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Short communication

The expression of mRNA for calcitonin receptor-like receptor/receptor-activity modifying proteins in rat peritoneal mast cells

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Abstract

Adrenomedullin is a hypotensive peptide secreted from various cells. Recently, we found that adrenomedullin, but not calcitonin gene-related peptide (CGRP), stimulates histamine release from rat peritoneal mast cells. In the present studies, we investigated the expression of mRNA for calcitonin-receptor-like receptor (CRLR) and receptor-activity modifying proteins (RAMPs), the components of proposed adrenomedullin receptors, in rat peritoneal mast cells by reverse transcription–polymerase chain reaction (RT–PCR). Results revealed that mRNA for CRLR, RAMP2 and RAMP3 was expressed in rat peritoneal mast cells, whereas mRNA for RAMP1 was not. These data suggest that adrenomedullin might stimulate histamine release via its proposed receptor (CRLR/RAMP2 or 3), rather than via the CGRP receptor (CRLR/RAMP1).

Keywords: Adrenomedullin; Calcitonin gene-related peptide (CGRP); CRLR (Calcitonin-receptor-like receptor); RAMP (Receptor-activity modifying protein); Mast cell; Peritoneal; (Rat)

1. Introduction

Adrenomedullin is a hypotensive peptide originally identified in a pheochromocytoma by monitoring its action to increase cyclic AMP (cAMP) levels in platelets (Kitamura et al., 1993b). Adrenomedullin consists of 52 amino acids with a single disulfide bridge between positions 16 and 21, and an amidated C-terminus (Kitamura et al., 1993a). Calcitonin gene-related peptide (CGRP) is a 37-amino acid hypotensive neuropeptide generated from an alternatively spliced transcript of the calcitonin gene with a single disulfide bridge between positions 2 and 7, and has an amidated C-terminus like adrenomedullin (Juaneda et al., 2000). Although adrenomedullin and CGRP share very little sequence identity (20%), adrenomedullin belongs to the calcitonin gene-related peptide (CGRP)/amylin superfamily, based on the common structure and biological effects (Juaneda et al., 2000; Hinson et al., 2000). It has been proposed that novel receptor complexes, consisting of receptor-activity modifying proteins (RAMPs) together with calcitonin-receptor-like receptor (CRLR), act as pharmacologically defined CGRP or adrenomedullin receptors in cells co-transfected with CRLR and RAMPs (McLatchie et al., 1998), i.e. RAMP1/CRLR as a CGRP receptor and RAMP2 or RAMP3/CRLR as an adrenomedullin receptor.

Recently, we have demonstrated that adrenomedullin induces histamine release from rat peritoneal mast cells, whereas CGRP does not (Yoshida et al., 2001). Moreover, it has been proposed that adrenomedullin might produce its action via a putative adrenomedullin receptor, since adrenomedullin-induced histamine release (1) occurs very rapidly (within 30 s), compared to the effect of immunoglobulin E (IgE) (Church et al., 1989), and (2) is inhibited by an adrenomedullin fragment that possesses antagonistic activity (Yoshida et al., 2001).

However, there are no reports indicating whether peritoneal mast cells possess adrenomedullin receptors. In the present study, we investigated the expression of mRNA for CRLR, RAMP1, RAMP2 and RAMP3 in peritoneal exudate cells, as well as purified mast cells.

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2. Methods

2.1. Animals

Male Wistar rats (300–400 g) were purchased from Japan SLC (Shizuoka, Japan). Animals were maintained at least 3 days before experiments in a temperature- (22–24 °C), humidity- (55 ± 5%) and light (12 h light–dark cycle; lights on at 07:00)-regulated room with food and water ad libitum.

2.2. Crude peritoneal mast cell preparation

Under pentobarbital anesthesia, the peritoneal cavity of a rat was washed with 10 ml of Ca^{2+}- and Mg^{2+}-free Tyrode's solution containing 0.1% (weight/volume) bovine serum albumin and 10 units/ml sodium heparin. After gentle massage of the abdomen for 3 min, peritoneal exudate cells were obtained. Collected peritoneal exudate cells were washed three times with Ca^{2+}- and Mg^{2+}-free Tyrode's solution containing 0.1% (w/v) bovine serum albumin and 10 units/ml sodium heparin by centrifugation (1200 rpm, 4 °C, 10 min). The pellets were then resuspended in ice-cold Ca^{2+}-, Mg^{2+}-free Tyrode's solution containing 20 mM HEPES buffer at a concentration of 5×10^6 mast cells/ml.

2.3. Purification of mast cells from peritoneal exudate cells

Purification of mast cells from peritoneal exudate cells was performed based on the previous report (Enerback and Svensson, 1980). Mast cells were purified to more than 95% by Percoll (Amersham Pharmacia Biotech, Piscataway, NJ) density gradient centrifugation at 1200 rpm for 10 min at 4 °C. Mast cells (3×10^6 cells) were washed three times in Ca^{2+}-, Mg^{2+}-free Tyrode's solution containing 20 mM HEPES buffer after Percoll treatment.

