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Adrenomedullin and Calcitonin Gene-Related Peptide Interact With the Same Receptor in Cultured Human Neuroblastoma SK-N-MC Cells

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ZIMMERMANN, U., J. A. FISCHER AND R. MUFF. *Adrenomedullin and calcitonin gene-related peptide interact with the same receptor in cultured human neuroblastoma SK-N-MC cells.* PEPTIDES **16**(3) 421–424, 1995.—Inhibition of human [^{125}I]calcitonin gene-related peptide-I ([^{125}I]hCGRP-I) binding by human adrenomedullin (hADM), its *N*-terminal truncated fragments, CGRP and amylin, and cyclic AMP accumulation were examined in the human neuroblastoma cell line SK-N-MC. Binding of [^{125}I]hCGRP-I (125 p*M*) was inhibited by hCGRP-I, hADM(1–52), hADM(13–52), and human amylin with IC_{50} of 0.32 ± 0.06, 2.11 ± 0.26, 3.45 ± 0.54, and 68.8 ± 6.6 n*M*, respectively. hCGRP-I(8–37) and hADM(22–52), which lack the *N*-terminal ring structure, inhibited [^{125}I]hCGRP-I binding with IC_{50} of 2.35 ± 0.45 and > 1000 n*M*. hCGRP-I, hADM(1–52), hADM(13–52) and human amylin stimulated cAMP accumulation with EC_{50} of 0.40 ± 0.05, 18.1 ± 2.6, 51.3 ± 9.0 and 925 ± 159 n*M*, respectively. hCGRP-I(8–37) (100 n*M*) antagonized hCGRP-I and hADM(1–52) stimulated cAMP production with the same K_i of 16.6 ± 1.2 and 16.8 ± 1.1 n*M*. In conclusion, human ADM, which is more distantly related to CGRP than amylin, interacts more potently with the CGRP receptor in SK-N-MC cells than amylin. The *N*-terminal ring structure of hADM, unlike that of hCGRP, is essential for binding to the CGRP receptor. Coupling of hADM binding to cAMP stimulation is less efficient than for hCGRP-I and is reduced by deletion of the unique 12 amino acid sequence of hADM *N*-terminal to the ring structure.

Adrenomedullin Amylin Antagonist Calcitonin gene-related peptide cAMP Receptors

HUMAN adrenomedullin (hADM) was isolated from pheochromocytoma (11). Sequence analysis revealed a 52 amino acid polypeptide with a six amino acid ring structure linked by a disulfide bridge between amino acids 16 and 21, and an amidated *C*-terminus (Fig. 1). The structures of hADM(1–52) and rat ADM(1–50) (rADM) were obtained by molecular cloning (12,17). hADM and rADM mRNA and immunoreactive hADM are expressed in several tissues, including adrenal medulla and pheochromocytoma, and heart, lung, and kidney (8,12,17). Immunoreactive ADM was found to be raised in the serum of hypertensive patients (13). Sequence homology between hADM(15–52) and human calcitonin gene-related peptide (hCGRP)-I(1–37) and human amylin(1–37), also with six amino acid ring structures and amidated *C*-termini, is 24% and 22%, respectively.

Human ADM and hCGRP-I had comparable hypotensive action in anesthetized rats (11). In the pulmonary vascular bed of the cat, the relative vasodilator activity of hADM was higher than that of hCGRP-I (2). Vasodilation in the precontracted rat mesenteric vascular bed was obtained with 10-fold higher concentrations of hADM than hCGRP-I (15). Suppression by the CGRP receptor antagonist hCGRP-I(8–37) suggests that the observed effects are mediated by a CGRP receptor. The *N*-terminal truncated hADM(13–52) and intact hADM(1–52) had comparable hypotensive properties in the anesthetized rat (6). hADM(1–52) and hADM(13–52), on the other hand, showed mild negative chronotropic and inotropic effects in the rat in vivo (9,16), which contrasts with the positive chronotropic and inotropic actions of CGRP (4,5). Distinct ADM receptors may therefore exist. To this end, specific ADM receptors have been considered in rat vascular smooth muscle cells (3,10).

