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Proadrenomedullin N-terminal 20 peptide (PAMP) inhibits proliferation of human neuroblastoma TGW cells

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Abstract We investigated the effects of proadrenomedullin N-terminal 20 peptide (PAMP) and adrenomedullin (AM) on the growth of human neuroblastoma TGW cells. Both PAMP and AM inhibited growth and DNA synthesis in neuroblastoma cells. Calcitonin gene-related peptide $(CGRP)_{8-37}$, an antagonist to CGRP, abolished the inhibitory effect of AM on growth and DNA synthesis of neuroblastoma cells but did not affect that of PAMP. AM_{22-52}, an antagonist to AM, also reversed the effect of AM. On the other hand, pertussis toxin (PTX) and ω-conotoxin GIVA blocked the effect of PAMP alone. Thus, PAMP inhibits the growth of neuroblastoma cells by inhibiting N-type Ca^{2+} channels through PTX-sensitive G protein-coupled receptors, which is different mechanism of AM-induced inhibition of the cell growth.

Key words: Adrenomedullin; Pertussis toxin; Calcitonin gene-related peptide; Cell growth; ω-Conotoxin GIVA

1. Introduction

Proadrenomedullin N-terminal 20 peptide (PAMP) [1] is a novel gene-related peptide of adrenomedullin (AM) [2], which is recently identified vasodilator peptide from human pheochromocytoma. Both PAMP and AM are hypotensive peptides generated from post-transcriptional enzymatic processing of the 185-amino acid prepro-AM molecule [1–3]. However, they exhibit the vasodilator effect through different ways. AM exhibited the direct vasodilator effect associated with increased cyclic AMP in vascular smooth muscle cells (VSMC) [4] whereas PAMP dilates vasculature through the sympathetic nerve suppression [5,6].

The vasodilator effect with AM is blocked by the antagonist of CGRP, $CGRP_{8-37}$, suggesting that the effect of AM is mediated through CGRP-related receptor [4]. However, increased cyclic AMP with AM is not inhibited by $CGRP_{8-37}$ in vascular endothelial cells [7] so the AM receptors in endothelial cells are different from VSMC. Study using ^{125}I-AM indicates that AM receptors exist in plenty of tissues such as lung and heart and that there are several kinds of AM receptors [8]. Recently, one of AM receptors that contains 7 transmembrane domains have been cloned and it is the orphan receptor that has been originally isolated from rat lung [9]. This receptor does not respond to CGRP and exhibits AM-specific elevation of cyclic AMP. On the other hand, PAMP has no homology of amino acid sequence to AM or CGRP and its vasodilator effect through the sympathetic suppression is not inhibited by $CGRP_{8-37}$ [5,6]. Pertussis toxin (PTX)-sensitive G protein may inhibit N-type Ca^{2+} channels and mediate the inhibition of norepinephrine release with PAMP [10,11]. Moreover, the recent report indicates that specific binding sites for PAMP are widely distributed in many organs such as brain, lung, aorta, kidney, adrenal glands [12] so PAMP may show the other effect than the sympathetic suppression.

In addition to vasodilator effect, AM has been reported to modify cell growth. For example, AM suppresses the growth of rat mesangial cells through enhanced cyclic AMP pathway [13], whereas it stimulates growth in Swiss 3T3 cells, which is also mediated by elevation of cyclic AMP [14]. However, there has been no data concerning the effect of PAMP on cell growth. Thus, we investigated the effect of PAMP as well as AM on the growth of human neuroblastoma TGW cells [15] because PAMP acts on neural cells [5,6,10,11] and because neuroblastoma cells have a receptor to be interacted by AM [16,17].

2. Materials and methods

2.1. Materials

Human AM, human PAMP, and $CGRP_{8-37}$ was purchased from the Peptide Institute, Inc. (Osaka, Japan). PTX was purchased from Seikagaku, Co. (Tokyo, Japan), MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) from Sigma Chemical Co. (St. Louis, MO, USA), AM_{22-52} from Phoenix Pharmaceuticals, Inc. (Mountain View, CA, USA), and ω-conotoxin GIVA (ω-CTX) from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA, USA).

