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ADRENOMEDULLIN STIMULATES CYCLIC AMP FORMATION IN RAT VASCULAR SMOOTH MUSCLE CELLS

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SUMMARY: A newly identified human peptide, adrenomedullin (AM) increased cAMP levels with dose and time dependencies in a manner similar to that of human calcitonin gene-related peptide (CGRP) in rat vascular smooth muscle cells (VSMC). The EC_{50} value of human AM is 2×10^{-8}M which is slightly higher than that of CGRP (8.5×10^{-9} M). In a receptor binding assay for AM in rat VSMC, the binding of [^{125}I] AM was competitively inhibited by human AM, but not by human CGRP. Thus, AM is thought to increase intracellular cAMP in rat VSMC via its specific receptor to evoke vasodilation.

Adrenomedullin (AM) is a new hypotensive hormone which was discovered in human pheochromocytoma by monitoring the elevating activity of platelet cAMP [1]. AM has little homology in amino acid sequence to calcitonin gene related peptide (CGRP) in a six residue ring structure formed by an intramolecular disulfide linkage and the C-terminal amide structure [1]. AM is found to be abundant in the normal adrenal medulla and is widely distributed in normal human tissues including lung and kidney. Furthermore, AM was found to circulate in blood in a considerable concentration. Thus, it is suggested that AM is a circulating hormone different from CGRP which functions as a neuropeptide [2]. The hypotensive activity of AM is comparable to that of CGRP [1], which is one of the strongest vasorelaxants known. The former study, the hemodynamic effect of human AM, indicated that AM reduced blood pressure mainly by vasodilatation. In addition, AM induces vasodilatation in the perfused rat mesenteric vascular bed [9]. Therefore, AM has been suggested to reduce blood pressure mainly by vasodilatation. However, the vasodilatation mechanism of AM has never been elucidated.

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Abbreviations: AM, adrenomedullin; CGRP, calcitonin gene-related peptide; VSMC, vascular smooth muscle cells; DMEM, Dulbecco's modified Eagle's medium; RIA, radioimmunoassay.

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In order to further elucidate the mechanism of vasodilation, we investigated the effect of AM in rat vascular smooth muscle cells.

MATERIAL AND METHODS

Peptides: Human synthetic AM was prepared by solid phase methods in the Peptide Institute, Inc. (Osaka, Japan). Human CGRP and human CGRP-(8-37) were purchased from Peptide Institute, Inc. (Osaka, Japan).

Culture of Rat Vascular Smooth Muscle Cells (VSMC): Rat VSMC was derived from an explant of the aorta by enzymatic dispersion as previously described [4] (male Wister - Kyoto rats) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco), 100 U/ml penicillin and 100 μg/ml streptomysin at 37 ℃ in a humidified atmosphere of 95% air / 5% CO_2 [5]. Cells were used for experiments between the 4th and 12th passages.

Receptor binding assay: Cells were cultured in 24-well multiplates (Corning) to be confluent (ca. 2 $\times 10^5$ cells/ well). Cells were incubated with 2.5×10^{-12} M [^{125}I] adrenomedullin (ca. 2×10^5 cpm) and unlabeled peptide ranging from 1×10^{-12} M to 1×10^{-5} M in 0.25 ml of DMEM containing 0.1% bovine serum albumin and 20mM HEPES, pH 7.4, for 2 hr at 4℃. After incubation, Cells were washed extensively with the same medium, and the bound [^{125}I] AM was solubilized with 0.5N NaOH. Radioactivity was measured by γ -spectrophotometry (ARC-500, Aloka). Total binding was defined by incubating the cells with [^{125}I] AM.

cAMP accumulation assay: VSMC was cultured in 24-well plates, and confluent cells were incubated with peptides in 0.5 ml DMEM containing 0.1% BSA, 20mM HEPES and 0.5mM 1- metyl 3-isobutylxanthine (IBMX), pH 7.4, at room temperature for 1 hr. The culture medium after incubation was added to 100 μl of 50mM sodium acetate buffer (pH 6.2). Cyclic AMP in the solution was succinylated by adding 30 μl of dioxan containing 650mM succinic anhydrate and 10 μl of triethylamine. After standing at room temperature for 30 min, the solution was lyophilized, dissolved in RIA buffer and analyzed by RIA for cAMP as reported previouly [6]. Assays were routinely performed in duplicate.

Statistics: All values were expressed as means $\pm$ S.E.M. The results was evaluated by a one-way analysis of variance, and significance of differences was subsequently determined by Anova's test.

