace levels of expression were observed in the brain. Amplification of GAPDH resulted in the generation of the predicted 983 bp transcript in all tissue samples studied. Amplification fidelity was confirmed by the observations that the PCR-generated bovine and human transcripts were: (a) of the predicted (310 bp) size, (b) lacking when *Taq* polymerase was omitted from the reaction mixture, and (c) lacking

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  1 MEKKFFLSFLFLLPFFMILVIAESEEENPDDLIQLGVTRNKIMTAQYECY 50   Bovine
    ||||  | || ||||||||| ||  ||.|:| ||||||||||||||||||
  1 MEKKCTLYFLVLLPFFMILVTAEL.EESPEDSIQLGVTRNKIMTAQYECY 49   Human
              .         .         .         .         .
 51 QKIMQDPVQQTEGIYCNRTWDGWLCWNDVAAGTESMQHCPDYFQDFDPSE 100
    |||||||:|| ||:|||||||||||||||||||||| ||||||||||||
 50 QKIMQDPIQQAEGVYCNRTWDGWLCWNDVAAGTESMQLCPDYFQDFDPSE 99
              .         .         .         .   I     .
101 KVTKICDQDGNWFRHPASNRTWTNYTQCNVNTHEKVKTALNLFYLTIIGH 150
    ||||||||||||||||||||||||||||||||||||||||||||||||||
100 KVTKICDQDGNWFRHPASNRTWTNYTQCNVNTHEKVKTALNLFYLTIIGH 149
              .         .         .         .II       .
151 VLSIASLLISLGIFFYFKSLSCQRITLHKNLFFSFVCNSVITIIHLTAVA 200
     |||||||||||||||||||||||||||||||||||||:|||||||||
150 GLSIASLLISLGIFFYFKSLSCQRITLHKNLFFSFVCNSVVTIIHLTAVA 199
              .         .         .         .III      .
201 NNQALVATNPVSCKVSQFIHLYLMGCNYFWMLCEGIYLHTLVVVAVFAEK 250
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200 NNQALVATNPVSCKVSQFIHLYLMGCNYFWMLCEGIYLHTLIVVAVFAEK 249
              .       IV    .         .         .    V   .
251 QHLMWYYFLGWGFPLIPACIHAVARRLYYNDNCWISSDTQLLYIIHGPIC 300
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250 QHLMWYYFLGWGFPLIPACIHAIARSLYYNDNCWISSDTHLLYIIHGPIC 299
              .         .         .         . VI       .
301 AALLVNLFFLLNIVRVLITKLKVTHQAESNLYMKAVRATLILVPLLGIEF 350
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300 AALLVNLFFLLNIVRVLITKLKVTHQAESNLYMKAVRATLILVPLLGIEF 349
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351 VLIPWRPEGKIAEEIYDYIINILMHYQGLLVSTIFCFFNGEVQAILRRNW 400
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350 VLIPWRPEGKIAEEVYDYIMHILMHFQGLLVSTIFCFFNGEVQAILRRNW 399
              .         .         .         .         .
401 NQYKIQFGNNFSHSDTLRSASYTVSTISDGTGYSHDCLSEHLNGKSIHDT 450
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400 NQYKIQFGNSFSNSEALRSASYTVSTISDGPGYSHDCPSEHLNGKSIHDI 449
              .
451 DNVVIKPEKLYD* 463
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450 ENVLLKPENLYN* 462
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Fig. 1. Amino acid sequence alignment of bovine and human CRLR cDNA clones. cDNA inserts encoding the bovine CRLR were sequenced by the dideoxy method. The deduced amino acid residues are indicated beginning with the initiator methionine. The optimal alignment of the deduced amino acid sequences was made with the Wisconsin GCG Software Package. The regions identifying the transmembrane as domains I–VII are underlined and numbered sequentially.

from RNA samples not subjected to RT. The full-length bovine CRLR sequence was cloned into pCDN and transfected into HEK-293 cells. Transient transfection of bovine CRLR in HEK-293 cells resulted in marked stimulation of cAMP accumulation in response to ADM and CGRP compared with vector-transfected HEK-293 cells (130 and 85% increase over basal for ADM and CGRP, respectively). Full characterization of the receptor (radioligand binding) with transient transfection was not possible because of the low level of expression. To characterize this clone further, we made stable cell lines of bovine CRLR in HEK-293 cells (BE-CRLR-293). Initial characterization of [^{125}I]hαCGRP (150 pM) binding to HEK-293 cells or membranes isolated from cells expressing bovine CRLR revealed no significant difference from mock-transfected HEK-293 cells. On the other hand, [^{125}I]rADM (150 pM) displayed significant specific binding. Saturation binding studies indicated that [^{125}I]rADM binding was saturable and of high-affinity (Fig. 3A). Scatchard transformation of the specific binding revealed a single class of

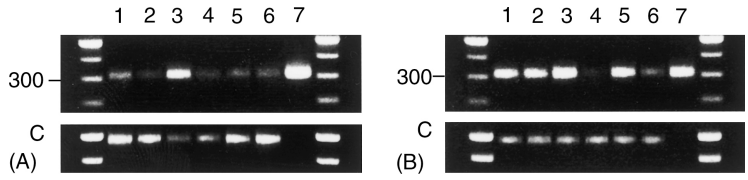

Fig. 2. RT–PCR product comparison of bovine CRLR transcripts in different bovine tissues (B) [heart (lane 1), brain (lane 2), lung (lane 3), kidney (lane 4), liver (lane 5), spleen (lane 6), and plasmid control (lane 7)] with those of human CRLR transcripts (A) in corresponding human tissues. PCR products were separated on a 1.5% agarose gel. The DNA fragment of 310 bp corresponds to bovine CRLR. Amplification of GAPDH cDNA did not differ significantly between bovine tissues (C).

