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Receptor activity modifying proteins regulate the activity of a calcitonin gene-related peptide receptor in rabbit aortic endothelial cells

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Abstract In *Xenopus* oocytes with an endogenous calcitonin gene-related peptide (CGRP) receptor, a receptor activity modifying protein (RAMP1) enhancing CGRP stimulated chloride currents of the cystic fibrosis transmembrane regulator was recently cloned [McLatchie, L.M. et al. (1998) Nature 393, 333–339]. Here, transient expression of RAMP1 in rabbit aortic endothelial cells (RAEC) brought about stimulation of cAMP accumulation by human (h) αCGRP with an EC_{50} of 0.41 nM. This was antagonized by a CGRP receptor antagonist αCGRP(8–37). Co-expression of RAMP3 together with RAMP1 reduced the maximal cAMP response to hαCGRP by 47% ($P < 0.05$). The cells also express RAMP2 encoding mRNA and an adrenomedullin (ADM) receptor coupled to stimulation of cAMP formation by hADM (EC_{50} 0.18 nM). The latter was antagonized by an ADM receptor antagonist hADM(22–52). In conclusion, expression of a CGRP receptor in RAEC requires RAMP1. The same receptor presumably recognizes ADM making use of endogenous RAMP2. The results reveal competition between the different RAMPs in the regulation of CGRP/ADM receptor activity.

Key words: Neuropeptide; Signal transduction; Receptor expression; Vascular cell

1. Introduction

Calcitonin gene-related peptide (CGRP) and adrenomedullin (ADM) are potent vasodilators [1]. Both peptides interact with receptors on vascular endothelial and smooth muscle cells [2,3]. In certain vascular beds the actions of ADM are inhibited by a CGRP receptor antagonist CGRP(8–37) and they are thought to be mediated by a CGRP receptor. However, CGRP(8–37) insensitive actions of ADM have also been recognized [1]. Therefore, vasodilatory actions of ADM and CGRP are presumably mediated by ADM and ADM/CGRP receptors.

Recently, a human orphan calcitonin receptor-like receptor (CRLR) [4] was found to be directed to the cell surface to become a functional CGRP receptor if co-expressed with a receptor activity modifying protein-1 (RAMP1) [5]. The same receptor has been identified as a CGRP receptor when stably transfected in a human embryonal kidney cell line [6]. Two other structurally related RAMPs, RAMP2 and -3, have also been cloned. When the CRLR was co-expressed with RAMP2 in HEK293T cells the receptor displayed the pharmacological profile of an ADM receptor [5]. Functional properties of RAMP3 have not so far been reported.

In the present study, cDNAs encoding RAMP1, -2 and -3 alone or in combination were transfected in a rabbit aortic endothelial cell (RAEC) line [7]. Stimulation of cAMP formation by CGRP in RAMP1 transfected cells is consistent with the activation of an endogenous rabbit CRLR homologue yielding a CGRP receptor. The RAEC also express endogenous RAMP2 and an ADM receptor which is not affected by transfection of RAMP1, -2 and -3 encoding cDNA. However, co-expression of RAMP3 reduced RAMP1 dependent CGRP receptor activity.

2. Materials and methods

2.1. Materials

Human (h) αCGRP(1–37) and -(8–37) were purchased from Bachem (Bubendorf, Switzerland), and hADM(1–52) and -(22–52) from Peptide Institute (Osaka, Japan). Geneticin, Lipofectamine and Optimem 1 medium were from Life Technologies (Gaithersburg, MD, USA).

