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Three receptor-activity-modifying proteins define calcitonin gene-related peptide or adrenomedullin selectivity of the mouse calcitonin-like receptor in COS-7 cells

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Abstract

Receptors for calcitonin gene-related peptide (CGRP) and adrenomedullin (AM) are heterodimeric complexes of the calcitonin-like receptor (CLR) together with associated receptor-activity-modifying proteins (RAMP)1, -2 or -3. The RAMP define the specificity of the CLR for CGRP or AM. Here, mouse (m)CLR/mRAMP1, -2 and -3 were expressed in COS-7 cells that lack detectable CGRP and AM receptors. myc epitope-tagged non-glycosylated mRAMP1 required V5-tagged mCLR for its translocation to the cell surface. The glycosylated myc-mRAMP2 and -3, on the other hand, were expressed at the cell surface in the absence of co-transfected mCLR. Selective binding of [^{125}I]hαCGRP to mCLR/mRAMP1 expressing cells was inhibited by rat (r)αCGRP(1–37) and the CGRP antagonist rαCGRP(8–37) with IC_{50} of 7.0 ± 1.6 nM and 1.0 ± 0.1 nM (mean ± SEM). rAM(1–50) and the AM antagonist rAM(20–50) inhibited [^{125}I]hαCGRP binding at over 36-fold higher concentrations than rαCGRP. In mCLR/mRAMP2 expressing cells, selective [^{125}I]rAM binding was inhibited by rAM(1–50) and -(20–50) with IC_{50} of 8.9 ± 2.6 nM and 34 ± 9 nM. rαCGRP(1–37) and -(8–37) displaced the binding at over 25-fold higher concentrations. mCLR/mRAMP3 expressing cells recognized both [^{125}I]hαCGRP and -rAM. The IC_{50} of rAM and rαCGRP(8–37) ranged between 5.8 and 7.0 nM, and those of rαCGRP and rAM(20–50) were only 4- to 8-fold higher. rαCGRP and rAM stimulated and rαCGRP(8–37) and rAM(20–50) antagonized mCLR/mRAMP1, -2 and -3 mediated cAMP formation with relative potencies that reflected the observed CGRP and AM selectivity of the three receptor types. In conclusion, mCLR/mRAMP1 and -2 are CGRP- and AM-selective receptors, respectively, whereas mCLR/mRAMP3 is an AM/CGRP receptor.

Keywords: Adrenomedullin; Calcitonin gene-related peptide; Calcitonin-like receptor; Receptor-activity-modifying protein; Antagonist

1. Introduction

CGRP and AM are potent vasodilators and as a consequence hypotensive peptides [1,2]. They exhibit limited structural similarities which include six amino acid ring structures, formed by a disulfide bridge between cysteine residues, followed by an alpha helix and amidated C-termini. As a result, the peptides crossreact to a different degree with the respective receptors in tissues and cell lines [3]. The corresponding CLR requires the interaction with one of three RAMP1, -2 and -3 for the recognition of CGRP and/or AM [4].

The amino acid sequences of the RAMP of man, mouse, rat, pig, and guinea pig have been revealed through molecular cloning [4–6]. The RAMP are single transmembrane domain proteins with an extracellular N-terminus of about 100 amino acids and an intracellular C-terminal tail of 10 amino acids. RAMP1, -2 and -3 exhibit approximately 30% amino acid sequence identity.

The CLR of man, rat, pig and cow have been characterized through co-expression with RAMP1, -2 or -3 [4,5,7,8]. Co-expression of cloned CLR with RAMP in cell lines of different species revealed variable CGRP and AM selectivity of expressed receptors. The variability can be explained in part by the presence of endogenous CLR and/or

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Abbreviations: AM, adrenomedullin; CGRP, calcitonin gene-related peptide; CLR, calcitonin-like receptor; RAMP, receptor-activity-modifying protein.

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RAMP in most of the cells examined (for review see [9]). Besides, a non-peptidic CGRP antagonist was active with human unlike rodent RAMP1/CLR [10]. This selectivity was determined by a tryptophan residue present in human but not in rodent RAMP1.

Human (h)αCGRP(8–37), unlike hAM(22–52) or rat (r)AM(20–50), is an antagonist at a CGRP receptor of human SK-N-MC neuroblastoma cells [11]. A particular AM receptor subtype expressed in rabbit aortic endothelial cells and in neuroblastoma × glioma hybrid NG108-15 cells is antagonized by hAM(22–52) but not by hαCGRP(8–37) [12,13]. Another AM receptor subtype found in rat astrocytes is more potently antagonized by hαCGRP(8–37) than hAM(22–52) [13]. Several CGRP and AM receptors expressed from cDNA encoding CLR and the corresponding RAMP transfected into various cells remain to be examined as far as the interaction with the antagonists CGRP(8–37) and hAM(22–52) or rAM(20–50) are concerned.

In the present study, the mCLR co-expressed with mRAMP1, -2 or -3 was examined in COS-7 cells which do not express detectable endogenous CLR and RAMP. The mCLR/mRAMP1 was a CGRP receptor antagonized by rαCGRP(8–37). The mCLR/mRAMP2, on the other hand, was an AM receptor antagonized by rAM(20–50). A mixed-type AM/CGRP receptor more potently antagonized by rαCGRP(8–37) than by rAM(20–50) was revealed with the mCLR co-expressed with mRAMP3. Translocation to the cell surface of an mCLR with an N-terminal V5 epitope-tag did not require RAMP. myc-mRAMP2 and -3, unlike myc-mRAMP1, were recognized at the cell surface in the absence of co-expressed mCLR.