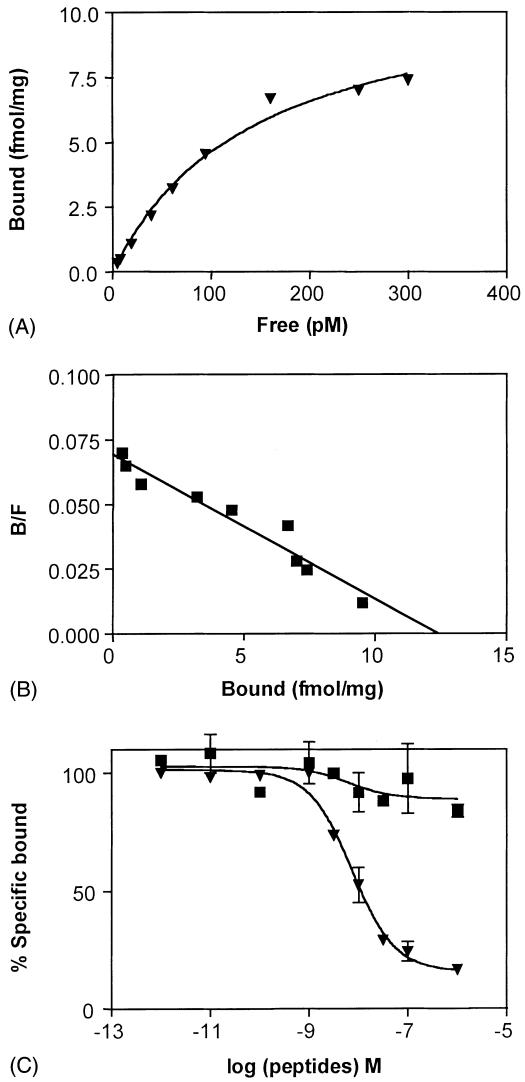

Fig. 3. (A) Specific binding of [^{125}I]rADM to BE-CRLR-293 membranes obtained from a saturation binding experiment. (B) Scatchard plot derived from the saturation binding data. The data represent one example of three different experiments. (C) Competition curves for rADM (▼) and hαCGRP (■) against [^{125}I]rADM binding in BE-CRLR-293 membranes. Data are the means (±range) of duplicate determinations and are representative results from one of three independent experiments.

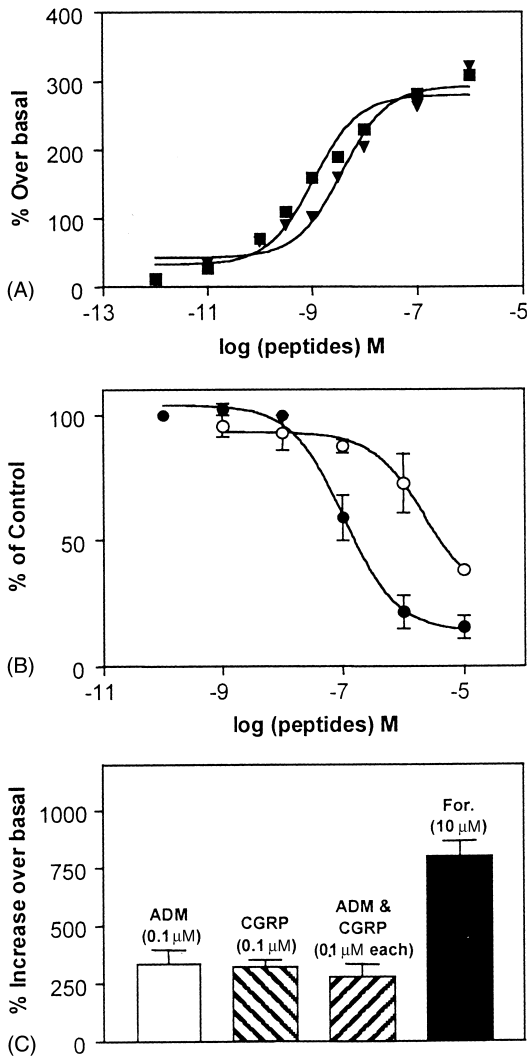

Fig. 4. (A) Stimulation of adenylyl cyclase activity in BE-CRLR-293 membranes by rADM (▼) and hαCGRP (■). Data are the means of duplicate determinations and are representative results from one of four independent experiments. (B) Inhibition by hαCGRP(8-37) (○) or hADM(22-52) (●) of ADM-stimulated adenylyl cyclase activity in BE-CRLR-293 membranes. Membranes were incubated with 5 nM hADM in the absence or presence of the indicated concentrations of hαCGRP(8-37) or hADM(22-52). Results are means (±range) from one representative experiment performed in duplicate. The experiment was repeated with similar results. Basal adenylyl cyclase activity was not affected by incubation with these antagonists (1 μM). (C) Additive effect of rADM (100 nM) and hαCGRP (100 nM) on adenylyl cyclase activity in BE-CRLR-293 membranes. Adenylyl cyclase activity was measured in BE-CRLR-293 membranes in response to 100 nM rADM, 100 nM hαCGRP, 100 nM rADM plus 100 nM hαCGRP, or 10 μM forskolin. The data presented are means ± SEM from one experiment done in triplicate, which is representative of two similar experiments.