2.2. Cell culture

Human neuroblastoma TGW cells [15] were cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS). For MTT or BrdU5-bromo-2′-deoxyuridine) study, the cells were seeded into 96-well polyvinylchloride plates at a density of approximately 10^5 cells/mL (100 μL/well) in RPMI medium with 1.0% FBS. After 24 h incubation, PAMP at dose of 10^{-7}, 3×10^{-7}, or 10^{-6} mol/L and AM at dose of 5×10^{-9}, 2×10^{-8}, or 5×10^{-8} mol/L were added into each well. Also, simultaneously administration of $CGRP_{8-37}$ (10^{-5} mol/L for PAMP, 10^{-6} mol/L for AM), AM_{22-52} (10^{-6} mol/L), PTX (1 mg/mL), and ω-CTX (10^{-7} mol/L) was done in some wells.

2.3. Assay

Cell growth was assayed by MTT method 24, 48, and 96 h after treatments. In brief, we added 10 μL of MTT (5 mg/mL) into wells. After 4 h incubation, we removed medium and cells was solubilized by 100 μL dimethylsulfoxide. The Bio-Rad microplate Manager plate reader (Model 3550) and software was used to determine the number of viable cells measured with excitation 570 nm and remission 630 nm.

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Abbreviations: AM, adrenomedullin; ANOVA, analysis of variance; BrdU, 5-bromo-2′-deoxyuridine; CGRP, calcitonin gene-related peptide; FBS, fetal bovine serum; MoAb-G6, polyclonal antibody against P072, a fragment of AM; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PAMP, proadrenomedullin N-terminal 20 peptide; PTX, pertussis toxin; TMB-8, 8-[*N*,*N*-dimethlyamino]octyl-3,4,5-trimethoxybenzoate hydrochloride; VSMC, vascular smooth muscle cells; ω-CTX, ω-conotoxin GIVA

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DNA synthesis was examined by BrdU incorporation assay kit (Cell Proliferation ELISA System, version 2; Amersham International plc., Buckinghamshire, UK) 96 h after treatments.

2.4. Statistical analysis

Each experiment was done with triplet and repeated 3 to 4 times. The average of these data are expressed as mean ± S.E.M. The data of MTT assay and BrdU incorporation was expressed as percentage of the data with control (untreated) cells. They were evaluated by one-way or two-way analysis of variance (ANOVA) and subsequent multiple comparison by Tukey's method. *P*-values of <0.05 level were accepted as significant.

3. Results

Both PAMP (10^{-6} mol/L) and AM (5×10^{-8} mol/L) inhibited cell growth as shown in Fig. 1: the significant inhibition was observed 48 h after treatment (PAMP: 72 ± 11%, AM: 88 ± 3%, $P<0.05$, respectively) and this effect was continued to at least 96 h (PAMP: 56 ± 16%, AM: 70 ± 5%, $P<0.05$). The inhibitory effect of 96 h treatment of PAMP and AM on growth of TGW cells was dose-dependent fashion (Fig. 2).

Simultaneous administration of 10^{-5} mol/L of $CGRP_{8-37}$ did not affect the suppression of PAMP (5×10^{-8} mol/L) on cell growth (81 ± 7% vs. 55 ± 3%, $P<0.05$) but 1 mg/mL PTX abolished it (60 ± 2% vs. 63 ± 1%, n.s.; Fig. 3). Also, the effect of PAMP was inhibited by 10^{-7} mol/L ω-CTX (72 ± 7% vs. 87 ± 12%, n.s.). In contrast, the effect of AM (10^{-6} mol/L) was blocked by $CGRP_{8-37}$ (88 ± 7% vs. 89 ± 5%, n.s.) and AM_{22-52} (105 ± 7% vs. 108 ± 6%, n.s.) but not by PTX (69 ± 4% vs. 52 ± 2%, $P<0.05$). The results of the BrdU incorporation showed similar tendency (Fig. 4). Both PAMP and AM inhibited growth of neuroblastoma cells (PAMP: 29 ± 19%, AM: 79 ± 4%, $P<0.05$, respectively). The PAMP-induced suppression of DNA synthesis was abolished by PTX alone (23 ± 11% vs. 23 ± 5%, $P<0.05$) whereas the effect of AM was blocked by $CGRP_{8-37}$ (88 ± 17% vs. 92 ± 7%, $P<0.05$).