2. Materials and methods

2.1. cDNA constructs for expression of epitope-tagged mRAMP and mCLR

The constructs encoding mRAMP1, -2 and -3 with an N-terminal myc-epitope (myc-mRAMP1, -2 and -3) were obtained as follows. DNA fragments encoding mRAMP1, -2 and -3 without the corresponding signal sequences were obtained by PCR from previously cloned cDNA [5]. The nucleotide sequences of the primer pairs were for mRAMP1 (5′-ATATCTCGAGTGCCGGGACCCTGAC-TATGGGACTC-3′ and 5′-ATATCTCGAGGAATTCCCA-GGTTTAAGAATACTCTTTTAA-3′), for mRAMP2 (5′-AAAACTCGAGTCTCCGGAGTCCCTGAACCAATC-3′ and 5′-TTTTCTCGAGGAATTCGTACAAAAACATAA-CTTTATTTTA-3′), and for mRAMP3 (5′-GGCTCGAG-TGCAACGAGACAGGGATGCTGG-3′ and 5′-GGT-CTAGATCCCAGCCTGCCAGGCATGG-3′). The PCR products were cloned into the pGEM®-T Easy vector (Promega). A DNA fragment encoding the human CD33 signal sequence (CD33) followed by the myc-epitope EQKLISEEDLL was amplified from a CD33-myc-hRAMP1-pcDNA3 expression construct provided by S. Foord (GlaxoSmithKline). The 5′-primer (5′-TTTTGGATCCGCCAC-CATGCCGCTGCTGCTACTGC-3′) and the 3′-primer (5′-ATATCTCGAGTCCATGCAACAAGTCCTCTTCAG-3′) contained 5′ *Bam*HI and 3′ *Xho*I restriction sites. Digestion of the PCR products with *Bam*HI and *Xho*I restriction enzymes and subcloning into *Bam*HI/*Xho*I-digested pcDNA3 expression vector (Invitrogen) revealed CD33-myc-pcDNA3. The DNA fragments encoding mRAMP1 and -2 without the signal sequence were excised with *Xho*I restriction enzyme from the pGEM-T® Easy vector constructs and cloned downstream and in frame with CD33-myc in *Xho*I-digested CD33-myc-pcDNA3. A DNA fragment encoding mRAMP3 without the signal sequence was removed from the corresponding pGEM-T® Easy vector construct by *Xho*I/*Xba*I restriction digestion and cloned downstream and in frame with CD33-myc in *Xho*I/*Xba*I-digested CD33-myc-pcDNA3.

The expression construct encoding the mCLR with the human CD33 signal sequence and the V5 epitope GKPIPWPLLGLDST (V5-mCLR) was obtained as follows. The CD33-myc-mRAMP1 insert was excised with *Bam*HI and *Eco*RI from the pcDNA3 vector and cloned into *Bam*HI/*Eco*RI digested pBluescript SK(+) (Stratagene). The myc-mRAMP1 coding fragment was excised with *Nco*I and *Cla*I restriction enzymes, resulting in linearized pBluescript SK(+)-CD33. A fragment encoding the V5-epitope was amplified by PCR using the following primer pair: 5′-TTTTCCATGGAAGAAGGTAAGCCTATCCCTAACCC-TCTCCTCGG-3′ and 5′-TTTTCCATGGATCGATACGC-GTAGAATCGAGACCGAGGAGAGGGTTAGGG-3′. The PCR product was digested with *Nco*I and *Cla*I and cloned into pBluescript SK(+)-CD33 to reveal SK(+)-CD33-V5.

A DNA fragment encoding the 80 N-terminal amino acids of the mCLR without the signal sequence between a *Cla*I restriction site, provided by the 5′-primer 5′-AA-AAATCGATGAAGAAGGCGTGAACCAAACAGAC-3′, and the *Bgl*II site in the mCLR sequence and contained in the 3′-primer 5′-AAAAAGATCTTTGTAACCTTCTCT-GAAGGATC-3′, was amplified by PCR from a mCLR-pcDNA3.1 construct, provided by Dr. J. Daut (Marburg University, Germany) and cloned into the pGEM-T® Easy vector. The mCLR-fragment was excised by *Cla*I and *Bgl*II digestion. The CD33-V5 fragment was excised from the pBluescript SK(+)-CD33-V5 with *Bam*HI and *Cla*I. Both fragments were cloned together into the *Bam*HI/*Bgl*II digested mCLR-pcDNA3.1. The nucleotide sequences of the PCR-amplified DNA fragments used in the described myc-mRAMP1, -2 and -3 and V5-mCLR constructs were confirmed by sequencing in both directions.

2.2. Cell culture and transfection

COS-7 cells were grown at 37° in a humidified atmosphere of 95% air and 5% CO_2 in HamF12/DMEM (4.5 g/L

glucose) medium (1:1) supplemented with 2 mM glutamine and 10% FCS. For DNA transfections, the cells were seeded in 24-well plates at a density of 15,000 cells/cm^2. The cells were transfected 48 hr later with the indicated expression constructs and linear polyethylenimine (PEI; 25 kDa) (Polysciences) [14]. Briefly, 0.4 μg per well plasmid DNA was diluted with 12.5 μL per well cell culture medium. One microgram per well PEI in 12.5 μL per well cell culture medium was prepared separately. The solutions were then combined, mixed by vortexing and incubated for 15 min at room temperature. Subsequently, the mixtures were adjusted to 250 μL per well with cell culture medium and added to the cells for 4–6 hr. After transfection, cell culture medium was added up to a final volume of 500 μL per well. The experiments were carried out 48 hr after transfection.

