The RAMP2/CRLR complex is a functional adrenomedullin receptor in human endothelial and vascular smooth muscle cells

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Abstract Adrenomedullin, a potently hypotensive peptide isolated from human pheochromocytoma, is known to elicit a rise in cAMP levels within mammalian endothelial and smooth muscle cells. Until now, however, little has been known about the adrenomedullin receptor. Recently, a group called receptor activity-modifying proteins that complex with the calcitonin receptor-like receptor, and thereby regulate its transport and ligand specificity, were identified. Here we show that mRNA for both the calcitonin receptor-like receptor and the receptor activity-modifying protein 2, but not the receptor activity-modifying protein 1 or receptor activity-modifying protein 3, are expressed in human endothelial and vascular smooth muscle cells. We also found that adrenomedullin increased cAMP levels in HeLa EBNA and 293 EBNA cells, expressing both the receptor activity-modifying protein 2 and calcitonin receptor-like receptor proteins. Thus, the receptor activity-modifying protein 2/calcitonin receptor-like receptor complex apparently serves as a functional adrenomedullin receptor in human endothelial and vascular smooth muscle cells.

Key words: Calcitonin gene-related peptide; Calcitonin receptor-like receptor; Receptor activity-modifying protein; cAMP; Adrenomedullin

1. Introduction

Adrenomedullin, which is isolated from human pheochromocytoma, is a member of the calcitonin gene-related peptide (CGRP) family [1]. It is known to be potently hypotensive in rat and it evokes a rise in the levels of cAMP within mammalian endothelial, smooth muscle [1–4] and mesangial cells [5,6]. At present, however, little is known about the adrenomedullin receptor. Kapas et al. isolated a G-protein-coupled receptor homologue of the canine orphan receptor (RDC-1) from rat lung and, on the basis of its pharmacology, identified it as an adrenomedullin receptor [7,8]. But Kennedy et al. showed that neither this putative rat adrenomedullin receptor nor its human counterpart [9] was functional when expressed in COS-7 cells, indicating that they were not authentic adrenomedullin receptors [10]. Recently, McLatchie et al. reported that the calcitonin receptor-like receptor (CRLR) could function as either a CGRP receptor or an adrenomedullin receptor, depending on the expression of receptor activity-modifying proteins (RAMPs) (members of a new family of single transmembrane domain proteins) [11]. They proposed that whereas the RAMP1/CRLR complex serves as a CGRP receptor, RAMP2/CRLR serves as an adrenomedullin receptor. However, confirmation of the model through demonstration of the co-expression of CRLR and RAMP2 mRNA in adrenomedullin-sensitive cells has until now not been reported. Our present study, therefore, is the first to show that expression of both CRLR and RAMP2 is required for constituting functional adrenomedullin receptors.

2. Materials and methods

2.1. Materials

Human aortic smooth muscle cells (HASMCs), human aortic endothelial cells (HAECs) and human umbilical vascular endothelial cells (HUVECs) as well as Smooth Muscle Cell Basal Medium (SmBM) and Endothelial Cell Basal Medium (EBM) were all purchased from Clonetics Corp/Sanko Junyaku (Tokyo, Japan). Human adrenomedullin and CGRP were obtained from Peptide Institute (Osaka, Japan). Bovine serum albumin and 3-isobutyl-1-methylxanthine (IBMX) were from Sigma (St. Louis, MO, USA). Other reagents were from Nacalai Tesque (Kyoto, Japan).

