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$Cys^{2,7}Et\alpha CGRP$ is a potent agonist for $CGRP_1$ receptors in SK-N-MC cells

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Abstract

The present study reveals that cystein^{2,7} ethyl-amideαCGRP ($Cys^{2,7}Et\alpha CGRP$), an advertised calcitonin gene-related peptide 2 ($CGRP_2$) receptor subtype-selective agonist, is also a potent agonist for the calcitonin gene-related peptide 1 ($CGRP_1$) receptors natively expressed in the SK-N-MC human neuroblastoma cell line. $Cys^{2,7}Et\alpha CGRP$ and α calcitonin gene-related peptide (αCGRP) promote cyclic AMP accumulation in intact SK-N-MC cells to the same extent with EC_{50} of 1.6 ± 0.2 and 0.4 ± 0.08 nM, respectively. The antagonist α calcitonin gene-related peptide-8-37 (αCGRP-(8-37)) produces a concentration-dependent rightward shift of the αCGRP- and $Cys^{2,7}Et\alpha CGRP$ concentration–response curves with K_B-values (71 ± 33 and 47 ± 21 nM, respectively). The competitive antagonism by αCGRP-(8-37) and the similar K_B-values suggests that αCGRP and $Cys^{2,7}Et\alpha CGRP$ stimulate the same receptor. In competition binding studies with [^{125}I]-αCGRP on SK-N-MC cell membranes, $Cys^{2,7}Et\alpha CGRP$ and αCGRP-(8-37) display high affinity for the majority of the binding sites with K_i-values of 0.030 ± 0.013 and 0.60 ± 0.013 nM, respectively. The present findings are at odds with the proclaimed utilization of $Cys^{2,7}Et\alpha CGRP$ as a $CGRP_2$ receptor-selective pharmacological tool. Differences between the agonistic profile of this ligand in this and other experimental systems might be species – or even cell type – dependent.

Keywords: Calcitonin gene-related peptide; $Cys^{2,7}Et$ calcitonin gene-related peptide; CGRP-(8-37); CGRP receptor subtypes; Receptor activity modifying protein

The calcitonin gene-related peptide (CGRP, 37 amino acids) is known to produce vasodilation and several other effects on the cardiovascular, gastrointestinal, bronchotracheal, endocrine and central nervous systems [1,2]. It comprises two isoforms; α calcitonin gene-related peptide (αCGRP), which is expressed in sensory neurons, and β calcitonin gene-related peptide (βCGRP), which bears high, sequence homology but not derived from the calcitonin gene. Based on pharmacological studies, mainly on peripheral tissues, the intervening receptors have been commonly divided in two subtypes denoted as calcitonin gene-related peptide 1 ($CGRP_1$) and calcitonin gene-related peptide 2 ($CGRP_2$) receptors. The $CGRP_1$ receptors were typically found in guinea pig atrium and they were regarded to display higher affinity for αCGRP-(8-37), i.e., a fragment of αCGRP displaying antagonistic properties [3–5]. On the other hand, $CGRP_2$ receptors were typically found in rat vas deferens and they were regarded to display higher affinity for the agonistic αCGRP analogues $Cys^{2,7}Acm\alpha CGRP$ and $Cys^{2,7}Et\alpha CGRP$ [4–7]. For example, whereas αCGRP showed a potent positive chronotropic effect in the guinea pig right atrium and potent inhibition of the twitch response of the electrically stimulated rat vas deferens, $Cys^{2,7}Et\alpha CGRP$ mimicked these effects at much higher concentration in atrium ($EC_{50} > 1$ μM) than in vas deferens ($EC_{50} = 3.4$ nM). Moreover, this latter effect was not inhibited by αCGRP-(8-37) [7].

Abbreviations: αCGRP, α calcitonin gene-related peptide; cAMP, cyclic AMP; αCGRP-(8-37), α calcitonin gene-related peptide-8-37; $Cys^{2,7}Et\alpha CGRP$, cystein2,7 ethyl-amideαCGRP; $Cys^{2,7}Acm\alpha CGRP$, cystein2,7 acetyl-methylαCGRP; $CGRP_1$, calcitonin gene-related peptide 1; $CGRP_2$, calcitonin gene-related peptide 2; CRLR, calcitonin receptor-like receptor protein; RAMP, receptor activity modifying protein