The biological effects of ADM may be due to direct involvement of an ADM receptor, but also to activation of CGRP receptors. Thus, interactions of ADM and of its fragments that lack

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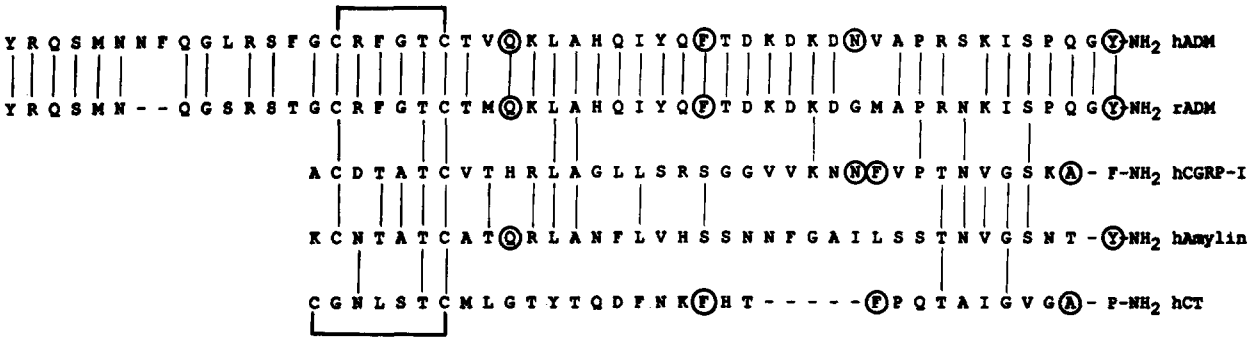

FIG. 1. Amino acid sequences of hADM(1–52), rADM(1–50), hCGRP-I, human amylin, and hCT. Identical amino acids are indicated by vertical lines and circles.

the unique linear sequence of amino acids *N*-terminal to the ring structure, and are therefore more closely related to CGRP, have been studied in the human neuroblastoma cell line SK-N-MC with a well-characterized CGRP receptor coupled to adenylyl cyclase (14,19). Our results indicate that ADM interacts with the CGRP receptor of SK-N-MC cells rather than with its own ADM receptor.

METHOD

Materials

Human ADM(1–52) was obtained from Peptide Institute (Osaka, Japan). hADM(1–52) was also donated by Dr. J.-K. Chang (Peninsula Laboratories, Belmont, CA, and Phoenix Pharmaceuticals, Mountain View, CA), together with hADM(13–52), (22–52), (26–52), and (29–52), rat (r)ADM(1–50), desamino-rADM(14–50), and desamino-human amylin(2–7)-hADM(22–52) [(amylin)-hADM] and desamino-hCGRP-I(2–7)-hADM(22–52) [(CGRP-I)-hADM] hybrids (Fig. 1). hCGRP-I, hCGRP-I(8–37), and human amylin were purchased from Bachem (Bubendorf, Switzerland). Human calcitonin (hCT) was obtained from Ciba (Basel, Switzerland).

Cell Culture

SK-N-MC cells were grown in HAM-F12 supplemented with 10% fetal calf serum. For binding studies and cAMP accumulation, the cells were seeded into 24-well plates at a density of approximately 40,000 cells/well and grown to confluence.

Radioiodination

Human CGRP-I was iodinated to a specific activity of approximately 200 Ci/mmol with use of the chloramine-T method modified as described (7,18). Purification by HPLC on a 4 × 250 mm Nucleosil C18 column with a linear gradient of 29–35% acetonitrile in 0.1% TFA over 60 min yielded a major and a minor peak of radioactivity with retention times longer than those of the nonlabeled peptide. The earlier eluting major peak had the retention time and the binding characteristics of monoiodinated [2-[^{125}I]iodohistidyl^{10}]hCGRP-I purchased from Amersham (Buckinghamshire, UK) (18). The specific radioactivity is approximately 2000 Ci/mmol.