2.2. Plasmids

A plasmid-cloned, complete CRLR cDNA was a gift from Dr Kangawa of the National Cardiovascular Research Institute (Suita, Japan). RAMP cDNAs were cloned from a human embryonic cDNA library (Clontech, Palo Alto, CA, USA) by PCR using the following sets of primers: RAMP1, 5′-CGA GCG GAC TCG ACT CGG CAC CGC T-3′ and 5′-GCC TGG GCC CCG CCT ACA CAA TGC C-3′; RAMP2, 5′-CTC CTT GCT GCA CGA TGG CCT C-3′ and 5′-TTG TTG AGA AGC TCG TGC CCC CTA G-3′; RAMP3, 5′-CAG CCA TGG AGA CTG GAG CGC TGC-3′ and 5′-ATC TCA CCG GGA CCC TCA CAG CAG C-3′. The RAMP cDNAs were then subcloned into a pCAGGS vector [12] carrying the neomycin resistance gene. CRLR cDNA was subcloned into a pDREF vector [13] also containing the EB virus nuclear antigen (EBNA-1) and the EB virus origin (Ori P), which enable high-copy episomal replication, and the hygromycin-B resistant gene, which is maintained extra-chromosomally and expresses high levels of recombinant proteins under the control of the human elongation factor 1α (EF-1α) promoter.

2.3. Cell culture and cDNA transfection

HASMCs were cultured in SmBM supplemented with human epidermal growth factor (0.5 ng/ml), human fibroblast growth factor-B (2 ng/ml), insulin (5 μg/ml), 5% fetal bovine serum, 50 ng/ml gentamicin and 50 μg/ml amphotericin-B. HAECs and HUVECs were cultured in EBM supplemented with human epidermal growth factor (0.5 ng/ml), human fibroblast growth factor-B (2 ng/ml), bovine brain extracts (12 μg/ml), hydrocortisone (1.0 μg/ml), 5% fetal bovine serum, 50 ng/ml gentamicin and 50 μg/ml amphotericin-B. HeLa EBNA cells and 293 EBNA cells (subclones of HeLa and 293 cells engineered to express EBNA-1 and to carry the neomycin resistance gene) were cultured in DMEM supplemented with 10% fetal bovine serum, 10% sodium glutamate, 100 μg/ml penicillin G, 100 U/ml streptomycin, 50 μg/ml amphotericin-B and 400 or 250 μg/ml geneticin. Cells were incubated at 37°C under an atmospheric 95% air/5% CO_2. HeLa EBNA cells

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and 293 EBNA cells expressing CRLR were generated by hygromycin-B selection of cells transfected with plasmids encoding CRLR and a selection marker. For transient expression, cells were plated in six well plates to a density of $1–5\times10^5$ cells/well and transfected with 1 μg plasmid/well. Transfection was carried out using LipofectAmine according to the manufacturer's instructions (Gibco BRL, Grand Island, NY, USA).

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared from the cultured cells using Trizol Reagent (Gibco BRL) and 5 μg aliquots were then subjected to reverse transcription using a Superscript II cDNA synthesis kit (Gibco BRL). CRLR and RAMP mRNAs were detected by nested PCR using a set of specific primers (Fig. 2A). All first PCRs were carried out on a Omnigene temperature cycler (HYBAID, Middlesex, TW, USA) using the following protocol: an initial denaturation step at 95°C for 5 min was followed by 30 cycles at 95°C for 30 s, 55°C for 30 s and 72°C for 30 s. Nested PCRs were then performed using the same protocol and 1 μl of the first PCR products as a template. A set of GAPDH primers was used as a control for the RT-PCR (Fig. 2A).

2.5. Measurement of cAMP

Intracellular cAMP was measured as reported previously [14]. Briefly, cells were washed twice with Hanks' balanced salts containing 20 mM HEPES (pH 7.4), 0.1% bovine serum albumin and 0.5 mM IBMX and then incubated in the same solution for 15 min in the presence or absence of ligand. The cells were then lysed in 6% trichloroacetic acid, after which the acid was removed by water-saturated diethyl ether extraction. The aqueous phase was then lyophilized and cAMP was measured using the Biotrack cAMP EIA assay kit (Amersham-Pharmacia, Little Chalfont, UK).

2.6. Statistics

All values were expressed as mean ± S.D. The statistical significance of differences among cells was evaluated using unpaired ANOVA and Students' *t*-tests. Values of $P<0.05$ were considered significant.