4. Discussion

We firstly demonstrated the effect of PAMP on cell growth: The proliferation of human neuroblastoma TGW cells was attenuated by PAMP as well as AM. However, PAMP inhibited cell growth at higher (about 20–50 times) doses than AM did: Similarly, hypotensive effect of AM is greater (about 50 times) than that of PAMP in conscious rats in our previous study [6]. The mechanism of the effects should be different between PAMP and AM. The inhibition of cell growth by PAMP was abolished by PTX and ω-CTX whereas that by AM was blocked by $CGRP_{8-37}$ and AM_{22-52}. Thus, PAMP attenuates the growth of neuroblastoma cells possibly by inhibiting N-type Ca^{2+} channels through PTX-sensitive G protein-coupled receptors but AM does not. In contrast to AM, cyclic AMP formation is not affected by rat PAMP in rat VSMC, which reveals PAMP binding sites [12].

We have already demonstrated that PAMP exhibits vasodilator effect through the peripheral sympathetic suppression, which is due to blockade of norepinephrine release [5,6]. The previous report suggests that PAMP inhibits the N-type Ca^{2+} channel through PTX-sensitive G protein in nerve growth factor-treated PC 12 cells [10]. In the present study, PTX and ω-CTX abolished the effect of PAMP on growth of neuroblastoma cells. Thus, changes in intracellular Ca^{2+} might be

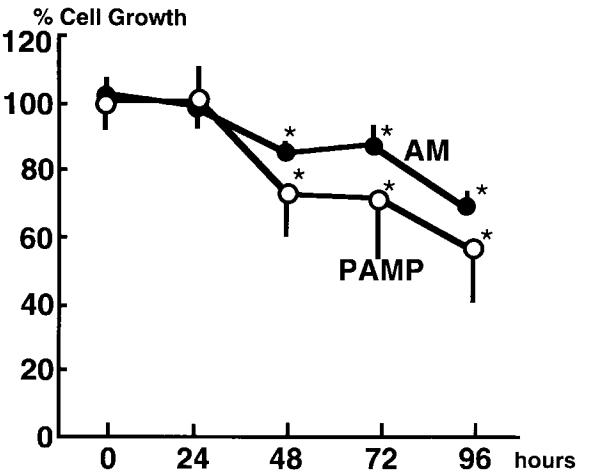

Fig. 1. Time-dependent effect of proadrenomedullin N-terminal 20 peptide (PAMP: 10^{-6} mol/L; open circle) and adrenomedullin (AM: 5×10^{-8} mol/L; closed circle) on growth of TGW cells. $^*P<0.05$.

intimately related to the cell growth inhibition because N-type Ca^{2+} channel has been demonstrated to be coupled to PTX-sensitive G protein in neuroblastoma cells [18,19]. In fact, Ca^{2+} channel antagonists, such as verapamil, nifedipine, diltiazem, Ni^{2+}, and Co^{2+}, inhibit growth of human neuroblastoma cells probably through interference with agonist-induced intracellular Ca^{2+} mobilization [20]. Also, K^+ channel modulators and high extracellular K^+ inhibit cell growth accompanied with blockade of intracellular Ca^{2+} mobilization in neuroblastoma cells [21]. Although we did not observed morphological changes in TGW cells, blockade of intracellular Ca^{2+} mobilization by TMB-8 (8-[*N*,*N*-dimethlyamino)octyl-3,4,5-trimethoxybenzoate hydrochloride) induces neuroblastoma cell differentiation [22]: PAMP might induce cell differentiation in the other experimental condition. Moreover, because PAMP receptors are widely distributed [12], PAMP may also exert antiproliferative effect in other cells than neural cells.