Binding Studies

Binding studies were performed on confluent SK-N-MC cells in a final volume of 200 μl DMEM with 0.45% glucose and HAM-F12 (1:1) supplemented with 1% bovine serum albumin as described (14). Briefly, the cells were incubated at 15°C for 2 h with 125 p*M* [^{125}I]hCGRP-I in the absence and presence of nonlabeled peptides. Monolayers were then washed once with 500 μl incubation medium and subsequently lysed with 500 μl 0.5% sodium dodecylsulfate. Lysates were then counted for radioactivity in a γ-counter (Kontron, Zurich, Switzerland).

cAMP Accumulation

cAMP accumulation was measured in confluent SK-N-MC cells for 15 min at 37°C as described (14). Briefly, cells were incubated in 200 μl of a medium containing 136 m*M* NaCl, 5.4 m*M* KCl, 1 m*M* Na_2HPO_4, 5.5 m*M* glucose, 1 m*M* $CaCl_2$, 1 m*M* $MgSO_4$, 1 m*M* isobutylmethylxanthine, 20 m*M* HEPES, pH 7.45, supplemented with 1% BSA, in the absence and presence of peptides. The medium was then aspirated and cAMP was extracted with 95% ethanol, pH 3, for 1 h at 4°C. Ethanol extracts were lyophilized and cAMP was determined by radioimmunoassay.

Statistical Analysis

Differences between means (±SEM) were determined by analysis of variance (ANOVA). A value of $p < 0.05$ was considered statistically significant.

RESULTS

Receptor Binding

Total binding of 50,000 cpm (125 p*M*) [^{125}I]hCGRP-I ranged from 10% to 30% of added tracer. Nonspecific binding in the presence of 1 μ*M* hCGRP-I was 2–4% of added tracer. Binding of [^{125}I]hCGRP-I was inhibited by hCGRP-I > hADM(1–52) = hADM(13–52) >> human amylin >> hADM(22–52) (Table 1, Fig. 2). Additional truncation of ADM in hADM(26–52), hADM(29–52), and hCT did not affect [^{125}I]hCGRP-I binding at up to 1 μ*M*. rADM(1–50) and hADM(1–52) were equipotent. The inhibitory concentration-50 (IC_{50}) of desamino-rADM(14–50), on the other hand, was three- and sixfold lower than those of hADM(1–52) and rADM(1–50) [$F(1, 4) = 14.71$ and $F(1, 5) = 13.03$, $p < 0.05$]. Desamino-rADM(14–50) was the most potent ADM tested. The IC_{50} of hADM(13–52) was similar to that of a hybrid peptide (amylin)-hADM. But another hybrid peptide (CGRP-I)-hADM was sevenfold less potent than (amylin)-hADM, although the only difference between the two hybrids was in position 2 with an Asn in human amylin vs. an Asp in hCGRP-I, $F(1, 6) = 15.66$, $p < 0.01$.

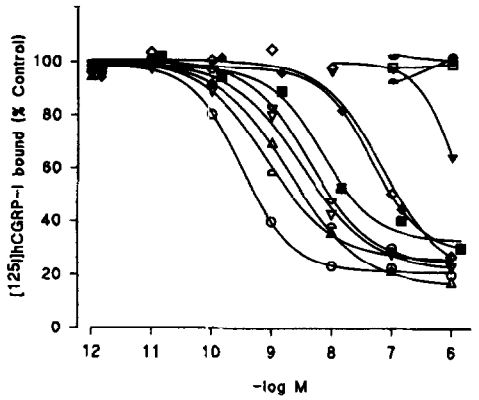

FIG. 2. Inhibition of [^{125}I]hCGRP-I binding by hCGRP-I (○), rADM(14–50) (◓), hADM(1–52) (△), hADM(13–52) (▽), rADM(1–50) (◡), desamino-amylin(2–7)-hADM(22–52) (■), desamino-h-CGRP-I(2–7)-hADM(22–52) (◆), human amylin (◇), hADM(22–52) (▼), hADM(26–52) (◒), hADM(29–52) (◓), and hCT (□) in human neuroblastoma cells (SK-N-MC). The cells were incubated with 125 p*M* [^{125}I]hCGRP-I for 2 h at 5°C. The results are the means of three to five experiments.