2.2. Northern blot analysis

Total RNA was isolated from RAEC with TRI reagent (Molecular Research Center, Cincinnati, OH, USA). The RNA was size fractionated on a formaldehyde/agarose (1% w/v) gel, electroblotted to Zeta-Probe membranes (Bio-Rad, Glattbrugg, Switzerland) and UV-crosslinked with a Stratalinker 2400 (Stratagene, Basel, Switzerland). Size markers in parallel lanes were stained after UV-crosslinking with methylene blue stain (Molecular Research Center, Cincinnati, OH, USA), marked with a waterproof pen and superimposed with the autoradiograms for size estimation of hybridizing RNA. RAMP1, -2 and -3 specific hybridization probes, encoding N-terminal extracellular domains of corresponding human proteins, were amplified by PCR from cloned cDNA and labeled with [^{32}P]dATP (3000 Ci/mmol, Amersham Life Science, Little Chalfont, UK) using the Prime-It II random primer labeling kit from Stratagene. Filters were prehybridized for 4 h and hybridized overnight at 55°C in 5×SSPE according to a Clontech (Palo Alto, CA, USA) protocol. Subsequently, filters were washed four times in 2×SSC, 1% SDS and two times in 0.1% SSC, 0.5% SDS at 55°C. A 1.8 kb human β-actin cDNA fragment (Clontech, Palo Alto, CA, USA) was labeled with the same kit and used for control hybridizations under the conditions recommended by Clontech. Filters were exposed to X-ray film at −80°C with intensifying screens.

2.3. Cell culture, transfections and cAMP measurements

RAEC [7] were grown at 37°C in Coon's modified F12 medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal calf serum and 250 μg/ml Geneticin in a humidified atmosphere containing 95% air and 5% CO_2. For the experiments the cells were seeded into 24-well plates, and at 50% confluence transfected with mammalian expression vector pcDNA3 (mock transfection) (Invitrogen, Carlsbad,

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Abbreviations: ADM, adrenomedullin; CGRP, calcitonin gene-related peptide; CRLR, calcitonin receptor-like receptor; RAEC, rabbit aortic endothelial cells; RAMP, receptor activity modifying protein

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CA, USA), or RAMP1, -2 or -3 expression constructs in pcDNA3 [5] or combinations thereof. The cells were incubated for 4–6 h in 500 μl Optimem 1 medium containing 400 ng/well plasmid DNA and 1.2 μl/well Lipofectamine.

After 48 h in the culture medium the cells were incubated for 15 min at 37°C with peptides in the presence of isobutylmethylxanthine and accumulated cAMP was measured in cell extracts as described [8].

2.4. Statistics

Results are mean values of pooled data of at least four experiments performed in duplicate. Standard errors ranging from 5 to 20% are not shown. Data in the text are means ± S.E.M. The values for half maximal effective concentrations (EC_{50}) were calculated by non-linear regression analysis using FigP 6.0 (Biosoft, Cambridge, UK). Differences between means were analyzed by ANOVA.

3. Results and discussion

3.1. Effects of RAMP1, -2, and -3 expression on CGRP and ADM stimulated cAMP production in endothelial cells

In RAEC transiently transfected with cDNA encoding RAMP1 hαCGRP stimulated cAMP production 6.3 ± 0.6-fold with an EC_{50} of 0.41 ± 0.08 nM ($n = 12$) (Fig. 1). Non- (not shown) or mock-transfected RAEC did not respond to hαCGRP and transfection of RAMP2 and -3 encoding cDNA did not evoke a CGRP response. ADM, on the other hand, increased cAMP accumulation 5.2 ± 0.4-fold with an EC_{50} of 0.18 ± 0.01 nM ($n = 4$) in non- (not shown) and mock-transfected cells, much like in bovine aortic and human umbilical vein endothelial cells [9,10]. In cells transfected with RAMP1, -2 and -3 the cAMP responses of up to 10 nM hADM were those of mock-transfected cells. In RAMP1 unlike RAMP2 and -3 transfected cells 100 nM ADM further increased the cAMP response by 53 ± 16% ($n = 12$; $P < 0.001$), presumably through crossreaction of ADM with the RAMP1 dependent CGRP receptor. Northern blot analysis of RNA isolated from non-transfected RAEC identified mRNA of approximately 1 kb hybridizing to a human RAMP2 encoding cDNA, but RAMP1 or -3 specific probes revealed no signal (Fig. 2).