high-affinity binding sites with an apparent K_d value of 145 ± 15 pM and a maximum binding capacity of 12 ± 2.5 fmol/mg protein (Fig. 3B). Specific binding of [^{125}I]rADM was inhibited in a concentration-dependent manner by ADM with K_i values of 5.4 ± 0.8 nM (Fig. 3C). hαCGRP displaced only 20% of the [^{125}I]rADM binding at 1 μM (Fig. 3C). No specific [^{125}I]rADM binding was observed for vector-transfected HEK-293 cells.

To determine the functional coupling of the recombinant bovine CRLR, agonist-stimulated adenylyl cyclase activity was determined in BE-CRLR-293 membranes. Exposure of these cell membranes to ADM or CGRP resulted in a concentration-dependent increase in adenylyl cyclase activity (Fig. 4A). Half-maximal activation was observed at 5.0 ± 1.2 and 2.5 ± 0.7 nM for rADM and hαCGRP, respectively (Fig. 4A). The linear CGRP peptide, [Cys(ACM^{2,7})] CGRP, and human and sCT failed to stimulate adenylyl cyclase activity in these membranes (data not shown). In the untransfected HEK-293 cell membranes, marginal (30–40%) stimulation of adenylyl cyclase activity over basal was observed with 1 μM CGRP or ADM. The CGRP receptor antagonist hαCGRP(8-37) and the ADM receptor antagonist hADM(22-52) were used

to study their effects on ADM-stimulated adenylyl cyclase activity in BE-CRLR-293 membranes. ADM-mediated activation of adenylyl cyclase was inhibited by hADM(22-52) and hαCGRP(8-37) with IC_{50} values of 110 ± 20 and 2156 ± 180 nM, respectively (Fig. 4B). There were no additive effects between rADM and hαCGRP, suggesting that both peptides act through the same receptor (Fig. 4C).

RT–PCR analysis was performed to determine the type of RAMP(s) present in HEK-293 cells. Specific primer sets for RAMP1, RAMP2, and RAMP3 were designed as reported by Kamitani *et al.* [24]. The total RNA was extracted from the vector and porcine CRLR- and bovine CRLR-transfected HEK-293 cells, and the PCR reaction was carried out for the synthesis of cDNAs by reverse transcriptase. The expression of only RAMP2 was observed from these HEK-293 cells (data not shown). This observation supports the data obtained in this investigation that bovine CRLR when expressed in HEK-293 cells displays a functional ADM receptor phenotype.

To study the interaction between bovine CRLR and RAMPs, the cDNAs from human RAMPs were used since bovine RAMPs are currently not available. cDNAs encoding bovine CRLR and individual human RAMPs (1, 2, and 3) were transiently transfected into HEK-293 cells for 48 hr and tested for their affinities to bind CGRP and ADM analogs. Binding of [^{125}I]hαCGRP and [^{125}I]rADM ^{125}I]rADM was examined in membranes prepared from transfected HEK-293 cells. In mock-transfected (pCDN) cells and cells transfected with human RAMP 1, 2, or 3 expression constructs alone (without CRLR), no specific binding was detected for [^{125}I]hαCGRP or [^{125}I]rADM. Co-transfection of bovine CRLR and RAMP1 conferred specific, high-affinity [^{125}I]hαCGRP binding (Fig. 5A). The apparent dissociation constant and maximal binding were 145 ± 30 pM and 94 ± 12 fmol/mg, respectively (Fig. 5B). Marginal specific binding of [^{125}I]ADM (6.0 ± 2.0 fmol/mg) also was observed in these membranes (data not shown). Examining the ability of CGRP and related peptides to compete for [^{125}I]hαCGRP binding (Fig. 5C) assessed the specificity of [^{125}I]hαCGRP binding