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This pharmacologic subclassification of the CGRP receptors has, however, been subject to growing criticism over the years. First, the potency of αCGRP-(8-37) in the same tissue has been found to vary considerably from one experiment to another and, in one report, this antagonist showed only a mere three-fold difference in affinity of between $CGRP_1$ receptors in guinea pig left atria and $CGRP_2$ receptors in rat vas deferens [4,8]. Moreover, while differences in αCGRP-(8-37) potency could be observed in some of the functional studies, this has never been clearly detected in radioligand binding experiments [4,9–11]. Among the potential reasons for these discrepancies, it has been evoked that the ''selectivity'' of CGRP-related peptides might result from an unequal distribution of tissue peptidases [12]. Rather than its putative selectivity for $CGRP_2$ receptors, tissue-dependent factors like the presence of a receptor reserve have also been evoked to explain differences in the agonistic behavior of $Cys^{2,7}$AcmαCGRP [13]. Taken together, it becomes increasingly evident that criteria like the potency of αCGRP-(8-37) and the agonist activity of $Cys^{2,7}$AcmαCGRP may not be sufficiently reliable to differentiate CGRP receptor subtypes.

In fact, pending the potential discovery of much more selective ligands, the present limitations even cast doubt on the very existence of multiple CGRP receptor subtypes. In this respect, it is also noteworthy that, till now, cloning studies only firmly established the existence of a single CGRP receptor [14]. This receptor is a heterodimer that is formed by the association of the heptahelical CRLR with the RAMP1 receptor activity modifying chaperone protein [15]. The existence of additional RAMPs has been evidenced as well and, while the CRLR–RAMP1 complex is characterized by its high affinity for CGRP, CRLR–RAMP2 and –RAMP3 complexes clearly prefer other bioactive peptides like adrenomedullin [5,14–16]. Because of their low affinity for CGRP, these latter complexes appear to be unrelated to any of the CGRP receptor subtypes defined in the earlier pharmacological studies.

The human neuroblastoma-derived SK-N-MC cell line express CRLR, RAMP1 and RAMP2 but do not respond to adrenomedullin [15]. RAMP 1 appears to have dominant activity and the resulting endogenous CGRP receptors display high affinity for αCGRP-(8-37) [15]. Based on this pharmacological criterion, they have been classified as $CGRP_1$ receptors [12,15,17,18]. In the present study, we have used this well established receptor model to investigate its interaction with $Cys^{2,7}$EtαCGRP both by radioligand binding and by measurement of the receptor-evoked cAMP production. Contrary to its proclaimed utilization as a $CGRP_2$ receptor-selective pharmacological tool, $Cys^{2,7}$EtαCGRP was found to display high affinity/potency for the $CGRP_1$ receptors in SK-N-MC cells.

1. Materials and methods

1.1. Chemicals

αCGRP and αCGRP-(8-37) were purchased from Bachem AG (Switzerland). $Cys^{2,7}$EtαCGRP was from Penisula laboratories Ltd. The radioligand [^{125}I]-αCGRP (2200 Ci/mmol) was obtained from Amersham Pharmacia Biotech. SK-N-MC cell membranes were from Receptor Biology. Other chemicals were obtained from Sigma–Aldrich and cell cultivation media and buffers from Life Technologies. Complete™ was from Boehringer Mannheim.

1.2. SK-N-MC cell membranes

Membranes (23.8 mg protein/ml) were kept in buffer (50 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$ and 10% sucrose). Upon arrival, aliquots of membranes were kept frozen at −80 °C. The protein concentration was determined using a Bio-Rad kit with lyophilized bovine plasma gamma globulin as standard.

1.3. [^{125}I]-αCGRP binding assay

Binding experiments were performed in 96-well micro titer plates (Nunc™). All components were diluted in binding buffer containing 50 mM Tris–HCl, pH 7.4, 5 mM MgCl2, 0.1% (w/v) bovine serum albumin and 100 μM GTP. Each assay contained 100 μl membrane suspension (24 μg protein per well), 50 μl [^{125}I]-αCGRP and 50 μl of buffer either alone or containing increasing concentrations (20 pM–50 μM) of non-labeled substances. For displacement experiments, a constant concentration of [^{125}I]-αCGRP (20 pM) was used. Non-specific binding was determined in the presence of 20–50 μM αCGRP-(8-37) Incubations were performed for 1 h at room temperature and were terminated by rapid filtration (Micrf6 Harvester, Skatron Instruments) using ice-cold filtration buffer (50 mM Tris–HCl (pH 7.4 at 20 °C), 5 mM $MgCl_2$). The filters (Filtermat B, Wallac) were soaked in 0.3% polyetylenimine for 15 min and dried. After the experiment the filters were dried and a scintillator sheet (MeltiLex B/HB, Wallac) was melted onto them (microsealer 1495-021, Wallac). The amount of bound [^{125}I]-αCGRP was measured by counting the β-radiation in a β-liquid scintillation counter (1450 Microbeta PLUS, Wallac).