Taken together these observations are consistent with the existence in RAEC of a rabbit CRLR homologue expressed as a CGRP receptor following transfection with RAMP1 encoding cDNA and an ADM receptor dependent on endogenous RAMP2. In HEK293 and COS-7 cells transfected with CRLR and RAMP1 a CGRP receptor was recognized, whereas co-transfection of the same CRLR with RAMP2 revealed an

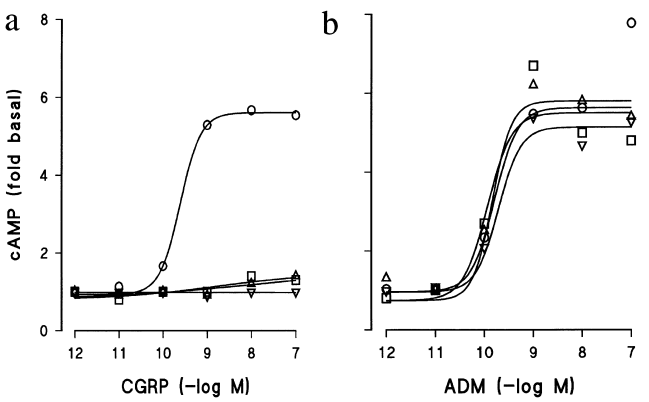

Fig. 1. cAMP accumulation in RAEC transfected with pcDNA3 and RAMP1, -2 and -3. The cells were transiently transfected with 400 ng pcDNA3 alone (▽) and 200 ng RAMP1 (○), -2 (□) and -3 (△) expression constructs together with 200 ng pcDNA3. 48 h later the cells were incubated with hαCGRP (a) and hADM (b) for 15 min. The results are mean values of pooled data of 4–14 experiments performed in duplicate.

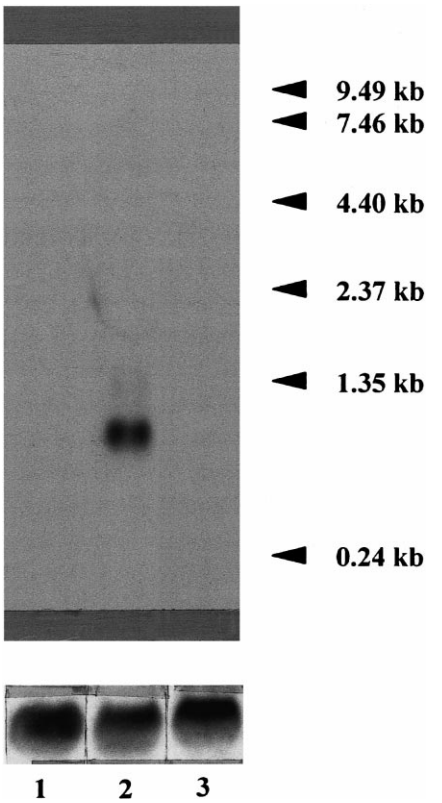

Fig. 2. Northern blot analysis of total RNA isolated from RAEC. 20 μg RNA was electrophoresed in individual lanes and positions of size markers in an adjacent lane are indicated. Individual filters were hybridized with RAMP1 (lane 1), RAMP2 (lane 2) and RAMP3 (lane 3) specific probes and exposed to X-ray films for 6 days (top panel). Subsequently, the filters were washed and rehybridized with a 1.8 kb human β-actin cDNA and exposed to X-ray films for 40 h (bottom panel).

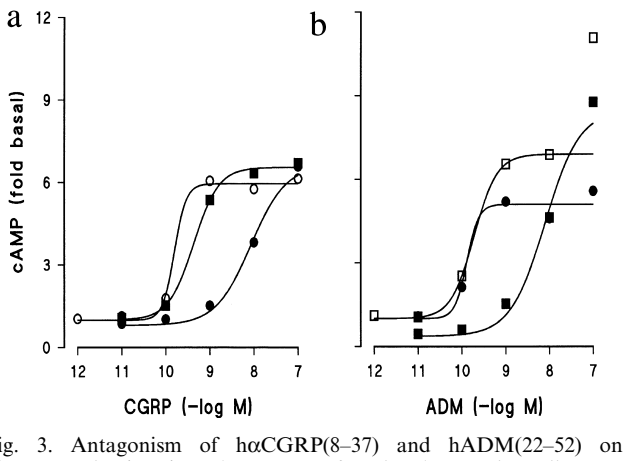

Fig. 3. Antagonism of hαCGRP(8–37) and hADM(22–52) on cAMP production in RAMP1 transfected RAEC. The cells were transiently transfected with 200 ng RAMP1 expression construct together with 200 ng pcDNA3 and incubated 48 h later for 15 min with hαCGRP (a) and hADM (b) in the absence (○, □) and presence of 100 nM hαCGRP(8–37) (●) and hADM(22–52) (■). The results are mean values of pooled data of five (a) and four (b) experiments performed in duplicate.