The inhibition of growth of neuroblastoma cells with AM was reversed by $CGRP_{8-37}$ and AM_{22-52}, which is similar to the results in cultured rat mesangial cells [23]: both $CGRP_{8-37}$ and AM_{22-52} inhibited the increased production of cyclic AMP by AM or CGRP. Thus, CGRP-related receptor may play an important role. However, $CGRP_{8-37}$ slightly inhibited the cell growth (control of PAMP: 81 ± 7%, $P<0.05$, control of AM: 88 ± 7%, n.s.) but AM_{22-52} did not affect it at all (105 ± 7%, n.s.). This might be due to intrinsic factors affected CGRP receptors other than AM, such as CGRP or amylin [24], which might stimulate growth of neuroblastoma cells. Although AM_{22-52} antagonizes vasodilator response to CGRP but not AM in the cat [25], AM_{22-52} might block the other receptors than CGRP receptors, resulting in unchanged cell growth. However, the present data did not clarify the different response of TGW cells between $CGRP_{8-37}$ and AM_{22-52}, so the further investigation should be required. Thus, multiple receptors might affect growth of neuroblastoma cells although the cells have a receptor to be interacted by both AM and CGRP [16,17]. AM has been reported to modify cell growth by several investigators, which is different be-

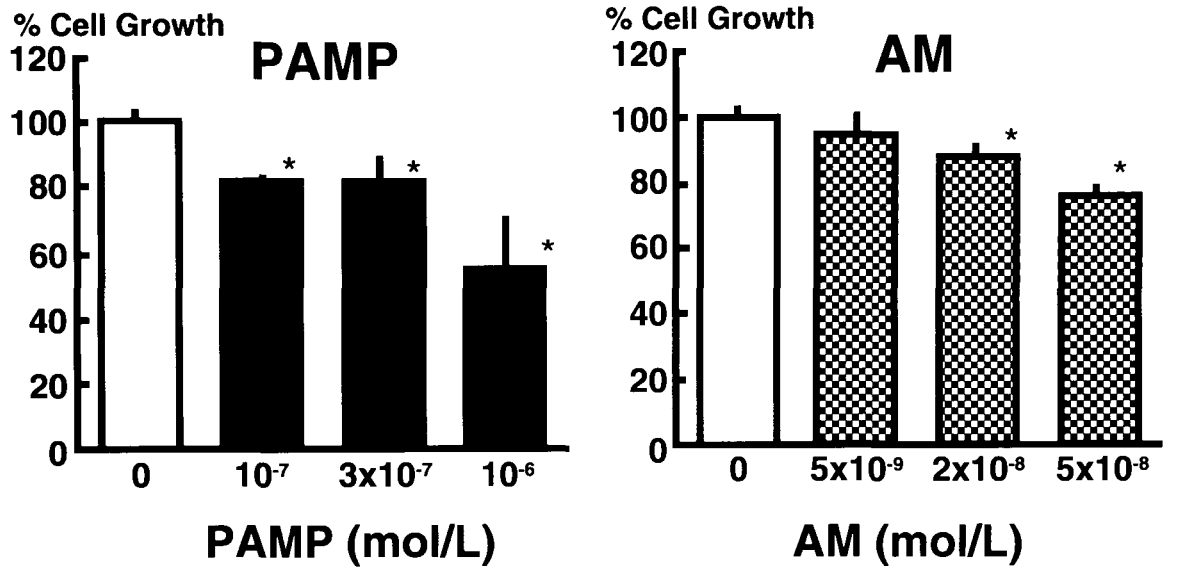

Fig. 2. Effect of proadrenomedullin N-terminal 20 peptide (PAMP; closed column) and adrenomedullin (AM; shaded column) at different dose on growth of TGW cells. $^{*}P < 0.05$.