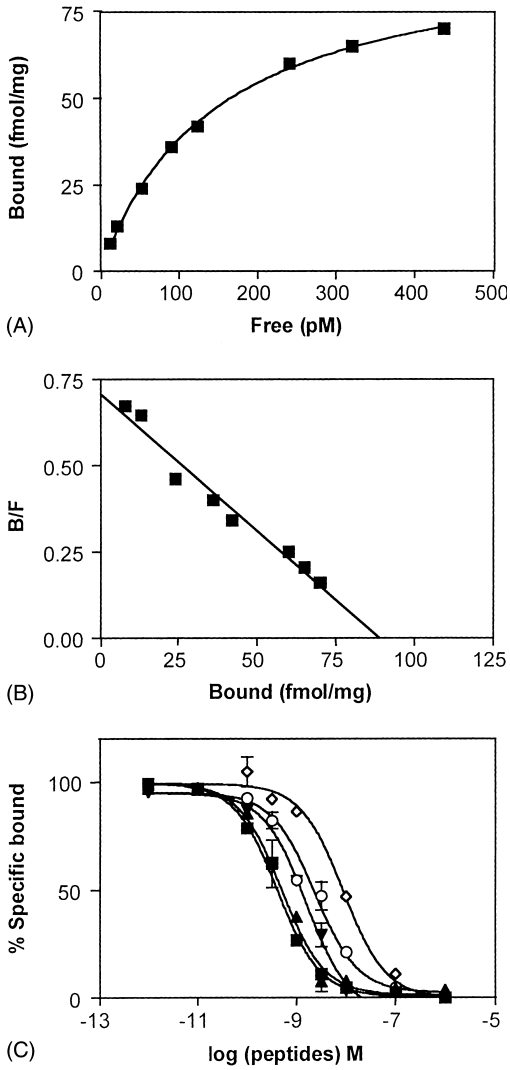

Fig. 5. Saturation binding curve (A) and Scatchard plot (B) of [^{125}I]hαCGRP binding to BE-CRLR/RAMP1 membranes. Experimental details are described in Section 2. Data are the means of duplicate determinations and are representative results from one of three independent experiments. (C) Competition curves for CGRP and ADM peptides against [^{125}I]hαCGRP binding to BE-CRLR/RAMP1 membranes. Key: hαCGRP (■), hαCGRP(8-37) (○), hβCGRP (▲), rADM (▼), and [Cys(ACM^{2,7})] CGRP (◇). Data are the means (±range) of duplicate determinations and are representative results from one of three independent experiments.

Table 1
K_i values for peptides calculated from competition binding experiments using [^{125}I]hαCGRP or [^{125}I]rADM and membranes isolated from BE-CRLR with individual RAMPs

	BE-CRLR/RAMP1 [^{125}I]hαCGRP	BE-CRLR/RAMP2 [^{125}I]rADM	BE-CRLR/RAMP3 [^{125}I]rADM
K_i (nM)			
hαCGRP	0.11 ± 0.03	> 1,000	> 1,000
hβCGRP	0.20 ± 0.05	ND	ND
hαCGRP(8-37)	0.90 ± 0.23	> 1,000	> 1,000
[Cys(ACM^{2,7})] CGRP	4.30 ± 0.6	35.67 ± 8.76	ND
hADM	ND	1.35 ± 0.08	0.26 ± 0.05
rADM	2.60 ± 0.42	0.52 ± 0.1	0.22 ± 0.08
hADM(22-52)	ND	35.55 ± 8.5	> 1,000
sCT	> 10,000	> 10,000	> 10,000

Values are means ± SEM, N = 3–5 independent experiments. ND = not determined.

to BE-CRLR/RAMP1 membranes. Both hα- and hβCGRP were equipotent in competing against [^{125}I]hαCGRP binding followed by hαCGRP(8-37) and rADM. The linear peptide [Cys(ACM^{2,7})] CGRP was 20- to 30-fold weaker than CGRP(8-37) or ADM (Fig. 5C, Table 1).

To determine the functional coupling to BE-CRLR/RAMP1, agonist-stimulated adenylyl cyclase activities were determined in these membranes. Both α and β CGRP displayed concentration-dependent increases in adenylyl cyclase activity, with EC_{50} values of 0.78 ± 0.09 and 0.69 ± 0.10 nM, respectively (Fig. 6A). The linear CGRP peptide [Cys(ACM^{2,7})] CGRP, rADM, and hADM also stimulated adenylyl cyclase activity, with EC_{50} values of 97.9, 4.3, and 7.0 nM, respectively (Fig. 6A, Table 2). sCT failed to stimulate adenylyl cyclase activity significantly in these membranes. While hαCGRP(8-37) was effective in inhibiting CGRP-mediated adenylyl cyclase activity in these membranes with an IC_{50} value of 110 ± 25 nM, hADM(22-52) was weaker, and inhibited only 60% at 10 μM (Fig. 6B). ADM-activated adenylyl cyclase activity was inhibited by hαCGRP(8-37) with an IC_{50} value of 35 ± 8 nM. hADM(22-52) inhibited ADM response with an IC_{50} value = 2600 ± 240 nM. The inhibition at 10 μM was 80% of the control (Fig. 6C). Activation of adenylyl cyclase and the binding affinity of [^{125}I]hαCGRP for BE-CRLR/RAMP1 were similar to those observed for endogenous CGRP receptors present in membranes prepared from human neuroblastoma SK-N-MC cells [26] as well as human CRLR/RAMP1 [10].