2.3. Radioligand binding and cAMP formation

[^{125}I]hαCGRP(1–37) ([^{125}I]hαCGRP) and [^{125}I]rAM(1–50) ([^{125}I]rAM) were prepared by a modified chloramine-T method and subsequent purification by high performance liquid chromatography [15,16]. In radioligand binding experiments transiently transfected COS-7 cells were incubated for 2 hr at 15° with 1700 Bq per well of [^{125}I]hαCGRP or [^{125}I]rAM in 200 μL HamF12/DMEM (1:1) supplemented with 0.1% BSA and 2 mM glutamine (binding medium) in the absence and presence of competing peptides. The cells were then washed with 500 μL binding medium and lysed with 500 μL 0.5% SDS and the lysates were counted in a γ-counter (Kontron). [^{125}I]hαCGRP and -rAM binding in the presence of 1 μM hαCGRP and rAM was considered as non-specific. Cyclic AMP stimulation was performed in binding medium supplemented with 1 mM isobutylmethylxanthine for 15 min at 37° as described [15].

2.4. Immunohistochemistry

COS-7 cells were seeded on cover slides in 24-well plates and transfected with the indicated combinations of myc-mRAMP1, -2 and -3 and V5-mCLR expression constructs as described under Section 2.1. Cell surface and total expression of the proteins was estimated 48 hr after transfection by V5- and myc-immunofluorescent staining of intact and saponin-permeabilized cells, respectively. Briefly, the cells were fixed with 4% formalin in PBS for 20 min at room temperature, washed with PBS and incubated with immunostaining medium (HamF12/DMEM (1:1), 0.1% BSA) in the absence or presence of 0.1% saponin for 30 min at room temperature. The cells were then incubated for 2 hr at room temperature with monoclonal mouse antibodies to myc or V5 (Invitrogen) diluted 1:300 in 200 μL immunostaining medium in the absence or presence of saponin. The cells were washed three times with the immunostaining medium and incubated with Cy3-labeled sheep anti-mouse antibodies (1:200 final dilution) (Sigma) for 30 min at room temperature. After three additional washes the slides were mounted with Immu-Mount (Shandon Scientific). The cells were viewed with an Eclipse E600 Nikon microscope equipped with a Plan Fluor 20×/0.5 DLL objective, a G-2A filter and a Kappa DX20 CCD camera connected to the microscope with a Nikon 0.45× projection lens. Immunofluorescence of intact and saponin-permeabilized cells was recorded with a Nikon U-III multipoint sensor system and respective reciprocal exposure times were taken as a measure for cell surface and total expression of V5-mCLR and myc-RAMP1, -2 and -3 [17].

2.5. Data analysis

The values for half-maximal inhibitory (IC_{50}) and effective concentrations (EC_{50}) were calculated by non-linear regression analysis using FigP 6.0 (Biosoft). Results are means ± standard error of the mean (SEM). Differences between mean values were analyzed by ANOVA. $P < 0.05$ was considered statistically significant. K_i were calculated from the ratio of the EC_{50} of the respective agonists in the absence and presence of 1 μM antagonists using the equation for competitive inhibition $EC_{50}/EC'_{50} = (1 + 1\,\mu M/K_i)$.

3. Results

3.1. Cell surface expression of V5-mCLR and myc-mRAMP1, -2 and -3

V5-mCLR and myc-mRAMP1, -2 and -3 immunofluorescent staining of non-permeabilized and saponin-permeabilized COS-7 cells indicated cell surface and total expression, respectively. V5-mCLR was expressed at the cell surface in the absence of co-transfected mRAMP1, -2 or -3 (Fig. 1, top panel). Cell surface expression of the V5-mCLR was approximately 50% of total expression in the absence and the presence of the RAMP (not shown). Moreover, the levels of the V5-mCLR at the cell surface in the presence of myc-mRAMP1, -2 and -3 were 114 ± 32%, 142 ± 32% and 95 ± 22%, respectively, of those in the absence of the RAMP. Therefore, RAMP independent cell surface expression of mCLR is apparent.

myc-mRAMP1 was not expressed at the cell surface in the absence of mCLR, but mCLR independent cell surface expression of myc-mRAMP2 and -3 was observed (Fig. 1, top panel). The total expression of myc-mRAMP1 alone was 14% of that in the presence of V5-mCLR (Fig. 1, bottom panel). The levels of expression of myc-mRAMP1 at the cell surface in the absence of V5-mCLR were only 3% of those in its presence. Total and cell surface expression of myc-mRAMP2 and -3, on the other hand, was not affected to any great extent by the presence of the V5-mCLR. Taken together, myc-mRAMP1 unlike myc-mRAMP2 and -3 was minimally expressed in the absence