1.4. Culturing of SK-N-MC cells

Stock cells obtained from ATCC were kept in 1-ml aliquots (10^7 cells) and frozen at −150 °C in a mixture of 10% DMSO in culture medium. Cells were seeded in 175 cm^2 flasks (Nunclon™, Nunc) in 40 ml culture medium (minimum essential medium with Earl's salts and

L-glutamine, 10% foetal bovine serum, 1% sodium pyruvate and 1% non-essential amino acids). Cells were grown at 37 °C for 2–3 days in humid atmosphere containing 5% CO_2 to reach 80% confluency and then either passaged or used for experiments. Culture medium was aspirated and the cells were washed with 10 ml PBS Dulbecco's medium without sodium bicarbonate. The cells were detached by adding 10 ml PBS Dulbecco's medium with 1 mM EDTA but without calcium, magnesium and sodium bicarbonate. After six to seven passages, fresh cells were thawed and seeded as described above.

1.5. cAMP experiments

SK-N-MC cells were seeded in 96-well cell culture cluster (3595, Costar). Forty thousand cells in 250 μl culture medium were added in each well and cultured for 24–28 h prior to the experiment.

Experiments were initiated by removing the medium. Each well was gently washed twice with 200 μl cAMP buffer (2.5 mM Tris–HCl, 2.5 mM HEPES (pH 7.4 at 20 °C)), 140 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 4.5 g/l glucose, 0.2% (w/v) bovine serum albumin and 0.1 mM isobutyl methyl xantine (IBMX). After washing, cells were pre-incubated for 60 min in 37 °C in cAMP buffer (240 μl/well). Then, 10 μl buffer with or without antagonist was added to each well and cells were further pre-incubated for 10 min at 37 °C. Finally, 50 μl buffer containing various concentrations of agonist was added to each well and cells were incubated for 20 min at 37 °C. The reaction was interrupted by quickly removing the reaction mixture and adding 200 μl acidified methanol to produce cell lysis. An aliquot (150 μl) of the mixture was transferred from each well to a new 96-well plate and the methanol was totally evaporated at 50 °C. The plate was then kept frozen (−18 °C) for later analysis of cAMP. The amount of cAMP produced was determined by using an enzyme immunoassay kit (RPM225, Amersham Pharmacia Biotech). All results were calculated as percent of maximum stimulation by 10 nM αCGRP.

1.6. Data analysis and calculation of results

Results were evaluated with XLfit and calculation models were used to determine IC_{50}- and EC_{50}-values. The total number of receptors (B_{max}) and radioligand equilibrium dissociation constants (K_d-values) were determined from saturation binding experiments. The equilibrium dissociation constants for non-labeled ligands (K_i-values) were calculated from the Cheng and Prusoff equation from their IC_{50}-values. For the cAMP experiments, antagonist K_B- and K_d-values were calculated by linear regression analysis of the Schild plots [19]. For each data set, a one site fit analysis was performed and for results from SK-N-MC, a two site fit analysis was obtained. Results are expressed as mean ± standard deviation (S.D.).

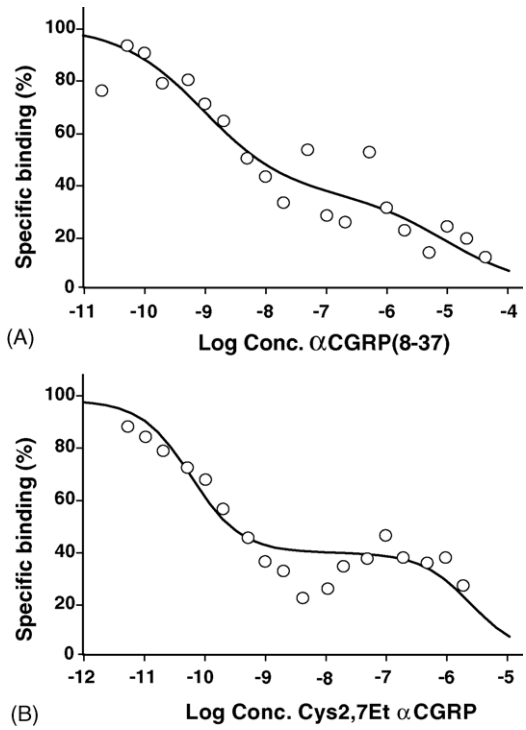

Fig. 1. Competition binding curves for αCGRP-(8-37) (A) and $Cys^{2,7}$-EtαCGRP (B) in SK-N-MC cell membranes.