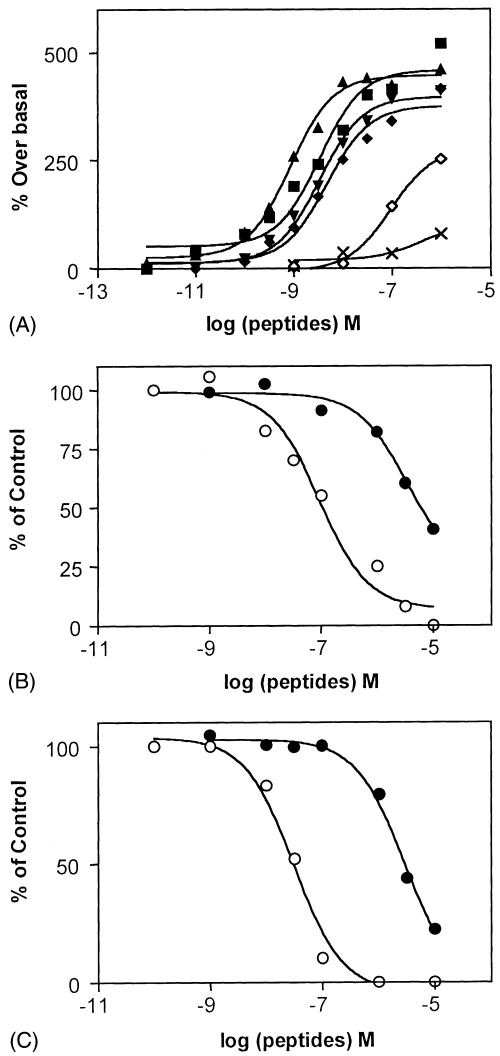

Fig. 6. (A) Stimulation of adenylyl cyclase activity in BE-CRLR/RAMP1 membranes. The membranes were incubated with increasing concentrations of hαCGRP (■), hβCGRP (▲), [Cys(ACM^{2,7})] CGRP (◇), hADM (◆), rADM (▼), and sCT (×) at 30° for 20 min and processed as explained in Section 2. Data are the means of duplicate determinations and are representative results from one of four independent experiments. (B and C) Inhibition of ADM-stimulated adenylyl cyclase activity by hαCGRP(8-37) or hADM(22-52) in BE-CRLR/RAMP1 membranes. Membranes were incubated with 3 nM hαCGRP (Fig. 6B) or 3 nM ADM (Fig. 6C) in the absence or presence of the indicated concentrations of hαCGRP(8-37) (○) or hADM(22-52) (●). Results are means from duplicate determinations. Similar results were obtained in another experiment. Basal adenylyl cyclase activity was not affected by these antagonists (1 μM).

Table 2
The EC_{50} values of CGRP, ADM, and related peptides for the activation of adenylyl cyclase in membranes isolated from BE-CRLR with individual RAMPs

	BE-CRLR plus		
	RAMP1	RAMP2	RAMP3
EC_{50} (nM)			
hαCGRP	0.78 ± 0.09	97.83 ± 32	52.4 ± 13.8
hβCGRP	0.69 ± 0.095	121.86 ± 37	65.56 ± 4.0
hADM	6.97 ± 0.82	0.86 ± 0.08	1.08 ± 0.16
rADM	4.33 ± 0.45	0.67 ± 0.12	0.77 ± 0.27
[Cys(ACM^{2,7})] CGRP	97.9 ± 9.26		

Values are means ± SEM, N = 3–5 independent experiments.

Co-transfection of HEK-293 cells with bovine CRLR and hRAMP2 or hRAMP3 displayed specific ADM binding. Saturation binding data obtained from [^{125}I]rADM binding to membranes prepared from BE-CRLR/RAMP2 and BE-CRLR/RAMP3 are presented in Figs. 7A and 8A, respectively. In both systems, high-affinity and high-density bindings for [^{125}I]rADM were observed. The K_d and B_{max} of [^{125}I]rADM were 158 ± 18 pM and 5087 ± 240 fmol/mg protein for BE-CRLR/RAMP2 (Fig. 7B) and 163 ± 32 pM and 3380 ± 310 fmol/mg protein for BE-CRLR/RAMP3 (Fig. 8B). Very little binding of [^{125}I]CGRP was observed in these membranes. Competition binding experiments of ADM and CGRP analogs against [^{125}I]ADM binding to BE-CRLR/RAMP2 or BE-CRLR/RAMP3 revealed the following rank order of potency: rADM > hADM > hADM(22-52). hαCGRP and hαCGRP(8-37) displayed K_i values >1000 μM (Figs. 7C and 8C, respectively; Table 1). sCT failed to displace [^{125}I]rADM binding (data not shown). In all cases, the peptides displayed monophasic competition curves with Hill coefficients not significantly different from unity, indicative of interaction with a single class of binding sites. hADM and rADM stimulated adenylyl cyclase activity in a concentration-dependent manner in BE-CRLR/RAMP2 (Fig. 9A) or BE-CRLR/RAMP3 (Fig. 10A). The EC_{50} values