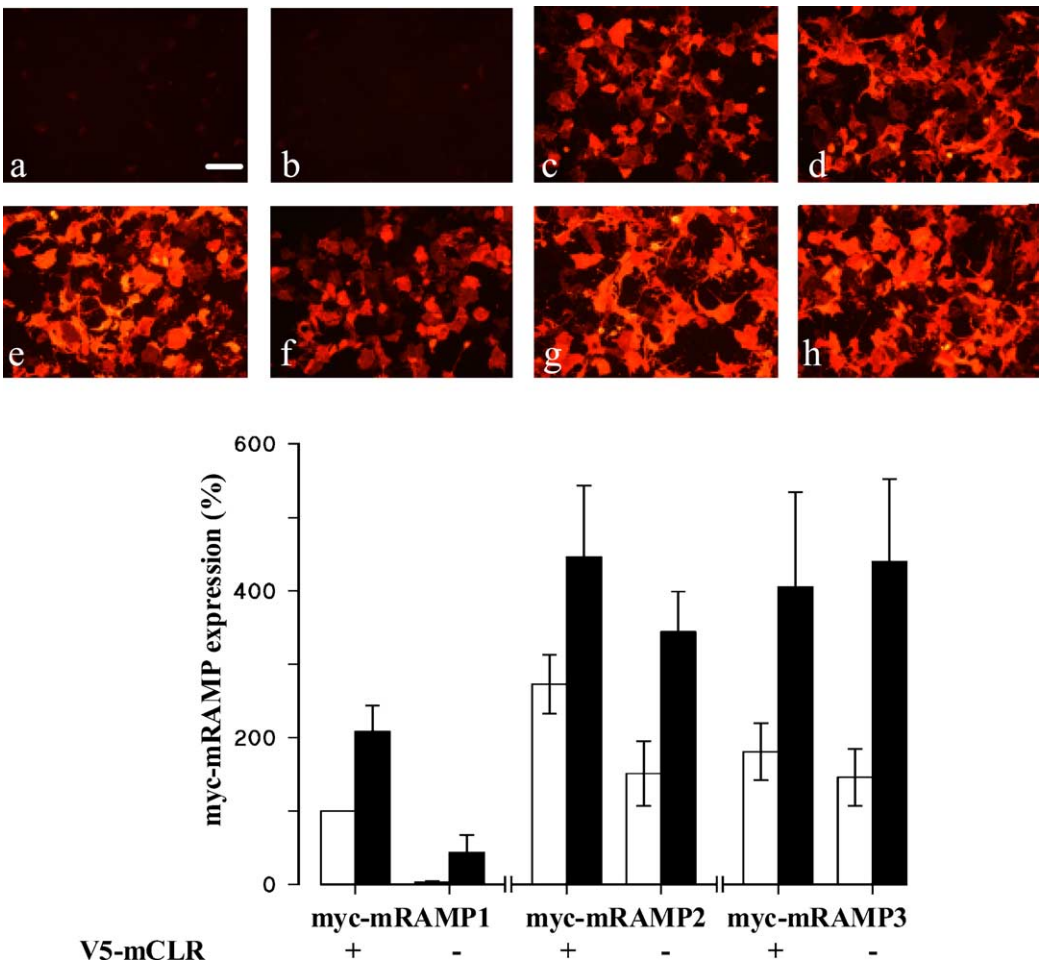

Fig. 1. Cell surface and total expression of V5-mCLR and myc-mRAMP in transiently transfected COS-7 cells. Top: cell surface expression of the V5-mCLR and myc-RAMP1, -2 and -3 was visualized by immunofluorescent staining of intact cells. The cells were transfected with pcDNA3 (a), myc-mRAMP1, -2 or -3 (b–d) or with V5-mCLR (e) alone, and with myc-mRAMP1, -2 or -3 together with V5-mCLR (f–h). Formalin fixed cells were stained with mouse V5 antiserum (a, e) or with mouse myc antiserum (b–d and f–h) combined with Cy3-labeled sheep anti-mouse serum. Images show areas of confluent cells at 20-fold magnification. Scale bar, 100 μm. Bottom: cell surface (open bars) and total expression (closed bars) of the myc-mRAMP were quantified as described under Section 2 by immunofluorescent staining of intact and saponin-permeabilized cells, respectively. myc-mRAMP1 co-expressed with V5-mCLR was set to 100%. Results are means ± SEM of at least four independent experiments.

of co-transfected mCLR and not translocated to the surface in COS-7 cells when expressed alone. With the co-transfection of the mCLR the expression of mRAMP1 was increased and its translocation to the cell surface became apparent.

3.2. [^{125}I]hαCGRP and -rAM binding

In COS-7 cells transfected with the empty pcDNA3 expression vector specific binding of [^{125}I]hαCGRP and [^{125}I]rAM in the absence and presence of 1 μM corresponding non-labeled peptides was below 0.1 fmol/100,000 cells (N = 3). Co-expression of the mCLR with mRAMP1 revealed 1.0 ± 0.3 fmol/100,000 cells specific [^{125}I]hαCGRP binding and below 0.1 fmol/100,000 cells specific [^{125}I]rAM binding (N = 3). With the mCLR together with mRAMP2 specific [^{125}I]rAM binding was 5.3 ± 0.8 fmol/100,000 cells, but specific [^{125}I]hαCGRP binding was below 0.2 fmol/100,000 cells (N = 3). Co-expression of the mCLR with mRAMP3 revealed 1.0 ± 0.3 fmol/100,000 cells specific [^{125}I]hαCGRP and 1.1 ± 0.2 fmol/100,000 cells specific [^{125}I]rAM binding (N = 3).

In mCLR/mRAMP1 expressing COS-7 cells binding of [^{125}I]hαCGRP was displaced by rαCGRP and the CGRP antagonist rαCGRP(8–37) with IC_{50} of 7.0 ± 1.6 nM and 1.0 ± 0.1 nM, and rAM was 36-fold less potent than rαCGRP (Fig. 2, Table 1). [^{125}I]hαCGRP was not displaced by up to 1 μM rAM(20–50). In cells co-expressing the mCLR and mRAMP2 binding of [^{125}I]rAM was inhibited by rAM with an IC_{50} of 8.9 ± 2.6 nM and rAM(20–50), rαCGRP(8–37) and rαCGRP were 4-, 25- and >100-fold less potent. [^{125}I]rAM binding in mCLR/mRAMP3 expressing cells was displaced by rAM and rαCGRP(8–37) with similar IC_{50} of 6.3 ± 0.4 nM and 6.2 ± 1.0 nM. rαCGRP and rAM(20–50) were 4–8 times less potent than rAM and rαCGRP(8–37). A similar profile was obtained with [^{125}I]hαCGRP.

