Proadrenomedullin NH₂-terminal 20 peptide (PAMP) and adrenomedullin bind to teratocarcinoma cells

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Received 14 June 1999; accepted 1 September 1999

Abstract

Proadrenomedullin NH₂-terminal 20 peptide (PAMP) and adrenomedullin (ADM) bind to teratocarcinoma cells. The effects of PAMP and ADM on teratocarcinoma cells were investigated. ¹²⁵I-PAMP bound to PA1 cells with moderate affinity (Kₐ = 110 nM) to a single class of sites (Bₘₒₓ = 110 000/cell). Specific ¹²⁵I-PAMP binding was inhibited by PAMP (IC₅₀ of 100 nM) but not ADM, calcitonin gene-related peptide (CGRP), or amylin. Specific ¹²⁵I-ADM binding was inhibited with high affinity by ADM, CGRP, and CGRP(8–37) (IC₅₀ values of 10, 10, and 15 nM respectively) but not PAMP or amylin. ADM elevated cAMP (ED₅₀ value of 100 nM), whereas PAMP had no effect on basal cAMP but inhibited the increase in cAMP caused by 10 nM ADM. Also, the increase in cAMP caused by ADM was inhibited by CGRP(8–37), suggesting that ADM is binding to CGRP receptors. ADM (100 nM) stimulated transiently c-fos mRNA, whereas PAMP (1000 nM) had little effect; however, PAMP inhibited the increase in c-fos mRNA caused by ADM. ADM stimulated [³H]thymidine uptake into PA1 cells, whereas PAMP inhibited the increase in thymidine uptake caused by ADM. These results indicate that ADM and PAMP are both biologically active in teratocarcinoma cells. Published by Elsevier Science Inc.

Keywords: PAMP; Adrenomedullin; Receptors; cAMP; C-fos mRNA; Growth

1. Introduction

Adrenomedullin (ADM) and its gene-related peptide proadrenomedullin N-terminal 20 peptide (PAMP) are derived from a 185-amino acid precursor molecule [12,14,39]. Originally isolated from pheochromocytoma, ADM has been localized to the adrenal gland, lung, cardiac ventricle, kidney, pancreas, and brain, especially the thalamus and hypothalamus [7,30]. In the periphery, ADM is a potent hypotensive agent interacting with receptors on endothelial cells and vascular smooth muscle cells [5] and regulating electrolyte homeostasis [32]. ADM inhibits ACTH secretion from the pituitary, insulin secretion from the pancreas, and catecholamine secretion from the adrenal gland [28]. PAMP is biologically active and it is a hypotensive agent [4,15] that decreases catecholamine secretion [13,29,36].

ADM has sequence homologies with calcitonin gene-related peptide (CGRP). The ADM receptor (G10D) binds ADM but not CGRP with high affinity and stimulates adenylyl cyclase [10]. The CGRP receptor binds the agonists ADM and CGRP as well as the antagonist CGRP [8–37] with high affinity [1,11,43]. Recently, it was found that RAMP proteins are essential for the biologic activity of CGRP and ADM receptors [20]. If the CRLR receptor was combined with RAMP-1, a CGRP response resulted, whereas if the CRLR receptor was transfected with RAMP-2, an ADM response resulted [20]. Thus, RAMP proteins are essential for proper CGRP and ADM receptor function.

Previously, we showed that ADM receptors are present on ovarian, lung, glioma, and breast cancer cells [18,19,21], and that many tumor cell lines express the peptide and receptor for ADM. The actions of ADM can be suppressed by monoclonal antibody (mAb)G6, which neutralizes ADM. Because mAbG6 reduces the growth of breast, lung, and ovarian cancer cells, ADM may function as an autocrine growth factor in cancer cells [21]. Here the effects of PAMP and ADM were investigated on teratocarcinoma cells.
2. Methods

Human teratocarcinoma (PA1) and choriocarcinoma (JAR) cells were obtained from ATCC and cultured in RPMI-1640 media containing 10% heat-inactivated fetal bovine serum (FBS). The cells were cultured in 5% CO2/95% air at 37°C and were used in exponential growth phase. PA1 and JAR cells, which are adherent, were split (1/20) weekly using trypsin-EDTA.

PA1 cells were fed twice weekly, and a day after feeding, binding experiments were conducted. The cells were washed three times with SIT medium (RPMI-1640 containing 3 × 10⁻⁸ M SeO₂, insulin (5 μg/ml) and transferrin (10 μg/ml)). The cells were incubated in receptor-binding medium (SIT medium containing 1% bovine serum albumin and 1 mg/ml bacitracin) with 0.2 nM ¹²⁵I-ADM or ¹²⁵I-PAMP (2200 Ci/mmol; Phoenix Pharmaceuticals, St. Joseph, MO, USA) in the presence or absence of competitor. After 1 h at 4°C, free peptide was removed by washing three times in receptor binding medium. Peptide bound to the cells was solubilized in 0.2 N NaOH and counted in a gamma counter.

The ability of ADM or PAMP to alter cAMP was investigated. Confluent PA1 cells in 24-well plates were washed three times in 0.5 ml of SIT medium and incubated with SIT medium containing 1% bovine serum albumin, 1 mg/ml bacitracin, and 200 μM isobutylmethylxanthine. After 20 min, peptides were added, and after a 5-min incubation at 37°C, 250 μl of supernatant was removed and 250 μl of iced ethanol was added. The cell suspensions were mixed, stored at −80°C until use, and assayed for cAMP by radio-immunoassay [16].