RESULTS

Figure 1A shows the generation of cAMP concentration in cultured VSMC by human AM and CGRP. Human AM increased cAMP at lower concentrations and produced a dose dependent increase in a manner similar to that of CGRP. The increase in cAMP concentration reached up to 15 times the basal level at 10^{-6}M of human AM. The EC_{50} value of human AM (2×10^{-8}M) is slightly higher than that of CGRP (8.5×10^{-9} M). Fig. 1B shows the time course of cAMP production by AM and CGRP. VSMC were exposed to each peptide at a concentration of 10^{-7}M during increased time periods, up to 120 min. AM increased cAMP concentration in a time dependent fashion up to 120 min in a way similar to that of CGRP.

To investigate whether VSMC has a specific receptor for AM, receptor binding assay for AM was performed in rat VSMC. As shown in Fig. 2, unlabeled AM competitively inhibited the binding of [^{125}I] AM and the IC_{50} value for human AM was 7.3×10^{-8} M in VSMC.

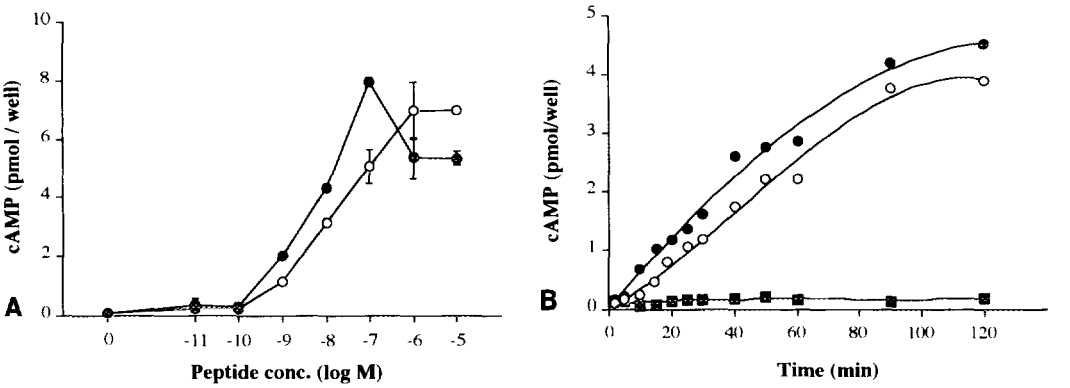

Figure 1. A: Augmentaion of cAMP concentrations by human AM (open circles) or human CGRP (closed circles) in rat vascular smooth muscle cells (VSMC). Rat VSMC were incubated with the peptide in the presence of IBMX as described in "Materials and Methods". Data represent mean values of triplicate determinations from three separate experiments in rat VSMC
1B: The time dependency of cAMP production by human AM (open circles), human CGRP (closed circles) and control (closed squares). Rat VSMC were exposed to a concentration of 10^{-7} M of each peptide during increased time periods, up to 120 min.

Figure 3 shows the inhibitory effect of human CGRP-(8-37) on cAMP production by human AM in rat VSMC. The cAMP production by human AM was inhibited by CGRP-(8-37). The inhibition of CGRP (8-37) was dependent on peptide concentration and IC_{50} value of CGRP (8-37) was estimated to be 9.3×10^{-8} M.

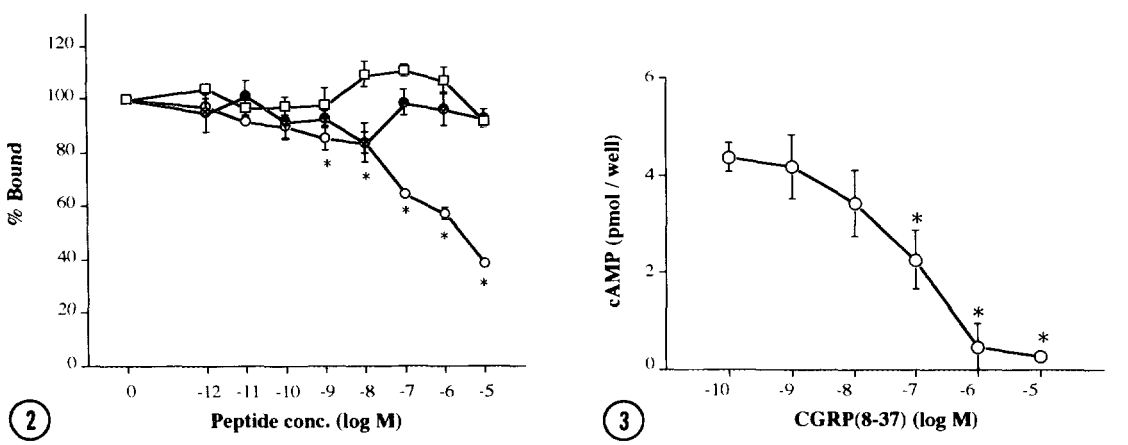

Figure 2. Displacement of [^{125}I] AM from binding sites by unlabeled AM (open circles) or CGRP (closed circles) or CGRP (8-37) (open squares) in rat VSMC. VSMC was incubated with 2.5×10^{-12}M[^{125}I] AM in the presence of unlabeled peptides. Data represent means of triplicate determinations in VSMC. *P<0.05, compared with control value.