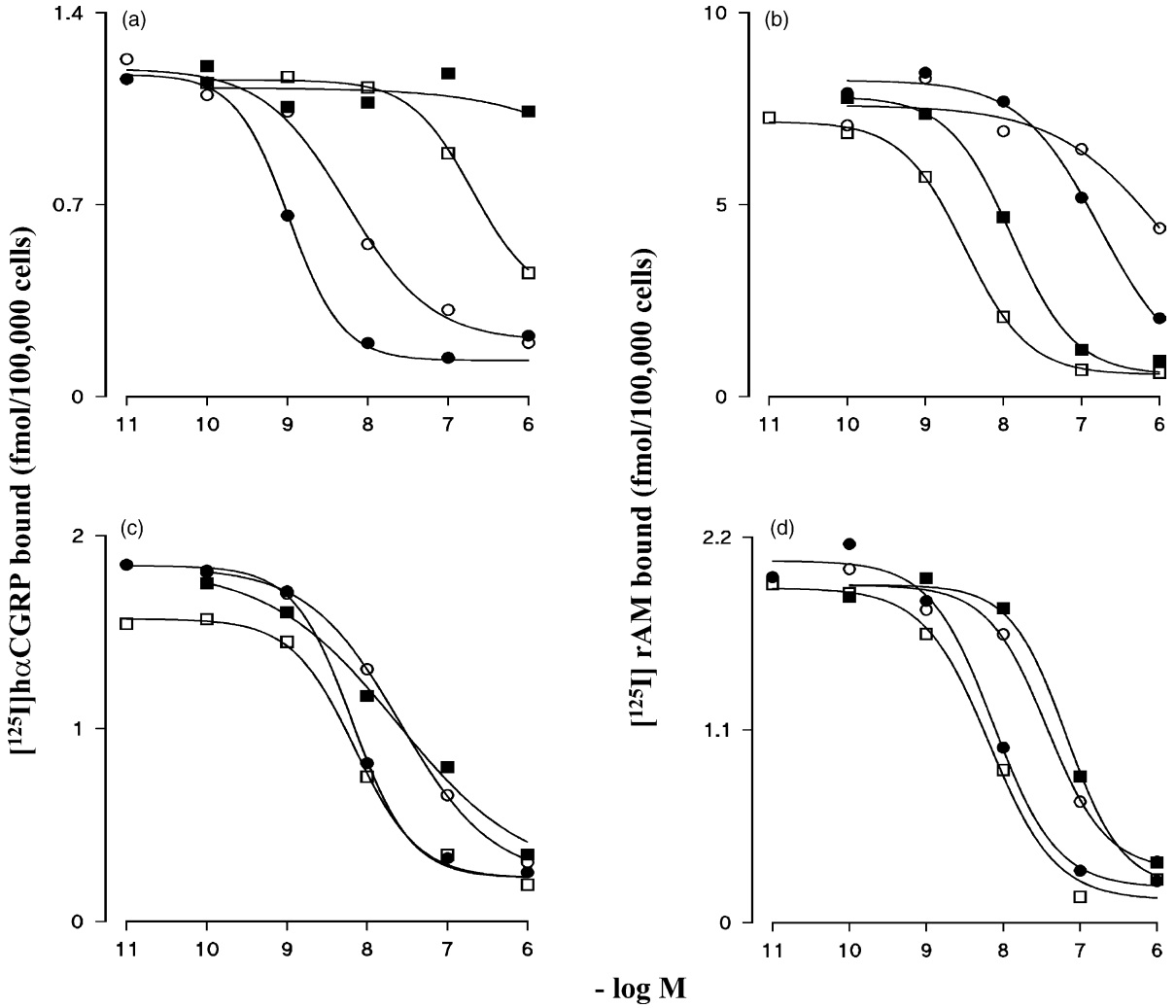

Fig. 2. Inhibition of [^{125}I]hαCGRP and [^{125}I]rAM binding in COS-7 cells. Cells were co-transfected with the mCLR and mRAMP1 (a), mRAMP2 (b) or mRAMP3 (c and d). Forty-eight hours later, the cells were incubated with 60–70 pM (148 GBq/μmol) [^{125}I]hαCGRP or 120–130 pM (74 GBq/μmol) [^{125}I]rAM in the absence and presence of rαCGRP (○), rαCGRP(8–37) (●), rAM (□) and rAM(20–50) (■). Representative experiment carried out at least three times in duplicates that varied by less than 10%.

3.3. cAMP formation

In mock transfected COS-7 cells basal cAMP levels were 0.8 ± 0.1 pmol/100,000 cells. They remained unchanged in the presence of rαCGRP(8–37) and rAM(20–50). In COS-7 cells co-transfected with mCLR and mRAMP1, -2 and -3 basal cAMP levels were 1.0 ± 0.2 ($P > 0.05$), 3.1 ± 0.5 ($P < 0.05$) and 2.6 ± 0.4 ($P < 0.01$) pmol/100,000 cells, respectively. Treatment of mCLR/mRAMP1 expressing cells with 1 μM rαCGRP(8–37) or rAM(20–50) did not affect cAMP formation (Fig. 3). But in mCLR/mRAMP2 expressing cells cAMP levels were lowered to $48 \pm 4\%$

Table 1
[^{125}I]hαCGRP and -rAM binding inhibition (IC_{50}, nM) in COS-7 cells co-expressing the mCLR and indicated mRAMP

	mRAMP1	mRAMP2	mRAMP3	
	[^{125}I]hαCGRP	[^{125}I]rAM	[^{125}I]rAM	[^{125}I]hαCGRP
rαCGRP	7.0 ± 1.6 (4)	>1000 (4)	23 ± 1 (3)[b]	47 ± 10 (5)[b]
rAM	255 ± 38 (3)[a]	8.9 ± 2.6 (5)	6.3 ± 0.4 (4)	5.8 ± 0.7 (3)
rαCGRP(8–37)	1.0 ± 0.1 (3)[a]	219 ± 49 (4)[b]	6.2 ± 1.0 (4)	7.0 ± 2.1 (4)
rAM(20–50)	>1000 (3)	34 ± 9 (4)[b]	51 ± 9 (4)[b]	43 ± 6 (3)[b]

Results are means ± SEM with numbers of experiments in parentheses.
[a] $P < 0.05$ vs. rαCGRP.
[b] $P < 0.05$ vs. rAM.