tween cell types: AM suppresses the growth in rat mesangial cells and VSMC [13] but stimulates it in Swiss 3T3 cells [14]. Also, MoAb-G6 (polyclonal antibody against P072, a fragment of AM) has been demonstrated to inhibit growth of several human tumor cell lines [26]. On the other hand, in hepatic pericytes, AM has negligible effects on DNA and protein synthesis [27]. These phenomena accompany with the elevation of cyclic AMP (even in hepatic pericytes) so these results suggest that the responses to cyclic AMP are different between cell types. In cultured human neuroblastoma cell lines, the increased generation of cyclic AMP by vasoactive intestinal peptide [28,29], pituitary adenylate cyclase activating peptide-38 [29], and Eudistoma alkaloids [30] induces the growth inhibition. Thus, increased cyclic AMP with AM treatment may also inhibit growth of the neuroblastoma cells. Recently, AM has been demonstrated in neural cells in mouse

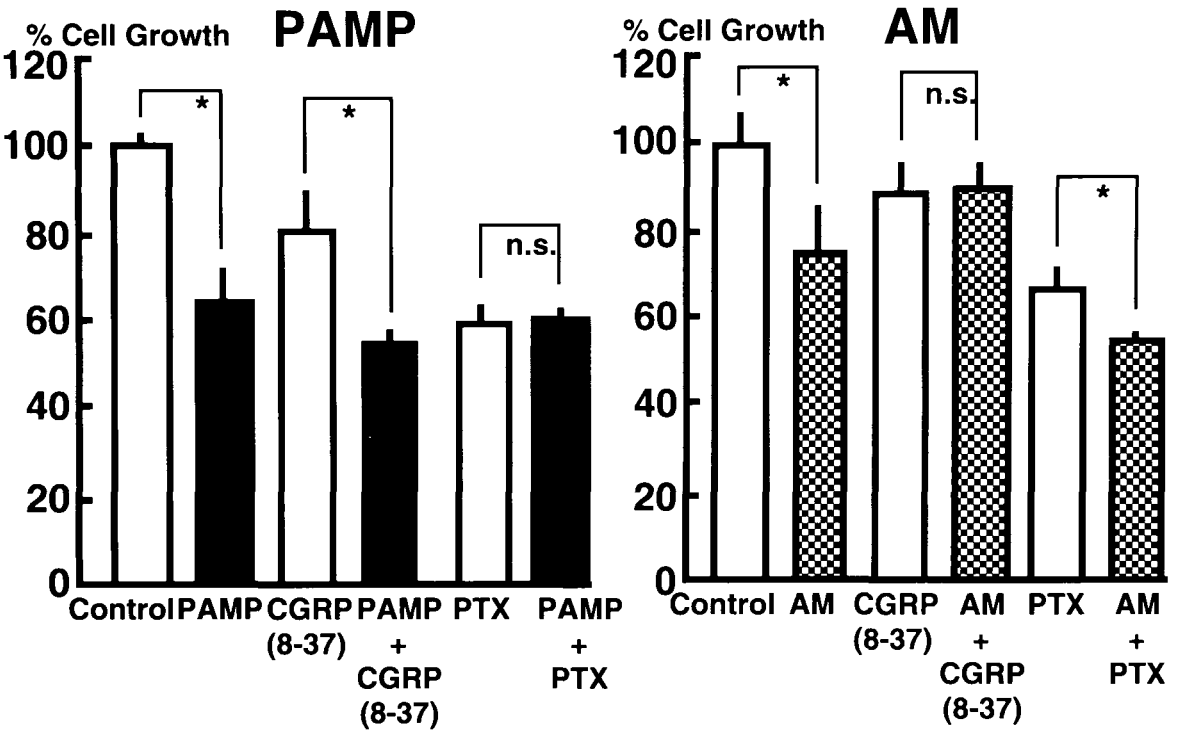

Fig. 3. Influence of calcitonin gene-related peptide $(CGRP)_{8-37}$ and pertussis toxin (PTX) on the effect of proadrenomedullin N-terminal 20 peptide (PAMP; closed column) and adrenomedullin (AM; shaded column) on growth of TGW cells. $^{*}P < 0.05$.