3. Results and discussion

3.1. Adrenomedullin selectively induced intracellular cAMP accumulation

We initially confirmed that adrenomedullin significantly and dose-dependently increased cAMP levels in HAECs, HUVECs and HASMCs (Fig. 1A). CGRP, by contrast, had no effect on the cAMP levels in human cells (Fig. 1B), even though it elevated cAMP in rat vascular smooth muscle cells (data not shown). In HeLa EBNA and 293 EBNA cells of human origin, neither adrenomedullin nor CGRP increased cAMP. Thus, the adrenomedullin receptor appears to be specifically expressed in HAECs, HUVECs and HASMCs.

3.2. RAMP2 and CRLR mRNA are both expressed in HAECs, HUVECs and HASMCs

To determine whether the adrenomedullin-sensitive, human endothelial and smooth muscle cells express both CRLR and RAMPs, we examined the expression of their mRNAs by RT-PCR. Specific primer sets for CRLR and RAMPs were designed (Fig. 2A) and total RNA was extracted from the cells assayed in Fig. 1. After cDNA synthesis by reverse transcription, nested PCR was carried out. Co-expression of CRLR and RAMP2 mRNA was detected in adrenomedullin-sensitive HAECs, HUVECs and HASMCs (Fig. 2B), but only RAMP2 mRNA was expressed in 293 EBNA cells and HeLa EBNA cells did not express either mRNA. This is consistent with the notion that when complexed, CRLR and RAMP2 comprise a functional adrenomedullin receptor. Neither RAMP1 nor RAMP3 mRNA was detected in any of the cells in this study in agreement with the results in Fig. 1.

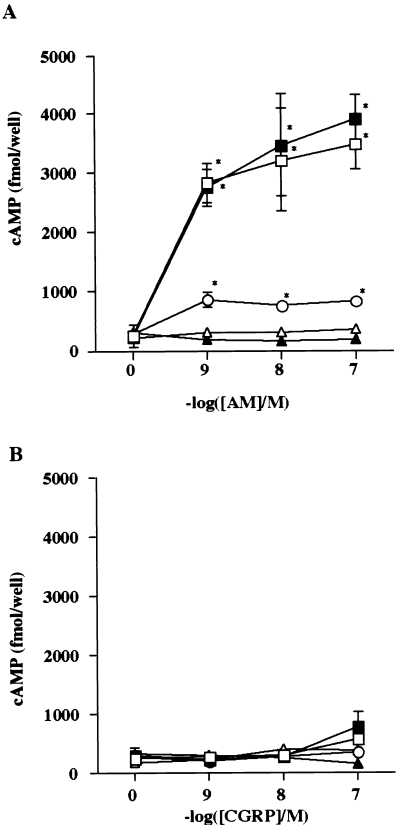

Fig. 1. The effect of adrenomedullin and CGRP on intracellular cAMP levels. Dose response curves showing intracellular cAMP accumulation in response to adrenomedullin (A) and CGRP (B). HAECs (open squares), HUVECs (closed squares), HASMCs (open circles), HeLa EBNA cells (open triangles) and 293 EBNA cells (closed triangles) were each incubated with $0–10^{-7}$ M adrenomedullin or CGRP for 15 min. Intracellular cAMP levels were then measured using a cAMP EIA kit. $*P<0.05$ versus basal levels.

3.3. The RAMP2/CRLR complex is a functional adrenomedullin receptor

To confirm that all else is equal, the specificity of the CRLR receptor could be defined entirely by the expression mode of RAMPs, cells constitutively expressing CRLR were constructed from HeLa EBNA and 293 EBNA cells as described in Section 2. These CRLR-expressing cells were transiently transfected with the respective RAMP cDNAs and the resultant transfectants were stimulated with either adrenomedullin or CGRP. Modulation of the receptor specificity was assessed on the basis of changes in intracellular cAMP levels. Fig. 3 shows that RAMP2 and CRLR reconstitute a functional adrenomedullin receptor in both HeLa EBNA and 293 EBNA cells, as indicated by the adrenomedullin-evoked accumulation of cAMP (A and C, solid bars). The RAMP2/CRLR complex was selective for adrenomedullin, making this the direct observation that the RAMP2/CRLR complex serves as a functional adrenomedullin receptor. The RAMP1/CRLR and RAMP3/CRLR complexes were sensitive to both adrenomedullin and CGRP (dotted and open bars), the former being equally sensitive or relatively more specific to CGRP and the latter more sensitive to adrenomedullin, but neither RAMP1 nor RAMP3 was detected in adrenomedullin-sensitive cells (Fig. 2B). We also observed that HAECs, endogenously expressing only RAMP2, became sensitive to CGRP