2. Results

2.1. Binding experiments

[^{125}I]-αCGRP saturation binding to SK-N-MC cell membranes revealed a K_d-value of 62 ± 15 pM and a B_{max} of 52 ± 5 fmol/mg protein ($n = 3$, data not shown).

In competition binding experiments, αCGRP-(8-37) and $Cys^{2,7}$EtαCGRP displayed biphasic curves when [^{125}I]-αCGRP was used as the radioligand (Fig. 1). Both peptides displaced most of the binding with high affinity. The Hill coefficient of the high affinity component of the curves was close to unity (nH = 0.93 ± 0.09 for αCGRP-(8-37) and 0.99 ± 0.4 for $Cys^{2,7}$EtαCGRP). Calculated K_i-values for this component were 0.60 ± 0.013 nM for αCGRP-(8-37) and 0.030 ± 0.013 nM for $Cys^{2,7}$EtαCGRP. Both peptides displaced the remaining [^{125}I]-αCGRP binding with low potency ($K_i > 1000$ nM) (Table 1).

Table 1
Mean affinity values (K_i, nM, $n = 4$) for αCGRP-(8-37) and $Cys^{2,7}$-EtαCGRP obtained from competition binding experiments in SK-N-MC cell membranes

Peptide	K_i (nM)		nH
	High affinity	Low affinity	
αCGRP(8-37)	0.92 ± 0.59	>2000	0.26
$Cys^{2,7}$EtαCGRP	0.044 ± 0.02	>1000	0.30

K_i-Values were determined from two site fit analysis and were calculated according to Cheng and Prusoff [35]. Hill coefficients (nH) were calculated for the entire curves.

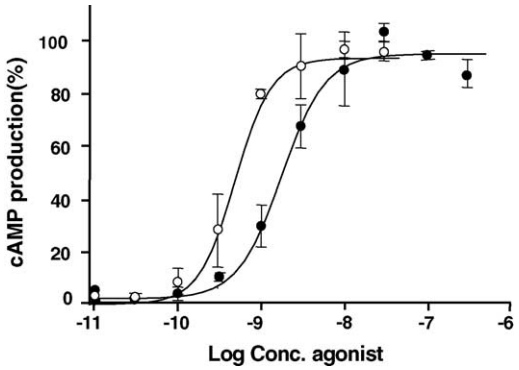

Fig. 2. Concentration–response curves for αCGRP (open circles) and $Cys^{2,7}$EtαCGRP (solid circles) on stimulation of cAMP production in SK-N-MC cells. Mean values ± S.D., $n = 3$.

Table 2
Effects of CGRP receptor agonists and antagonists on cAMP production in SK-N-MC cells

Peptide	Stimulation of cAMP production	Antagonistic effect of αCGRP-(8-37)	
	EC_{50} (nM)	K_B (nM)[a]	Schild slope
αCGRP	0.4 ± 0.08	47 ± 21	0.9
$Cys^{2,7}$EtαCGRP	1.6 ± 0.23[b]	71 ± 34	1.0

Results are mean ± S.E.M., $n = 3$–4.
[a] K_b-Values for αCGRP and $Cys^{2,7}$EtαCGRP was not significantly different.
[b] EC_{50}-values for αCGRP and $Cys^{2,7}$EtαCGRP were significantly different ($p < 0.001$).

2.2. In vitro cAMP experiments

αCGRP and $Cys^{2,7}$EtαCGRP concentration versus cAMP production curves in cultured SK-N-MC cells are shown in Fig. 2. Both peptides acted as agonists, producing nearly the same maximal increase in cAMP production. $Cys^{2,7}$EtαCGRP was only about four-fold less potent as αCGRP (Table 2). Although small, this difference was significant ($p < 0.001$).