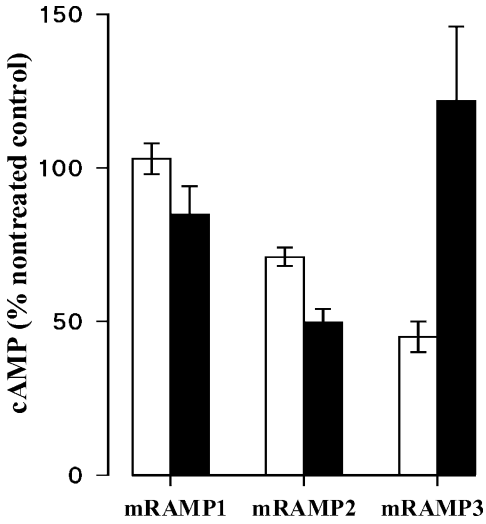

Fig. 3. Inhibition of basal cAMP formation in COS-7 cells. The cells were co-transfected with the mCLR and mRAMP1, -2 or –3, and left untreated (controls) or treated with 1 μM rαCGRP(8–37) (open bars) or rAM(20–50) (closed bars). cAMP in cell extracts of untreated control cells was set to 100%. Results are means ± SEM of three to four experiments.

and 77 ± 4% ($P < 0.005$) in the presence of 1 μM rAM(20–50) or rαCGRP(8–37), respectively. In mCLR/mRAMP3 expressing cells cAMP was decreased to 45 ± 3% ($P < 0.001$) with 1 μM rαCGRP(8–37), but not with rAM(20–50).

In COS-7 cells co-transfected with the mCLR and mRAMP1 cAMP formation was maximally stimulated 23 ± 5- and 30 ± 2-fold ($P > 0.05$) by rαCGRP and rAM with EC_{50} of 0.05 ± 0.01 nM and 5.6 ± 0.6 nM ($P < 0.01$), respectively (Fig. 4, Table 2). The EC_{50} of rαCGRP and rAM were increased 100- and 209-fold in the presence of 1 μM rαCGRP(8–37), but 1 μM rAM(20–50) was ineffective. The calculated K_i of rαCGRP(8–37) were 12 ± 4 nM and 5 ± 1 nM ($P > 0.05$) for rαCGRP and rAM stimulated cAMP accumulation, respectively. In cells co-transfected with mCLR and mRAMP2 rAM and rαCGRP maximally stimulated cAMP accumulation 12 ± 3- and 11 ± 2-fold ($P > 0.05$) with EC_{50} of 0.26 ± 0.06 nM and 73 ± 29 nM ($P < 0.05$), respectively. Here, 1 μM rAM(20–50) raised the EC_{50} of rAM and rαCGRP 11- and >14-fold, and the corresponding K_i

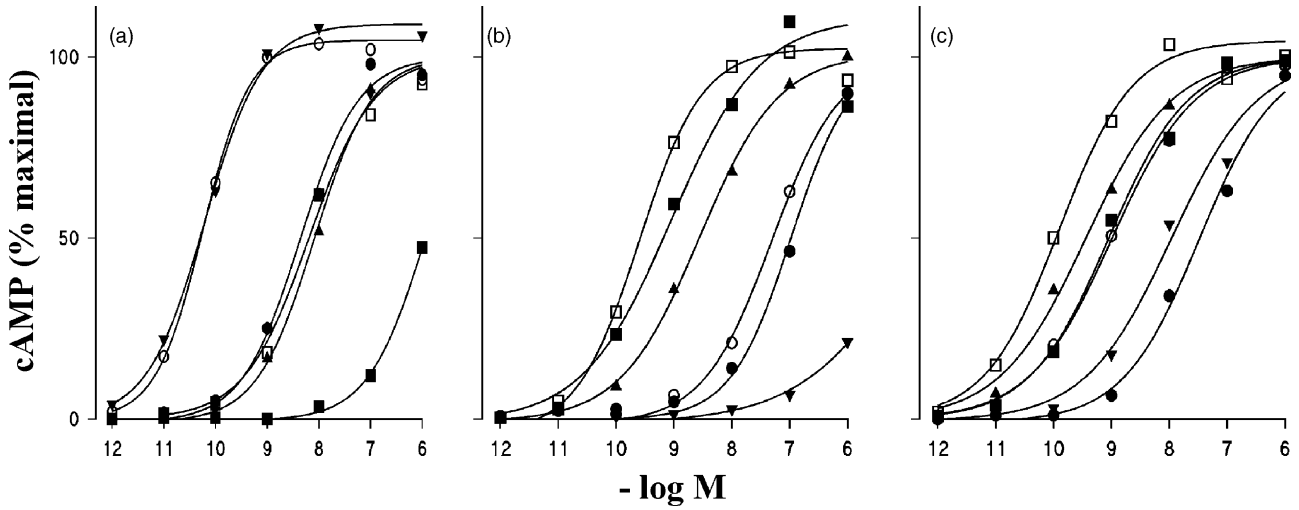

Fig. 4. Stimulation of cAMP formation in COS-7 cells. The cells were co-transfected with the mCLR and mRAMP1 (a), mRAMP2 (b) or mRAMP3 (c), and stimulated with rαCGRP alone (○) or together with 1 μM rαCGRP(8–37) (●) or rAM(20–50) (▼), or with rAM alone (□) or together with 1 μM rαCGRP(8–37) (■) or rAM(20–50) (▲). Means from at least three independent experiments with SEM below 10%.