Figure 3. Effect of human CGRP-(8-37) to inhibit an increase of cAMP concentration in rat VSMC induced by human AM (10^{-7}M; open circles). Rat VSMC were incubated with either human AM in the presence or absence (control) of increased concentrations of human CGRP-(8-37) for 1 hr at room temperture. Data is mean±SE of 4 separate experiments, with each experiment done in duplicate.
*P<0.05, compared with control value.

DISCUSSION

The present study clealy demonstrates for the first time that AM stimulates cAMP generation in vascular smooth muscle cells in a dose- and time-dependent manner. The EC_{50} value of human AM (2×10^{-8}M) is comparable to that of CGRP which is one of the strongest vasorelaxants known [7]. It is well accepted that CGRP employs cAMP as a second messenger [8], and receptors for CGRP in rat VSMC are linked to the adenylate cyclase - cAMP system. Therefore, we think that AM also employs cAMP as a second messenger to dilate vessels, sharing physiological functions in circulation control with CGRP.

Although the sequence homology between AM and CGRP is very low, pharmacological effects of both hormones are very similar. However it has never been elucidated whether AM has its specific receptor. The present study also demonstrate the presence of specific binding sites for AM in cultured rat VSMC. The binding sites for AM are different from those of CGRP because the binding of [^{125}I] AM was not inhibited by human unlabelled CGRP or CGRP (8-37). The IC_{50} value for human AM was 7.3×10^{-8} M in VSMC, which is comparable to that of EC_{50} for AM as shown in Fig 1A, suggesting that the binding site may contribute to accumulation of cAMP in VSMC.

In our previous study [9], we demonstrated that AM relaxes precontracted rat mesenteric vascular bed in vitro, whose effect is inhibited by CGRP(8-37), which is antagonist for CGRP. In the present study, we can show nonconflicting data that CGRP (8-37) inhibit cAMP generation by AM in rat VSMC. Therefore, our data indicate that CGRP (8-37) antagonize cAMP production by AM, but does not antagonizes specific AM binding. We cannot explain the reason of this phenomenon at present, but this discrepancy may be due to the specificity of AM (8-37) as an antagonist for CGRP. Because it has been reported that CGRP (8-37) also inhibit several biological effects induced by calcitonin [10], amylin [11] and capsaisin [12]. In order to explain these curious results and to elucidate the physiogical role of AM, cDNA cloning of AM's receptor is now undergoing.

In conclusion, it is evident that AM increase intracellular cAMP in rat VSMC via its specific receptor to evoke vasodilation.

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REFERENCES

1) Kitamura, K., Kangawa, K., Kawamoto, M, Ichiki, Y., Nakamura, S, Matsuo, H.and Eto, T. (1993) Biochem. Biophys. Res. Commun. 192, 553-560.
2) Morris, HR., Panio, M., Etienne, T., Tippins, J., Girgis, SI. and MacIntyre, I. (1984) Nature, 309, 746-748.
3) Ishiyama, Y., Kitamura, K., Ichiki, Y., Nakamura, S, Kida, O., Kangawa, K. and Eto, T. (1993) Eur. J. Pharmacol. 241, 271-273.

4) Chamley, J.H., Campbell, G.R., McConnell, J.D. and Groschel-Stewart, U. (1977) Cell Tissue Res. 177, 503-522.
5) Ross, R.(1971) J. Cell Biol., 50, 172-186.
6) Kitamura, K., Kangawa, K., Kawamoto, M, Ichiki, Y., Matsuo, H. and Eto, T. (1992) Biochem. Biophys. Res. Commun. 185, 134-141.
7) Brain, S. D., Williams, T. J., Tippins, J. R., Morris, H. R., and MacIntyre, I. (1984) Nature, 54-56.
8) Kubota, M., Moseley, J. M., Butera, L., Dusting, G. J., MacDonald, P. S. and Martin, T. J. (1985) Biochem. Biophys. Res. Commun. 132, 88-94.
9) Nuki, C., Kawasaki, H., Kitamura, K., Takenaga, M., Kangawa, K., Eto, T., and Wada, A. (1993) Biochem. Biophys. Res. Commun. 192, 553-560.
10) Han, SP., Naes, L. and Westfall, TC. (1990) Biochem. Biophys. Res. Commun. 168, 786-791.
11) Deems, R. O., Cardinaux, F., Deacon, R. W. Young, D. A.(1991) Biochem. Biophys. Res. Commun. 181, 116-120.
12) Zochodne, D. W. J. Physiol. (1991) 444, 615-630.