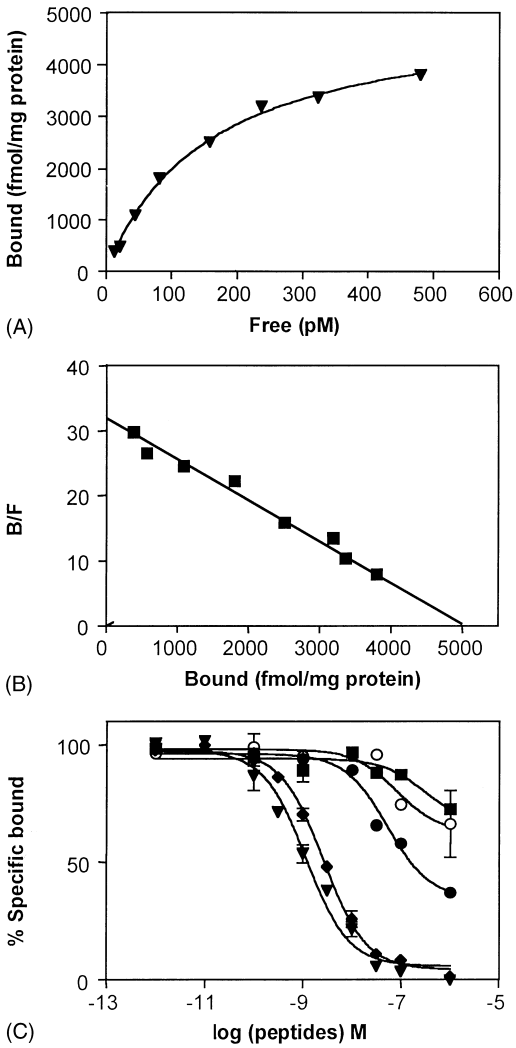

Fig. 7. Saturation binding curve (A) and Scatchard plot (B) of [^{125}I]rADM binding to BE-CRLR/RAMP2 membranes. Experimental details are described in Section 2. Data are the means of duplicate determinations and are representative results from one of three independent experiments. (C) Competition binding curves for ADM and CGRP peptides against [^{125}I]ADM (0.3 nM) binding to BE-CRLR/RAMP2 membranes. Key: hαCGRP (■), CGRP(8-37) (○), hADM (◆), rADM (▼), and hADM(22-52) (●). Data are the means (±range) of duplicate determinations and are representative results from one of five independent experiments. The K_i values for pooled data are shown in Table 1.

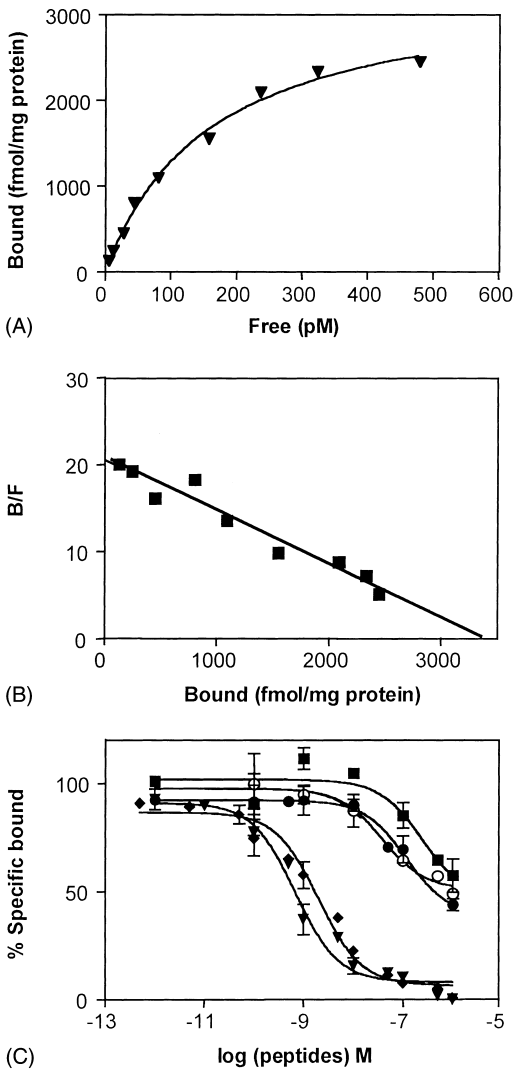

Fig. 8. Saturation binding curve (A) and Scatchard plot (B) of [^{125}I]rADM binding to BE-CRLR/RAMP3 membranes. Experimental details are described in Section 2. Data are the means of duplicate determinations and are representative of one of three independent experiments. (C) Competition binding curves for ADM and CGRP peptides against [^{125}I]rADM (0.3 nM) binding to BE-CRLR/RAMP3 membranes. Key: hαCGRP (■), hαCGRP(8-37) (○), hADM (◆), rADM (▼), and hADM(22-52) (●). Data are the means (±range) of duplicate determinations and are representative results from one of three independent experiments. The K_i values for pooled data are shown in Table 1.

for hADM and rADM were similar in both preparations (Table 2). Alpha and beta CGRP were much weaker than ADM peptides in stimulating adenylyl cyclase activity (Figs. 9A and 10A, Table 2). The effects of hADM(22-52) on ADM-stimulated adenylyl cyclase activity in BE-CRLR/RAMP2 or BE-CRLR/RAMP3 membranes are shown in Figs. 9B and 10B, respectively. While hADM(22-52) was potent (IC_{50} values were 125 ± 15 and 85 ± 12 nM, respectively, for BE-CRLR/RAMP2 and BE-CRLR/RAMP3) in inhibiting ADM-stimulated adenylyl cyclase activity in these membranes (Figs. 9B and 10B), hαCGRP(8-37) was 20-fold weaker. Also, in BE-CRLR/RAMP2, the inhibition by CGRP(8-37) was incomplete (plateau at 10 μM). Thus, the data presented in the present study suggest that RAMP2 and RAMP3 are functionally identical, with both proteins presenting bovine CRLR as ADM receptors. The pharmacology of the ADM receptor created by expression of CRLR with RAMP2 or RAMP3 appears to be insensitive to hαCGRP(8-37) [27].