Table 2
cAMP formation (EC_{50}, nM) by CGRP and AM agonists and together with antagonists in COS-7 cells co-expressing the mCLR with indicated mRAMP

Antagonists	mRAMP1		mRAMP2		mRAMP3	
	rαCGRP	rAM	rαCGRP	rAM	rαCGRP	rAM
–	0.05 ± 0.01 (5)	5.6 ± 0.6 (4)[a]	73 ± 29 (4)[b]	0.26 ± 0.06 (4)	1.56 ± 0.44 (6)[b]	0.10 ± 0.01 (7)
rαCGRP(8–37)	5.0 ± 2.0 (3)[c]	>1000 (3)	107 ± 26 (3)	0.92 ± 0.27 (3)[c]	34 ± 2 (4)[c]	1.03 ± 0.20 (4)[c]
rAM(20–50)	0.05 ± 0.01 (3)	8.3 ± 1.9 (3)	>1000 (3)	2.8 ± 0.6 (3)[c]	16 ± 6 (4)[c]	0.35 ± 0.08 (4)[c]

Results are means ± SEM with number of experiments in parentheses.
[a] $P < 0.05$ vs. rαCGRP.
[b] $P < 0.05$ vs. rAM.
[c] $P < 0.05$ vs. the respective agonist alone.

was 131 ± 65 nM for rAM evoked cAMP stimulation. The EC_{50} of rAM and rαCGRP were increased 2–4-fold by 1 μM rαCGRP(8–37) with a K_i of 380 ± 120 nM for rAM stimulated cAMP formation. In COS-7 cells co-transfected with mCLR and mRAMP3 cAMP formation was maximally stimulated 16 ± 6-fold by rAM and rαCGRP with EC_{50} of 0.10 ± 0.01 nM and 1.56 ± 0.44 nM ($P < 0.01$), respectively. Here, both rAM(20–50) and rαCGRP(8–37) were antagonists. One micromolar rαCGRP(8–37) increased the EC_{50} of rAM and rαCGRP 10- and 25-fold with K_i of 127 ± 49 nM and 47 ± 21 nM ($P > 0.05$). With 1 μM rAM(20–50) as an antagonist the EC_{50} of rAM and rαCGRP increased 4- and 12-fold and the K_i were 480 ± 149 nM and 120 ± 32 nM ($P > 0.05$), respectively. Interestingly, both rαCGRP and rAM were more potently antagonized by rαCGRP(8–37) than by rAM(20–50).

4. Discussion

Here we have functionally characterized homologous mCLR/mRAMP1, -2 and -3 CGRP and AM receptors in COS-7 cells. The mCLR has been characterized with mRAMP1 [18]. The human, rat, porcine and bovine CLR were mainly analyzed with RAMP from different species and in HEK cells with endogenous RAMP, CLR and calcitonin receptors that interfere with the analysis [9]. COS-7 cells do not express functional RAMP and CLR [15]. To this end, neither transfection of myc-mRAMP1, -2 or -3 or V5-mCLR alone increased cAMP production in response to 1 μM rαCGRP or rAM (not shown).

Cell surface expression of the CLR and the RAMP was thought to be interdependent [4]. Here we have shown that the V5-mCLR and myc-mRAMP2 and -3 expressed alone are at the cell surface. myc-mRAMP1, on the other hand, was largely retained inside the COS-7 cells in the absence of the mCLR. Related findings were reported in *Xenopus* oocytes with FLAG-tagged mRAMP and mCLR [19]. CLR and RAMP2 and -3 unlike RAMP1 are N-glycosylated. Introduction of N-glycosylation consensus sites in FLAG-mRAMP1 resulted in mCLR independent cell surface expression [19]. CLR independent cell surface expression was also observed with C-terminally truncated myc-hRAMP1 mutants [17]. Taken together, the nonglycosylated RAMP1 requires CLR for cell surface expression, but the glycosylated CLR and RAMP2 and -3 are expressed at the cell surface independently. The CLR independent expression of RAMP2 and -3 suggests unknown functions of these proteins at the cell surface.

Together with mRAMP1 the mCLR revealed specific binding of [^{125}I]hαCGRP but not of -rAM. This is in line with co-transfected rat, human, porcine and bovine CLR together with RAMP1 [5,7,8,15,20]. rαCGRP(8–37) was 7-fold more potent than rαCGRP, and rαCGRP was 36-fold more potent than rAM in [^{125}I]hαCGRP binding inhibition. The profile is similar to that described with the rat CLR and RAMP1 [5,15,20]. rαCGRP was 100-fold more potent than rAM in the stimulation of cAMP formation. This was also observed with the rat, human and porcine CLR associated to RAMP1 [5,7,15,20]. But in certain studies using HEK cells or osteoblast-like UMR106 cells which express endogenous functional RAMP2, CGRP was only 3- to 8-fold more potent than AM [8,15,21,22]. There, CLR/RAMP1 and CLR/RAMP2 CGRP and AM receptors co-expressed in the same cells have presumably been analyzed. Along with the potent inhibition of [^{125}I]hαCGRP binding rαCGRP(8–37) inhibited rαCGRP and rAM stimulated cAMP formation with similar K_i. This was also seen in human neuroblastoma SK-N-MC cells expressing $CGRP_1$ receptors [11,23]. The observed K_i of 10 nM is similar to those obtained with rat, human, porcine and bovine CLR and RAMP1 [7,8,24]. Consistent with the low potency of rAM(20–50) for CGRP binding inhibition, rAM(20–50) failed to antagonize rαCGRP and rAM stimulated cAMP formation. Taken together, the mCLR/mRAMP1 CGRP receptor recognizes CGRP over AM with a selectivity of about two orders of magnitude, and it is antagonized by rαCGRP(8–37) but not by rAM(20–50).