A

	1st PCR primer set	nested PCR primer set
CRLR	CTCCTCTACATTATCCATGG CCTCCTCTGCAATCTTTCC	ATTTGTGCTGCTTTACTGG ATTCAATGCCAAGCAATGG
RAMP1	AAGCTGGGCTGCTTCTGG CACAATGCCCTTCAGTGC	AATGCAGAGGTGGACAGG TGCCAGACCACCAGTGC
RAMP2	ATTGCCTGGAGCACTTTGC GCCTCACTGTCTTTACTCC	TGTTTGACCTGGGCTTCC ATGAGGAAGGGGATGAGG
RAMP3	TCGTGGGCTGCTACTGG CTCACAGCAGCGTGTCG	CCCAGGGCTTCATCATCACC GTTTGCTGCGCCACACC
GAPDH	AGACAGCCGCATCTTCTTGTGC CTCCTGGAAGATGGTGATGG	

B

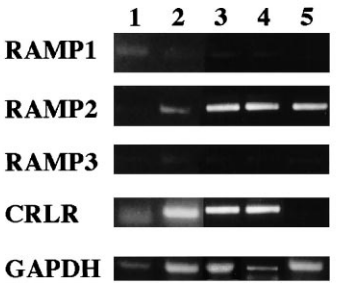

Fig. 2. Expression of RAMP and CRLR mRNA in human endothelial and smooth muscle cells. A: Primer sets used in the experiment. B: RT-PCR showing expression of RNAs for RAMP1, RAMP2, RAMP3 and CRLR in HeLa EBNA cells (lane 1), HASMCs (lane 2), HUVECs (lane 3), HAECs (lane 4) and 293 EBNA cells (lane 5). Total RNA from 293 EBNA cells transfected with the respective cDNAs was used as a positive control. After cDNA synthesis, nested PCR was performed using four sets of specific primers. Primers for GAPDH served as an internal control. The final PCR products were cloned and identified by DNA sequencing.