4. Discussion

In this paper, we describe the cloning and characterization of bovine CRLR. Cloning was accomplished using hCGRP receptor cDNA as a probe to screen the bovine

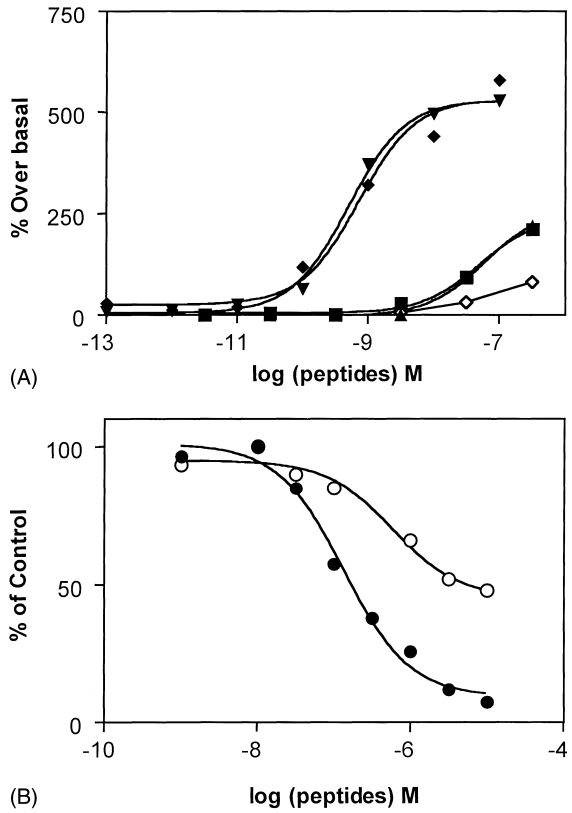

Fig. 9. (A) Stimulation of adenylyl cyclase activity in BE-CRLR/RAMP2 membranes by hαCGRP (■), hβCGRP (▲), rADM (▼), hADM (◆), and [Cys(ACM^{2,7})] CGRP (◇). Data are the means of duplicate determinations and are representative results from one of five independent experiments. (B) Inhibition of ADM-stimulated adenylyl cyclase activity by hαCGRP(8-37) or hADM(22-52) in BE-CRLR/RAMP2 membranes. Membranes were incubated with 3 nM hADM in the absence or presence of increasing concentrations of hαCGRP(8-37) (○) or hADM(22-52) (●). Results are means from one experiment performed in duplicate. Similar results were obtained in another experiment. Basal adenylyl cyclase activity was not affected by incubation with these antagonists (1 μM).

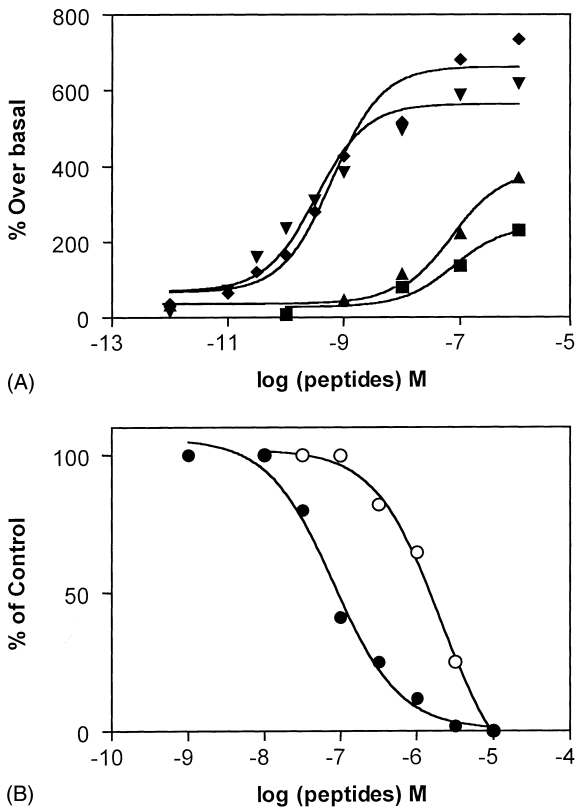

Fig. 10. (A) Stimulation of adenylyl cyclase activity in BE-CRLR/RAMP3 membranes by hαCGRP (■), hβCGRP (▲), rADM (▼), and hADM (◆). Data are the means of duplicate determinations and are representative results from one of five independent experiments. (B) Inhibition of ADM-stimulated adenylyl cyclase activity by hαCGRP(8-37) or hADM(22-52) in BE-CRLR/RAMP3. Membranes were incubated with 3 nM hADM in the absence or presence of increasing concentrations of hCGRP(8-37) (○) or hADM(22-52) (●). Results are means from one experiment performed in duplicate. Similar results were obtained in another experiment. Basal adenylyl cyclase activity was not affected by these antagonists (1 μM).