The inhibitory action of αCGRP-(8-37) on the αCGRP- and $Cys^{2,7}$EtαCGRP induced cAMP formation was evaluated by the Schild method (Fig. 3A and B). αCGRP-(8-37) shifted the concentration–response curves of both agonists to the right without producing a decline in the maximal response. Hence, αCGRP-(8-37) acted as a competitive antagonist in both instances. The shifts were αCGRP-(8-37) concentration-dependent (Fig. 3). The K_B-value for αCGRP-(8-37) for competing with αCGRP was slightly lower than in the case of $Cys^{2,7}$EtαCGRP but this difference was not significant (Table 2).

3. Discussion

The major finding of the present study is that both αCGRP and $Cys^{2,7}$EtαCGRP stimulate the $CGRP_1$ receptor subtype in SK-N-MC cells to the same degree and with

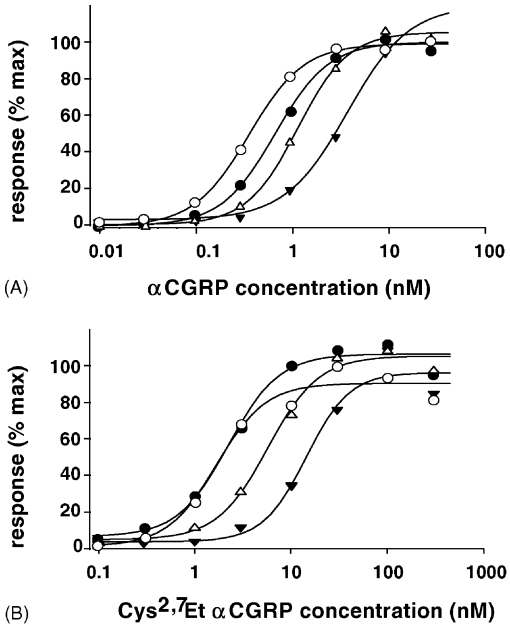

Fig. 3. Concentration–response curves of αCGRP (A) and $Cys^{2,7}$EtαCGRP (B) in the absence (control, open circles) and in the presence of 30 nM (solid circles), 100 nM (open triangles) and 300 nM (solid triangles) αCGRP-(8-37). Mean K_B-values from Schild analysis are shown in Table 2.

quite similar potency. This is in contrast to previous reports suggesting that $Cys^{2,7}$EtαCGRP is a selective $CGRP_2$ receptor agonist.

SK-N-MC cells represent a convenient model system to study endogenously expressed human CGRP receptors. In this respect, these cells have already been extensively used to determine the $CGRP_1$ receptor binding and activation profile of peptide αCGRP analogues and novel non-peptide antagonists [20,21]. CGRP receptor stimulation produces an elevation of the cAMP concentration in SK-N-MC cells [21] and, based on this property, the present data suggest that $Cys^{2,7}$EtαCGRP behaves as a potent CGRP receptor agonist. Indeed, it elevates the cAMP concentration to the same degree as the reference agonist αCGRP and its potency is only four times lower as that of αCGRP (Fig. 2). The implication of CGRP receptors is evidenced by the ability of the selective antagonist CGRP-(8-37) to competitively inhibit the effect of both agonists with the same potency. A high affinity interaction between $Cys^{2,7}$EtαCGRP and CGRP receptors is also evidenced by binding studies on SK-N-MC cell membranes. As shown in Fig. 1, αCGRP-(8-37) and $Cys^{2,7}$EtαCGRP were able to displace a similar, large portion of the [^{125}I]-αCGRP binding with high affinity. $Cys^{2,7}$EtαCGRP was even more potent than αCGRP-(8-37) and a similar behaviour of both peptides has also been reported (but not commented) in recent binding studies on SK-N-MC cell membranes with the non-peptide CGRP receptor antagonist [^{3}H]-BIBN4096BS [22,20]. Biphasic $Cys^{2,7}$EtαCGRP competition curves were also reported in this latter study but the difference in potency between high- and low-affinity sites was much

smaller as in the present study and, above all, it was only observed for agonists. This is compatible with the occurrence of shallow/biphasic agonist versus radiolabeled antagonist competition curves for many GPCRs [23]. In the present study, low affinity sites are also observed for the antagonist αCGRP-(8-37). Hence, they are unlikely to represent CGRP receptors.