2.4. RT–PCR

Total RNA was extracted from rat kidney, lung, peritoneal exudate cells, and purified mast cells with Trizol reagent (Invitrogen, Carlsbad, CA), and then subjected to reverse transcription using random hexmers and SuperScript II.

The polymerase chain reaction (PCR) utilized primers specific to CRLR, RAMP1, RAMP2 and RAMP3 genes. Details of the primers used are summarized in Table 1. The predicted sizes of the PCR products between two primers were 504 bp for CRLR, 230 bp for RAMP1, 175 bp for RAMP2, and 265 bp for RAMP3, respectively.

PCR mixes contained 0.2 μM primers, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, Taq polymerase, buffer supplied, and cDNA in 20 μl. The tubes were incubated in a GeneAmp 9700 (Perkin-Elmer, Norwalk, CT) at 94 °C for 2 min to denature the primers and cDNA and to activate the enzyme. The cycling program was 94 °C for 30 s, 54 °C for 30 s, 72 °C for 1 min, and for 7 min in the last cycle, and comprised 33 cycles. mRNA, from kidney and lung was extracted, transcribed and amplified as positive controls using the same thermal profile.

Table 1
Primers used for RT–PCR

Gene	Primer sequences (5′→3′)	PCR products (bp)
CRLR		
Forward	CAG CAG GAA CCG AGT CAA TG	504
Reverse	CAA ACA CAG CCA CCA CAA TG	
RAMP1		
Forward	ACT GGG GAA AGA CCA TAG GGA G	230
Reverse	AGT CAT GAG CAG TGT GAC CGT A	
RAMP2		
Forward	AGA CTT CCA TGG ACT CTG TCA AG	175
Reverse	GAG CAG TTG GCA AAG TGT ATC A	
RAMP3		
Forward	CTG TCG GAG TTC ATC GTG TA	265
Reverse	GCC GGT CAG TGT GCT TGC TA	

The primers for CRLR and RAMP3 were designed based on published cDNA sequences (Accession No. NM_012717, AB030944). The primers for RAMP1 and RAMP2 were used from previously reported (Qing et al., 2001).

The PCR products with the exception of RAMP2 in purified mast cells were analyzed electrophoretically on 2% agarose gels and visualized with ethidium bromide. The PCR products of RAMP2 in purified mast cells were electrophoresed on 8% polyacrylamide gels stained with silver.

2.5. cDNA analysis

For sequencing PCR products, kidney mRNA with the primers for CRLR and lung mRNA with the primers for RAMP1, RAMP2 and RAMP3 were electrophoresed on 3% NuSieve agarose gel (FMC BioProducts, Rockland, ME), and extracted by using QIAquick gel extraction kit (QIAGEN, Tokyo, Japan). All DNA cycle sequencing reactions were done using the ABIPRISM BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA). The sequencing reactions were performed by adding: 500 ng PCR products, 3.2 pmol primer, 8 μl BigDye terminator ready reaction premix containing AmpliTaq DNA polymerase, and diethylpyrocarbonate-treated water; the total reaction volume was 20 μl. All cycle sequencing was performed on GeneAmp 9700 (Perkin-Elmer) and 25 cycles of polymerase chain reaction (PCR) were performed, each consisting of denaturation at 96 °C for 10 s, annealing at 50 °C for 5 s, and extension at 60 °C for 4 min. The excess dye terminators were removed by using Centri-Sep spin columns (Applied Biosystems). The purified products were dried and resuspended in template suppression reagent (Applied Biosystems). After the last denaturation at 94 °C for 2 min, the products were run for 1

h on ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

3. Results

In the series of experiments, we chose kidney and lung as positive controls (Nagae et al., 2000; Ono et al., 2000; Totsune et al., 2000). Fig. 1 shows the PCR products amplified by the primers for CRLR, RAMP1, RAMP2 and RAMP3 from kidney, lung, peritoneal exudate cells, and purified mast cell cDNA templates. In control tissues, the primers for CRLR and RAMP1, RAMP2 and RAMP3 gave single bands of 504, 230, 175 and 265 bp, respectively. Sequence analysis of these PCR products revealed that they were identical to each cDNA from the libraries. The primers for CRLR amplified its production in peritoneal exudate cells and purified mast cells. The primers for RAMP2 and RAMP3 yielded amplification products of 175 and 265 bp, respectively, in peritoneal exudate cells and purified mast cells. Staining intensity in purified mast cells with the primers for RAMP2 appeared to be much lower, although we applied three times more cDNA templates from purified mast cells, compared to peritoneal exudate cells. No PCR product with the primers for RAMP1 was found from cDNA from both peritoneal exudate cells and purified mast cells.