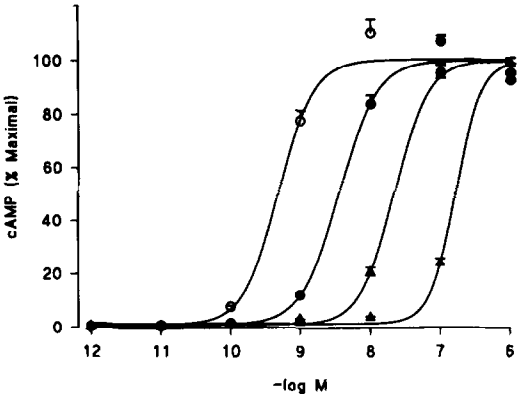

FIG. 3. Stimulation of cellular cAMP accumulation by hCGRP-I (○, ●) and hADM(1–52) (△, ▲) in the absence (open symbols) and presence (closed symbols) of 100 n*M* hCGRP-I(8–37) in human neuroblastoma cells (SK-N-MC). The cells were incubated for 15 min at 37°C. The results are means ± SEM of three experiments.

cAMP Stimulation

Basal levels of cAMP ranged from 1 to 5 pmol/well per 15 min, and they were maximally increased to between 200 and 400 pmol/well per 15 min by 1 μM hCGRP-I. The rank order of the EC_{50} of cAMP accumulation of hCGRP-I, different ADMs, and of amylin was related to their IC_{50} of receptor binding (Table 1). Here, hADM(1–52) was threefold more potent than hADM(13–52), $F(1, 7) = 22.18, p < 0.01$. hADM(22–52), further truncated ADMs, and hCT did not stimulate cAMP production at up to 1 μM. Again, desamino-rADM(14–50) was the most potent ADM examined. Coupling of binding of hCGRP-I and cAMP stimulation expressed as the ratio between EC_{50} and IC_{50} (EC_{50}/IC_{50}) was about 1 and was more efficient than that of the ADMs and of amylin (EC_{50}/IC_{50}, 2–15).

The CGRP receptor antagonist hCGRP-I(8–37) inhibited [^{125}I]hCGRP-I binding with an IC_{50} of 2.35 ± 0.45 n*M* ($n = 3$), but did not stimulate cAMP production in SK-N-MC cells (14). With 100 n*M* hCGRP-I(8–37) the EC_{50} of hCGRP-I was shifted from 0.51 ± 0.04 n*M* to 3.59 ± 0.30 n*M*, $F(1, 4) = 98.47, p <$ 0.01 (Fig. 3). A similar sevenfold shift of the EC_{50} of hADM(1–52) from 23.6 ± 0.7 n*M* to 165 ± 4 n*M* was observed in the presence of 100 n*M* hCGRP-I(8–37), $F(1, 4) = 1024.0, p <$ 0.01. Corresponding K_is for hCGRP(8–37) of hCGRP-I- and hADM(1–52)-stimulated cAMP accumulation were 16.6 ± 1.2 and 16.8 ± 1.1 n*M* ($n = 3$).

DISCUSSION

A [^{125}I]hCGRP-I receptor binding protein with an apparent molecular weight of 60,000 has been identified in the human neuroblastoma cell line SK-N-MC (14). The recently discovered ADM and amylin have the characteristic six amino acid ring structure of CGRP, and the *C*-termini are amidated. The overall homology of the corresponding sequence of amino acids between human ADM(15–52), CGRP(1–37), and amylin(1–37) is 22–24%. They therefore belong to the same family of peptides. Moreover, CGRP, ADM, and amylin have vasodilatory and, as a result, hypotensive properties (1,4,5,15).

The IC_{50} of [^{125}I]hCGRP-I binding inhibition of hADM(1–52) was sevenfold and the EC_{50} of cAMP accumulation 45-fold higher than that of hCGRP-I in SK-N-MC cells. The CGRP receptor antagonist hCGRP-I(8–37) competitively inhibited

TABLE 1

[^{125}I]hCGRP-I BINDING AND CYCLIC AMP ACCUMULATION IN SK-N-MC CELLS

	IC_{50} (n*M*)	RP (%)	EC_{50} (n*M*)	RP (%)	EC_{50}/IC_{50}
hCGRP-I	0.32 ± 0.06 (5)	100	0.40 ± 0.05 (6)	100	1
Human amylin	68.8 ± 6.60 (3)	0.5	925 ± 159 (3)	0.04	13
hADM (1–52)	2.11 ± 0.26 (3)	15	18.1 ± 2.61 (6)	2	9
hADM (13–52)	3.45 ± 0.54 (3)	9	51.3 ± 9.02 (3)	0.8	15
hADM (22–52)	>1000 (3)	<0.03	N (3)	—	—
hADM (26–52)	N (3)	—	N (3)	—	—
hADM (29–52)	N (3)	—	N (3)	—	—
rADM (1–50)	4.62 ± 0.87 (4)	7	19.6 ± 2.54 (3)	2	4
Desamino-rADM (14–50)	0.84 ± 0.21 (3)	38	1.79 ± 0.11 (3)	22	2
(Amylin)-hADM	6.94 ± 1.39 (3)	5	33.3 ± 4.03 (3)	1	5
(CGRP-I)-hADM	49.8 ± 8.08 (5)	0.6	284 ± 16.0 (3)	0.1	6