aortic endothelial cell library. The deduced sequence of bovine CRLR is 462 amino acids with a predicted molecular mass of 50.8 kDa. This sequence was 92, 88, and 94% identical to human, rat, and porcine CRLR, respectively. Several structural features of the cloned receptor demonstrate that it belongs to the family of seven-transmembrane G-protein-coupled receptors. The pharmacological properties of recombinant bovine endothelial cell CRLR stably expressed in HEK-293 cells compare well with those described for ADM receptors present on endothelial cells.

ADM has been shown to cause vasorelaxation in isolated vascular beds without the endothelium by binding to ADM and CGRP receptors and stimulating the accumulation of cAMP. ADM and CGRP initiate their biological responses through their interaction with CRLR that has been cloned from humans, pigs, and rats. McLatchie *et al.* [17] reported the cloning and characterization of cDNAs for a class of proteins (RAMPs), which, when co-expressed with CRLR, dictate the pharmacology profile of the receptor. Co-expression of CRLR with RAMP1 resulted in a receptor that displayed CGRP receptor pharmacology, whereas co-expression of CRLR with RAMP2 or RAMP3 resulted in a receptor that displayed ADM receptor pharmacology. HEK-293, which is a common cell line used for stable expression of GPCRs, has been shown to contain RAMP2. Stable transfection of bovine CRLR in HEK-293 cells in the absence of exogenous RAMPs resulted in high-affinity binding to [^{125}I]ADM with an apparent dissociation constant and maximum binding of 145 ± 15 pM and 12 ± 2.5 fmol/mg protein, respectively. Untransfected or mock-transfected cells did not display any [^{125}I]ADM binding, confirming that the bovine CRLR clone used for stable expression in HEK-293 cells displayed ADM receptor pharmacology in the absence of RAMP co-transfection. We have reported previously that stable expression of human or porcine lung CRLR in HEK-293 cells in the absence of exogenous RAMP transfection resulted in CRLR that displayed CGRP receptor pharmacology [10,12]. The same cell line, when used for bovine

endothelial cell CRLR transfection, produced a receptor that displayed ADM receptor pharmacology. The endogenous levels of RAMPs at mRNA level in vector- and stable CRLR-transfected HEK-293 cells were studied by RT–PCR. In the present study, we could detect mRNA for RAMP2 only. The mechanism in which the RAMPs convert the CRLR to a particular receptor is not well understood. The endogenous G-protein may also play a role in the display of receptor pharmacology [28]. It is also possible that the pharmacology displayed by the CRLR may be based upon certain critical amino acids present in the sequence of the receptor, which may dictate the interaction of the receptor with the endogenous RAMPs. As mentioned above, bovine CRLR is highly homologous to human and porcine CRLRs (92 and 94%, respectively), yet when expressed stably in HEK-293 cells, it displays ADM receptor pharmacology. This is in contrast to the CGRP receptor pharmacology that is displayed by human and porcine CRLRs. These data suggest that while human and porcine CRLRs preferred to interact with endogenous RAMP1 present in HEK-293 cells, bovine CRLR preferred to interact with endogenous RAMP2 present in HEK-293 cells.

The current study also confirmed that exogenous RAMPs, when co-transfected with CRLR, modulated bovine CRLR in a manner very similar to that of human and porcine CRLRs. Co-transfection of bovine CRLR with human RAMP1 led to the expression of CGRP receptor activity in HEK-293 cells, and co-transfection of bovine CRLR with RAMP2 or RAMP3 led to ADM receptor activity. One major difference between bovine and human CRLRs plus RAMP1 transfection is that while human CRLR plus RAMP1 transfection resulted in affinity for only the CGRP receptor, bovine CRLR plus RAMP1 also displayed high-affinity for ADM in both binding and functional studies in addition to high-affinity for CGRP. As expected, CRLR plus RAMP2 or RAMP3 displayed specific high-affinity for ADM receptors. Co-transfection of all three RAMPs along with bovine CRLR resulted in a receptor that displayed high-affinity for both CGRP and ADM, which was very similar to CRLR plus RAMP1 transfection.

These observations demonstrate that bovine endothelial cell CRLR fits the profile of an ADM receptor. This is in contrast to human and porcine CRLRs, which fit the profile of CGRP receptors when heterologously expressed in HEK-293 cells in the absence of exogenous RAMPs. In addition, even when RAMP1 was co-transfected with bovine CRLR, this receptor gained the pharmacology for the CGRP receptor while maintaining ADM receptor pharmacology. Thus, the data presented in this manuscript, along with previously published data on human and porcine CRLR [27], suggest that the bovine CRLR has the innate ability to present itself as an ADM receptor in the absence of exogeneous RAMPs, just as human and porcine CRLRs have the innate ability to present themselves as CGRP receptors in the absence of exogenous RAMPs. The factors that govern the innate abilities of these receptors will be the focus of future research.

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