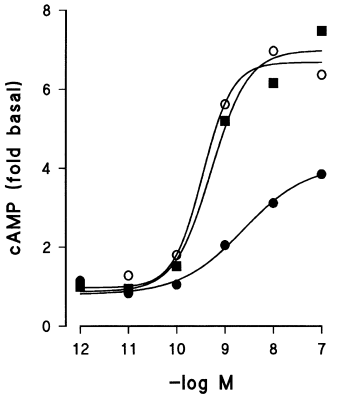

Fig. 4. Effects of co-expression of RAMP1 with RAMP2 or -3 on CGRP stimulated cAMP in RAEC. The cells were transiently co-transfected with 200 ng RAMP1 together with 200 ng pcDNA3 (○), 200 ng RAMP2 (■) and 200 ng RAMP3 (●) expression constructs, and incubated 48 h later with hαCGRP for 15 min. The results are mean values of pooled data of 5–6 experiments performed in duplicate.

ADM receptor ([5]; in preparation). However, expression in RAEC of an ADM receptor different from a RAMP2 dependent rabbit CRLR homologue cannot be excluded.

3.2. *Effects of receptor specific antagonists, and RAMP1 and RAMP3 co-expression on CGRP and ADM stimulated cAMP in endothelial cells*

In RAMP1 transfected RAEC, 100 nM hαCGRP(8–37) inhibited the stimulation of cAMP by hαCGRP with a 50 ± 8-fold ($P < 0.01$) increase in the EC_{50} and a K_i of 3.2 ± 1.3 nM ($n = 5$), and lowered the additional stimulation of cAMP accumulation of 100 nM ADM by $51 \pm 6\%$ ($n = 4$; $P < 0.005$) (Fig. 3). hADM(22–52) did not affect hαCGRP stimulated cAMP accumulation. Stimulation of cAMP production by hADM, on the other hand, was antagonized by 100 nM hADM(22–52), but not by hαCGRP(8–37), as shown by a 55 ± 16-fold ($P < 0.05$) increase in the EC_{50} corresponding to a K_i of 2.6 ± 0.9 nM ($n = 4$). Agonist specificity of the RAMP1 dependent CGRP receptor in RAEC is similar to that in SK-N-MC cells which are also responsive to ADM [8]. There hαCGRP(8–37) antagonized CGRP and ADM stimulated cAMP production with similar K_i, but ADM(22–52) failed to inhibit CGRP binding. The RAEC ADM receptor exhibits the ligand specificity of that in neuroblastoma×glioma NG108-15 hybrid cells [11]. There ADM(22–52), but not CGRP(8–37), antagonized ADM stimulated cAMP production. Our results reveal the co-existence of an ADM receptor, presumably dependent on endogenous RAMP2, and a RAMP1 dependent CGRP receptor in RAEC.

In RAEC co-transfected with RAMP1 and -3 but not with RAMP2 the cAMP response to 100 nM hαCGRP was reduced by $47 \pm 12\%$ ($n = 5$; $P < 0.05$) as compared to RAEC transfected with RAMP1 alone (Fig. 4). Therefore, for the first time competition between RAMP1 and -3 has been demonstrated. The underlying mechanism(s) remain to be investigated.

In conclusion, RAEC present endogenous RAMP2 and an ADM receptor linked to cAMP production antagonized by ADM(22–52) but not by CGRP(8–37). Transfection of RAMP1 encoding cDNA revealed the presence of a CGRP receptor, and CGRP(8–37), but not ADM(22–52) antagonized hαCGRP stimulated cAMP production. Competition between human RAMP3 and RAMP1 inhibits CGRP evoked effects. Distinct responses of CGRP and ADM in different vascular beds may be explained by the differential expression of G protein coupled CGRP and ADM receptors modulated by RAMP1, -2 or -3 proteins.

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