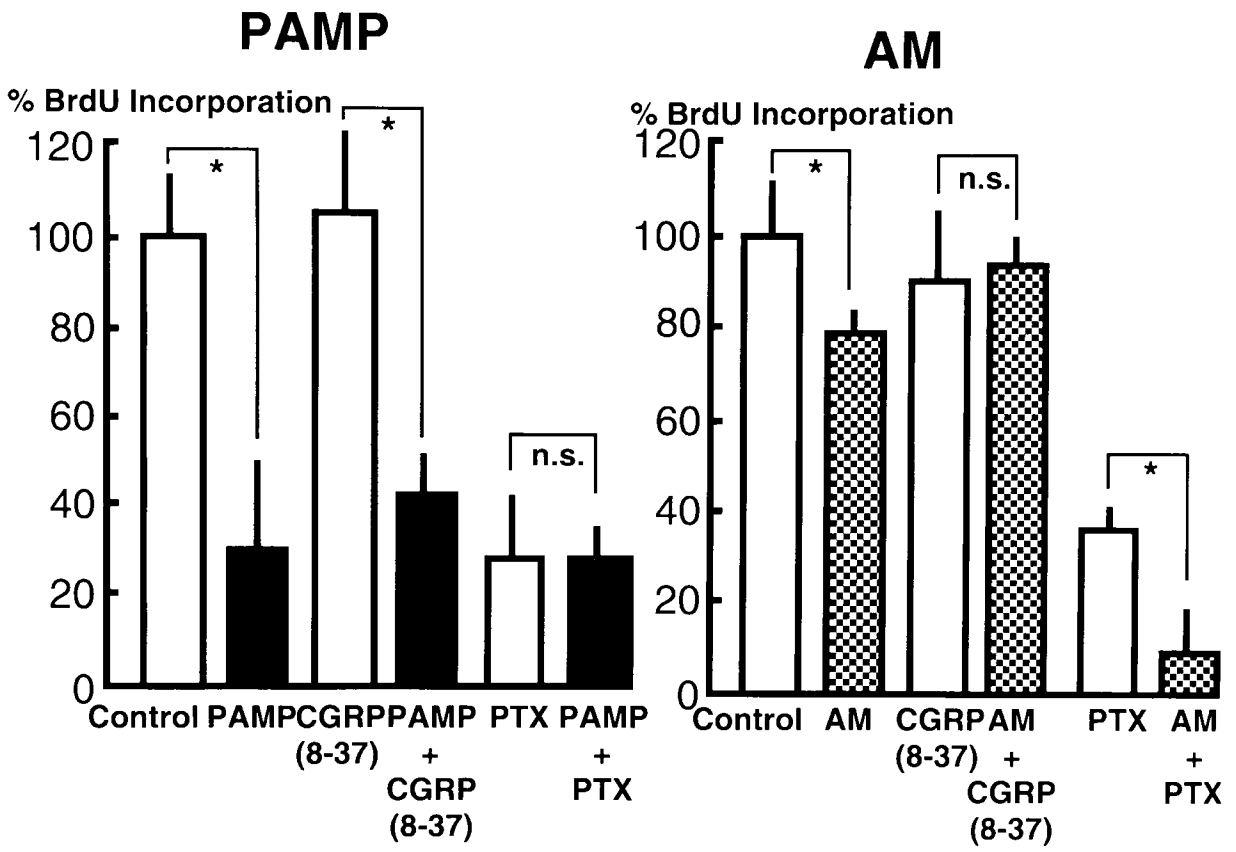

Fig. 4. Influence of calcitonin gene-related peptide $(CGRP)_{8-37}$ and pertussis toxin (PTX) on the effect of proadrenomedullin N-terminal 20 peptide (PAMP; closed column) and adrenomedullin (AM; shaded column) on DNA synthesis (BrdU incorporation) of TGW cells. $^*P < 0.05$.

and rat embryo, suggesting that AM plays a role in differentiation of neural tissues [31].

In conclusion, PAMP inhibits the neuroblastoma cell growth through PTX-sensitive G protein-coupled receptors whereas AM induces the inhibition of cell growth through the other mechanism(s). Both peptides may contribute to the regulation of neural cell growth and differentiation through different ways.

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