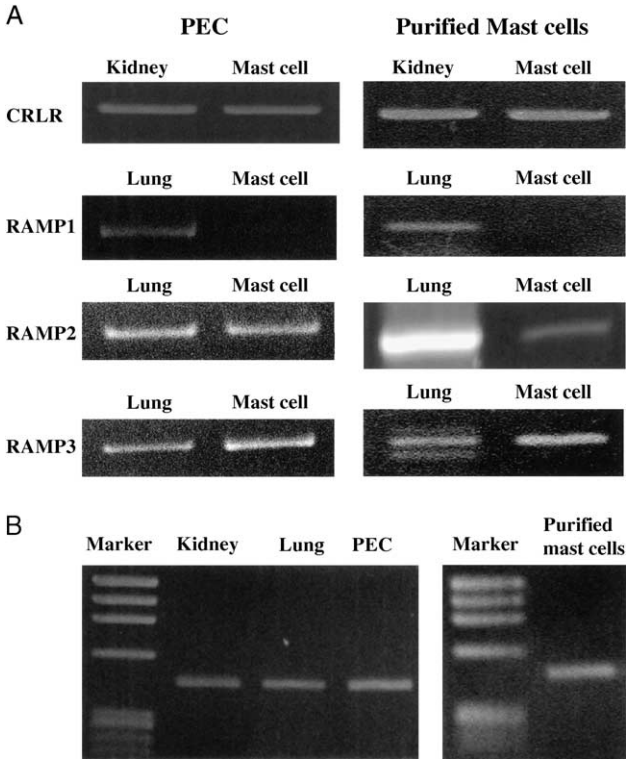

Fig. 1. RT–PCR analysis of CRLR, the RAMPs (A), and β-actin (B) mRNA expression in rat kidney, lung, peritoneal exudate cells, and purified mast cells. The sizes of PCR products were 504 bp for CRLR, 230 bp for RAMP1, 175 bp for RAMP2, and 265 bp for RAMP3, respectively.

4. Discussion

Adrenomedullin, but not CGRP, stimulates the release of histamine from rat peritoneal mast cells (Yoshida et al., 2001). In the present study, we examined the expression of mRNA for CRLR, RAMP1, RAMP2 and RAMP3, components of putative CGRP and adrenomedullin receptors (McLatchie et al., 1998), in peritoneal exudate cells and purified mast cells by RT–PCR. Our data indicate that PCR products of mRNA for CRLR, RAMP2 and RAMP3 were found in peritoneal exudate cells, as well as in purified mast cells. No PCR products of RAMP1 mRNA were detected in either peritoneal exudate cells or purified mast cells, although the primers for RAMP1 mRNA showed its band in rat lung. Taken together, these data suggest that adrenomedullin might induce histamine release via a proposed adrenomedullin receptor (CRLR/RAMP2 or 3). Moreover, the lack of CGRP (CRLR/RAMP1) receptor could explain the lack of effect of CGRP on histamine release from peritoneal mast cells.

The PCR signal of RAMP3 mRNA in purified mast cells was higher than that of RAMP2 mRNA. Thus, it is likely that RAMP3, but not RAMP2, forms a complex with CRLR in rat peritoneal mast cells, subsequently acting as an adrenomedullin receptor, although another study using rat tissues has demonstrated relatively low expression of RAMP3 mRNA compared to that of RAMP2 mRNA (Totsune et al., 2001).

The results of RT–PCR presented here show for the first time the expression of mRNA for CRLR and the RAMPs in rat peritoneal mast cells. The adrenomedullin receptor on rat peritoneal mast cells is most likely functional, since the histamine-releasing effect of adrenomedullin was inhibited by an adrenomedullin receptor antagonist, adrenomedullin (22–52) (Yoshida et al., 2001). The other receptors for CGRP and adrenomedullin have been identified: RDC-1 and L1 orphan receptor, respectively (Hinson et al., 2000), although their identity as functional adrenomedullin/CGRP receptors has been questioned (Chakravarty et al., 2000; Ladoux et al., 2000). Thus, it would still be of interest to further elucidate which receptor(s) is (are) crucial to produce the effect of adrenomedullin in rat peritoneal mast cells.

It is still unknown whether the existence of adrenomedullin receptors on peritoneal mast cells is definitive proof that adrenomedullin exerts its effects via its receptor. For example, substance P receptors are found on rat peritoneal mast cells (Okada et al., 1999), and substance P-induced histamine release is inhibited by tachykinin NK_1 receptor antagonists (Okada et al., 1999). However, histamine release induced by substance P is also inhibited by Gi protein inhibitor (Mousli et al., 1989) and high Ca^{2+} (Mori et al., 1994; Mousli et al., 1989). These data suggest the involvement of Gi protein-dependent mechanisms, but not specific receptor-mediated ones, similar to those for basic secretagogues (Ferry et al., 2002). Indeed, adrenomedullin-induced histamine release is sensitive to Gi protein inhibitor and

extracellular Ca^{2+}. Thus, further studies should be pursued to further elucidate this issue.

In summary, the present study has revealed that mRNA for CRLR, RAMP2 and RAMP3, but not RAMP1, is present in both peritoneal exudate cells and purified mast cells, as detected by RT–PCR. These results suggest that ADM might stimulate the release of histamine via its putative receptor (CRLR/RAMP2 or 3), although this may not be the case for CGRP because of the lack of expression of its receptor (CRLR/RAMP1). Thus, a putative ADM receptor antagonist could become useful as a drug to treat allergic disease such as asthma.

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