The ability of $Cys^{2,7}$EtαCGRP to produce a maximal $CGRP_1$ receptor mediated elevation of the cAMP concentration in SK-N-MC cells contrasts with earlier findings by others using experimental systems also claimed involve CGRP receptors. For example, $Cys^{2,7}$EtαCGRP only produced a modest stimulation of the guinea pig atrium at concentrations above 1 μM [7]. Also, whereas αCGRP was a potent modulator of the aldosteron secretion by zona glomerulosa cells and catecholamine secretion by adrenal medulla cells of the rat, $Cys^{2,7}$EtαCGRP was unable to do so [24]. The molecular mechanisms that are responsible for the above mentioned differences in $CGRP_1$ receptor behaviour with regard to $Cys^{2,7}$EtαCGRP are presently unknown but, among the potential explanations, it is reasonable to assume that they might be species- or even cell type-related.

Species-related differences in CGRP receptor binding affinity have been observed in the case of BIBN4096BS and other non-peptide antagonists [20,25] and evidence has been provided for the implication of the RAMP1 chaperone in this phenomenon [26]. Indeed, mutation studies pinpointed that BIBN4096BS displays high affinity for the CGRP receptor when its RAMP1 constituent contains Trp (as in human RAMP1) and only low affinity when RAMP1 contains a basic amino acid at this position (as in rat or porcine RAMP1) [26]. However, while having a profound effect on the affinity of BIBN4096BS, the nature of the amino acid at location 74 of RAMP1 had only little influence on the affinity of [^{125}I]-αCGRP and αCGRP-(8-37) [26]. So far, this hypothesis has not been verified in the case of CGRP receptor subtype-selective peptides like $Cys^{2,7}$EtαCGRP and $Cys^{2,7}$AcmαCGRP. Therefore, it cannot yet be excluded that species-related differences in RAMP1 and/or CRLR amino acid sequence may account for the some of the variability of the observed effects of these peptides.

An alternative potential explanation relies on the current thinking that receptors exist in a large number of conformational states, each of them constituting a minimum in an ''energy landscape'' [27]. In this respect, an increasing number of observations (e.g., [28]) support the notion that GPCRs are capable to adopt different ligand-specific states/conformations and that these may trigger distinct receptor activities, including the activation of distinct species of G protein [29,30]. In this vein, it has been shown that deletion of the first seven C-terminal amino acids of αCGRP results in an antagonistic peptide, CGRP-(8-37), with high affinity for the CGRP receptor [31]. Thus, the seven C-terminal amino acids are of importance to create activation of the receptor. In $Cys^{2,7}$AcmαCGRP and $Cys^{2,7}$EtαCGRP acetyl-methyl or ethyl-amide groups have been coupled to the cysteins in positions 2 and 7 in αCGRP. The disulfide bridge normally formed in αCGRP can now not be established and the bent conformation seen in αCGRP is abolished. Due to this major structural difference, it is quite plausible that $Cys^{2,7}$AcmαCGRP- and $Cys^{2,7}$EtαCGRP-bound CGRP receptors adopt an 'active' conformation that is distinct from the αCGRP-induced one. Assuming that both active receptor conformations may be differently affected by factors related to receptor structure or its environment (such as its presence in lipid rafts and/or caveolae [32]), this model not only explains the existence of αCGRP-preferring [7,24] and αCGRP versus $Cys^{2,7}$EtαCGRP-indifferent receptors (present study) but also the occurrence of $Cys^{2,7}$EtαCGRP-preferring receptors [33] in functional studies.

Besides the above-mentioned potential differences in the generation of the ''stimulus'', its relationship to the response (i.e., the occurrence of a ''receptor reserve'') may also be function of the tissue and the assay [13]. Provided that the intrinsic efficacy of these αCGRP-analogues is less than that of αCGRP, such potential differences in receptor reserve might profoundly affect their potency as well as the maximal response they can generate [34]. While such ligands might only behave as partial agonists in systems without receptor reserve, they might become full agonists in the case of an appreciable receptor reserve. These considerations could also explain why BIBN4096BS was more potent in antagonizing $Cys^{2,7}$EtαCGRP-induced responses than αCGRP-induced responses in rat vas deferens [33].

In conclusion, the present study indicates that $Cys^{2,7}$EtαCGRP acts as an agonist for the clearly established $CGRP_1$ receptor in human SK-N-MC cells. Besides invoking the occurrence of distinct CGRP receptor subtypes, it is also plausible that the different activation profiles by this ligand in other experimental systems may be related to the nature and/or strength of the stimulus as well as to the stimulus–response coupling.

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