Together with mRAMP2 the mCLR revealed specific binding of [^{125}I]rAM but not of -CGRP. This is in line with the human, rat and bovine CLR, but distinct from the porcine CLR where [^{125}I]hαCGRP binding was also observed [4,5,7,8]. With the mCLR and mRAMP2 rAM(1–50) displaced [^{125}I]rAM binding at 4-fold lower concentrations than rAM(20–50). rαCGRP(8–37) and rαCGRP were 25- and >100-fold less potent than rAM. Similar profiles were obtained for the human, rat and bovine CLR together with RAMP2 [4,5,15,20,21]. rAM was 280-fold more potent than rαCGRP in the stimulation of cAMP accumulation. A similar selectivity was observed with the rat and bovine CLR [5,8,15,20,25] . With the human and porcine CLR co-transfected with RAMP2 in HEK cells the selectivity of AM over CGRP was less evident because of the functional endogenous RAMP1 [7,21]. rAM(20–50) antagonized rAM and rαCGRP stimulated cAMP accumulation with similar K_i. Comparable results have been obtained with the human, porcine and bovine CLR co-transfected with RAMP2 [7,8]. rαCGRP(8–37) failed to antagonize rαCGRP and rAM stimulated cAMP stimulation to any great extent. Taken together, the mCLR/mRAMP2 AM receptor recognizes AM over CGRP with a selectivity of two to three orders of magnitude, and is more potently antagonized by rAM(20–50) than by rαCGRP(8–37).

Together with mRAMP3 the mCLR revealed similar specific binding of [^{125}I]rAM or [^{125}I]hαCGRP as observed with the porcine CLR co-transfected with human RAMP3 [7]. With the human and bovine CLR and human RAMP3 [^{125}I]AM binding alone was recognized [7,8]. The rat CLR together with mRAMP3, on the other hand, bound [^{125}I]hαCGRP [5]. Species differences in the radioligand recognition are apparent with the CLR and RAMP3.

With the mCLR and mRAMP3 rAM and rαCGRP(8–37) were equipotent and rAM(20–50) and rαCGRP 4- to 8-fold less potent, irrespective of the radioligand used. Similar data have been obtained with the rat CLR co-expressed with mRAMP3 and [^{125}I]hαCGRP as ligand [5]. The profile with rodent CLR and RAMP3 of rAM = rαCGRP(8–37) > rAM(20–50) = rαCGRP differs from that with human CLR and human RAMP3 of hAM ⩾ hAM(22–52) = hαCGRP(8–37) > hαCGRP [7,21]. The porcine and bovine CLR were even more selective for AM and CGRP(8–37) insensitive [7,8].

rAM was 14-fold more potent than rαCGRP in the stimulation of cAMP formation in COS-7 cells transfected with mCLR and mRAMP3. This is consistent with the radioligand inhibition data and with the results in COS-7 cells co-transfected with rCLR and mRAMP3 [5]. Here again, rodent CLR and RAMP3 are one order of magnitude less selective for AM over CGRP than human, porcine and bovine CLR and RAMP3 [7,8,21,22]. Both rαCGRP(8–37) and rAM(20–50) antagonized rAM and rαCGRP stimulated cAMP accumulation, but rαCGRP(8–37) was more potent than rAM(20–50). This is consistent with the higher potency of rαCGRP(8–37) in radioligand binding inhibition compared to rAM(20–50). Again, with the human, porcine and bovine CLR and RAMP3 the AM antagonists were more potent than CGRP(8–37) [7,8]. Taken together, the mCLR/mRAMP3 AM receptor recognizes AM over CGRP with a selectivity of one order of magnitude, and is more potently antagonized by rαCGRP(8–37) than by rAM(20–50).

In *Xenopus* oocytes co-expressing human CLR and RAMP1 pertussis toxin sensitive constitutive activation of potassium channels has been revealed [26]. Apparently, the constitutively active conformation of CLR/RAMP1 preferentially couples to G_i. Here, higher cAMP levels in the absence of added agonists were observed in COS-7 cells co-expressing the mCLR together with mRAMP2 or -3 than with mRAMP1. The secretion of AM unlike CGRP by COS-7 cells and an autocrine activation cannot be ruled out. But it remains to be demonstrated whether COS-7 cells secrete AM. Coupling to G proteins in the absence of agonists may also be caused by the overexpressed receptors. This seems not to be relevant since the cell surface expression of the V5-mCLR in the absence and presence of the individual RAMP was similar. The constitutive activity of the mCLR together with mRAMP2 was antagonized more potently by rAM(20–50) than by rαCGRP(8–37). Conversely, in mCLR/mRAMP3 expressing cells rαCGRP(8–37) unlike rAM(20–50) antagonized the constitutive cAMP formation. This reflects the relative activity of the two antagonists for the stimulation of cAMP formation with the two receptors. The constitutively active mCLR/mRAMP2 and -3 receptors couple to G_s in COS-7 cells.

In conclusion, RAMP independent CLR and CLR independent RAMP2 and -3 expression at the cell surface was observed in COS-7 cells lacking functional endogenous RAMP and CLR. Expression of mCLR and mRAMP1 reveals a CGRP-specific receptor antagonized by CGRP(8–37). Co-expression of mRAMP2 demonstrates a selective AM receptor antagonized by AM(20–50). With mRAMP3 an AM over CGRP preferring receptor is revealed which is more potently antagonized by CGRP(8–37) than by AM(20–50).

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