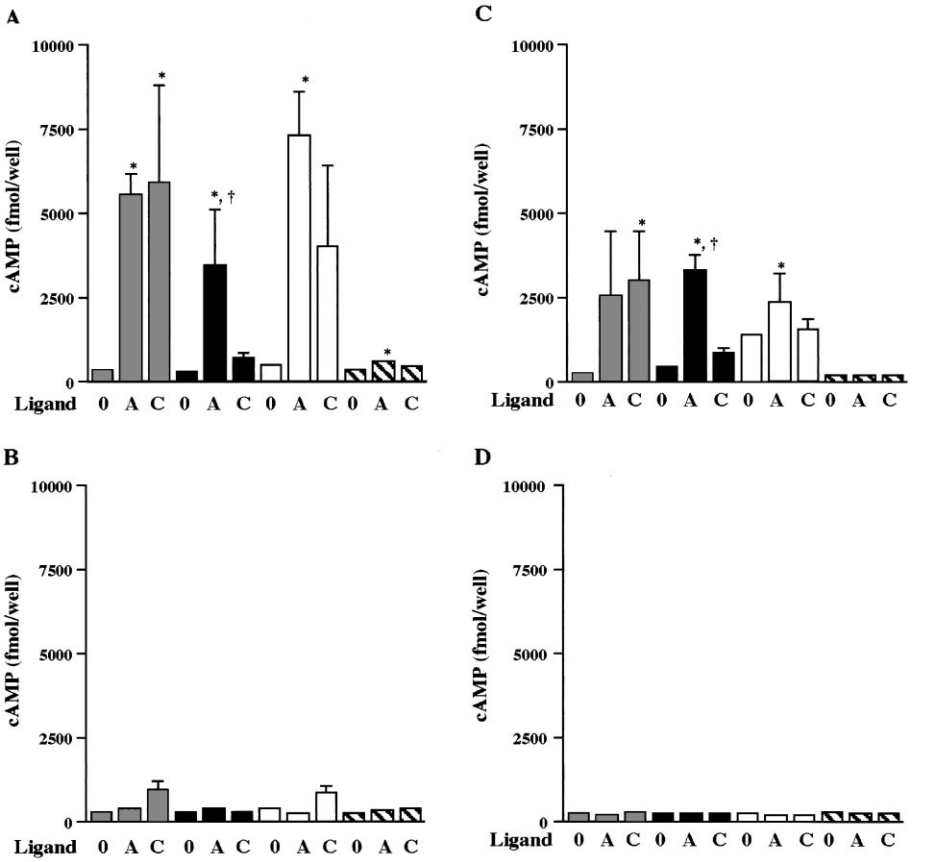

Fig. 3. RAMP-dependent modulation of the ligand specificity. RAMPs were expressed in 293 EBNA and HeLa EBNA cells either together with CRLR (A and C, respectively) or alone (B and D, respectively). Cells were then transiently transfected with RAMP1 (dotted bars), RAMP2 (solid bars), RAMP3 (open bars) or vector plasmid (hatched bars). The transfectants were incubated for 15 min in the absence of ligand (0) or in the presence of 10^{-8} M adrenomedullin (A) or 10^{-8} M CGRP (C) and intracellular cAMP levels were measured by the cAMP EIA kit. *$P < 0.05$ (A or C versus 0); †: $P < 0.05$ (A versus C).

by transfection with RAMP1 cDNA (data not shown). These results suggest that the degree of relative expression levels of the RAMPs family regulates the receptor specificity of CRLR. The magnitudes of the maximal responses to adrenomedullin were greater in endothelial cells than in smooth muscle cells (Fig. 1), a difference that likely reflects the respective levels of RAMP2 mRNA expression. Using Northern blots, RAMP2 was detected in endothelial cells but not in other cell types (data not shown). Moreover, 293 EBNA cells expressed RAMP2 mRNA prior to transfection, so that adrenomedullin, but not CGRP, dose-dependently evoked cAMP accumulation in cells constitutively expressing CRLR (data not shown). When the same cell type was then transiently transfected with RAMP2 cDNA, both the RAMP2 mRNA level and the evoked accumulation of cAMP were augmented. Thus, the stoichiometric ratio between RAMP2 and CRLR may be crucial in determining the responsiveness of cells to adrenomedullin. CRLR has three putative *N*-glycosylation sites at the amino-terminal and it has been suggested that RAMPs regulate the CRLR transport by modifying the level of its glycosylation [1]. In that regard, co-expression of RAMP1 and CRLR leads to terminal glycosylation of the CRLR, whereas expression of RAMP2 results in only core glycosylation. Receptor specificity may also be determined by glycosylation. For example, selectivity of the calcitonin receptor for either amylin or calcitonin is apparently glycosylation-dependent [15]. In analogous fashion, the heterodimerization required for the formation of functional gamma-aminobutyric acid $(GABA)_B$ receptors is dependent on the co-expression of $GABA_BR1$ and $GABA_BR2$. Of those, $GABA_BR1$ is terminally glycosylated and expressed at the cell surface where it complexes with $GABA_BR2$ [16]. Thus, RAMPs appear to contribute to the oligomerization, the functionality and the ligand specificity of CRLR receptors. The precise characterization of specific relationships between the expression of RAMP and of CRLR and the role of CRLR *N*-glycosylation in the receptor function will require further study.

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