Abbreviations: RP, relative potency; N, no [^{125}I]hCGRP-I binding inhibition and no cyclic AMP stimulation at up to 1 μM peptide. Results are means ± SEM with number of experiments in parentheses.

hCGRP-I- and hADM(1–52)-stimulated cAMP production with indistinguishable K_is. In rat platelets, ADM stimulated cAMP accumulation at three times higher concentrations than those of CGRP (11). Together, the results suggest that ADM interacts with the CGRP receptor of SK-N-MC cells and platelets.

In cultured vascular smooth muscle cells, low-affinity binding ($IC_{50} \sim 100$ n*M*) of [^{125}I]ADM was not inhibited by hCGRP-I (10). There the EC_{50} of cAMP stimulation of CGRP was slightly lower than that of ADM, and was antagonized by CGRP(8–37). In another report, ADM was somewhat more potent than CGRP in raising cAMP (3). The results are consistent with a specific ADM receptor. But with respect to vasodilation, ADM was 10-fold less potent than CGRP in rat mesenteric vascular beds and was similar in the pulmonary vascular bed of the cat (2,15).

In contrast to CGRP and amylin, hADM(1–52) has additional 14 amino acids *N*-terminal to the characteristic six amino acid ring structures. Removal of 12 amino acids from the *N*-terminus did not affect vasodilation in the anesthetized cat (6). Inhibition of [^{125}I]hCGRP-I binding by hADM(1–52) and hADM(13–52) remained unchanged in the SK-N-MC cells, but the EC_{50} of cAMP stimulation was raised threefold with the *N*-terminally truncated hADM(13–52). To this end, hADM(13–52) with phenylalanine and glycine and human amylin with a single lysine residue *N*-terminal of the ring structures reveal the highest EC_{50}/IC_{50} ratios, and therefore reduced coupling between receptor binding and G-protein-linked cAMP accumulation. rADM(1–50) has 13 amino acids *N*-terminal to the ring structure. Deletion of the first 13 amino acids together with the *N*-terminal NH_2 group in desamino-rADM(14–50) lowered the IC_{50} of [^{125}I]hCGRP-I binding and the EC_{50} of cAMP accumulation 6- and 11-fold, respectively, making it the most potent ADM used to date. It would seem that introduction or deletion of a positive charge near the ring structure differentially affects receptor binding and cAMP stimulation in hADM(13–52) and desamino-rADM(14–50). Substitution of Asn as the second amino acid of the ring structure of (amylin)-hADM by an Asp in the (CGRP-I)-hADM hybrid increased the IC_{50} of [^{125}I]hCGRP-I binding and the EC_{50} of cAMP stimulation seven- and ninefold, respectively. Therefore, the ring structure of ADM contributes importantly to binding in SK-N-MC cells.

hCGRP-I(8–37), which lacks the *N*-terminal ring structure, inhibited [^{125}I]hCGRP-I binding sevenfold less well than intact hCGRP-I in SK-N-MC cells. But hADM(22–52), also lacking the ring structure, was 500-fold less potent than intact ADM. Therefore, CGRP has an important binding domain *C*-terminal to the ring structure that is not detectable in ADM. Moreover, positively charged amino acids in the *C*-terminal region of CGRP and ADM that are not present in amylin may be responsible for the higher affinity of CGRP and ADM compared to amylin.

In conclusion, we have demonstrated that ADM interacts with a CGRP receptor in SK-N-MC cells. With ADM, but not with CGRP, the ring structure is essential for receptor binding. But stimulation of cAMP production requires the presence of the ring structure in both peptides. Indeed, its removal changes CGRP from agonist to antagonist. The indistinguishable K_is with respect to the actions of ADM and CGRP indicate that ADM interacts with a CGRP receptor in SK-N-MC cells rather than with a specific ADM receptor.

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