The ability of ADM or PAMP to stimulate nuclear oncogene (c-fos) production was investigated. PA1 cells were cultured with SIT medium containing 0.5% FBS. After 4 h, the cells were treated with increasing doses of ADM. After 1 h, total RNA was isolated by using guanidine isothiocyanate. Ten micrograms of denatured RNA was separated in a 0.66 M formaldehyde 1% agarose gel. The gel was treated with ethidium bromide to assess RNA integrity. The RNA was blotted onto a nylon membrane overnight, and the membrane hybridized with cDNA probes (1.25 kb cDNA of human c-fos labeled with ³²P-dCTP with a Bethesda Research Laboratories random priming kit [23]). The membrane was exposed to Kodak XAR-2 film at −80°C for 1 day and the autoradiogram developed.

The ability of ADM to alter proliferation of PA1 cells was investigated. When a monolayer of cells formed, SIT medium containing 0.5% FBS was added followed by ADM and/or PAMP. After 16 h, ³H-deoxyribose thymidine (10⁶ cpm) was added for 2 h. The 24-well plates were washed three time with cold PBS, one time with cold 5% trichloroacetic acid, and one time with cold ethanol/ether (2:1). After air drying, the plates were treated with 0.2 N NaOH and counted in a β counter.

<table>
<thead>
<tr>
<th>Table 1 Specific binding of ADM and PAMP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell line</strong></td>
</tr>
<tr>
<td>Pituitary cancer</td>
</tr>
<tr>
<td>GH3</td>
</tr>
<tr>
<td>A2f-20</td>
</tr>
<tr>
<td>Glioblastoma</td>
</tr>
<tr>
<td>U87</td>
</tr>
<tr>
<td>U138</td>
</tr>
<tr>
<td>U373</td>
</tr>
<tr>
<td>Teratocarcinoma</td>
</tr>
<tr>
<td>PA1</td>
</tr>
<tr>
<td>Choriocarcinoma</td>
</tr>
<tr>
<td>JAR</td>
</tr>
</tbody>
</table>

The mean cpm ± SD of four determinations each repeated in quadruplicate is indicated using 0.5 × 10⁶ cells.

3. Results

Table 1 shows that ¹²⁵I-ADM and ¹²⁵I-PAMP bound specifically to a wide variety of cells including pituitary cancer, glioblastoma, choriocarcinoma, and teratocarcinoma cells. Because ¹²⁵I-PAMP bound best to PA1 teratocarcinoma cells, its binding was further characterized.

¹²⁵I-PAMP binding to PA1 cells was time dependent. Total binding increased rapidly during the first 10 min and then slowly for the next 50 min (Fig. 1). In contrast, nonspecific binding changed little as a function of time. The difference between the two is specific binding, which increased rapidly the first 10 min and slowly the next 50 min. The ratio of specific/nonspecific binding was approximately 1:1.

¹²⁵I-PAMP binding was investigated as a function of ligand concentration. Fig. 2 shows that ¹²⁵I-PAMP bound to PA1 cells with moderate affinity (Kd = 110 nM) to a high density of sites (Bmax = 110 000/cell). Table 2 shows the specificity of binding. ¹²⁵I-PAMP binding to PA1 cells was inhibited by PAMP but not ADM, amylin, CGRP, or calcitonin. In contrast, ¹²⁵I-ADM binding was inhibited by 1000 nM ADM or CGRP, but not amylin or PAMP.

¹²⁵I-ADM binding was investigated as a function of competitor concentration (Fig. 3). Specific ¹²⁵I-ADM binding was inhibited by ADM in a dose-dependent manner by unlabeled ADM and was half-maximally inhibited (IC50) by 10 nM ADM. In contrast, PAMP had no effect on ¹²⁵I-ADM binding even at 1000 nM. CGRP and CGRP [8–37] had IC50 values of 10 and 15 nM.

The effect of ADM and PAMP on cAMP was investigated. Fig. 4 shows that ADM elevated cAMP in a dose-dependent manner with little stimulation at 1 nM and strong stimulation at 1000 nM. The half-maximal effective dose (ED50) was 100 nM for ADM, whereas PAMP had no effect on basal cAMP. PAMP inhibited the ADM stimulation of cAMP. Table 3 shows that PAMP, CGRP [8–37] and somatostatin (SST) had little effect on basal cAMP; however,
they significantly inhibited cAMP stimulated by 10 nM ADM.

The effects of ADM and PAMP on nuclear oncogene expression were investigated. Fig. 5 shows that 100 nM ADM stimulated c-fos mRNA. The increase in c-fos caused by 100 nM ADM was inhibited by 1000 nM PAMP, whereas PAMP had little effect on basal c-fos.

The ability of ADM and PAMP to alter the proliferation of PA1 cells was investigated. Table 4 shows that the addition of 100 nM ADM increased the [3H]thymidine uptake from 8825 to 14742 cpm. PAMP, in a dose-dependent manner, reversed the increase in [3H]thymidine uptake caused by ADM. As a positive control, 10% FBS increased the [3H]thymidine uptake to 26263 cpm.

The difference between the two (▲) represents specific binding. This experiment is representative of two others, and the lines are drawn point-to-point.

Fig. 1. [125I]-PAMP binding. Total (●) and nonspecific (○) [125I]-PAMP binding were determined. The mean value ± SD of four determinations is indicated.

Fig. 2. Scatchard plot. Specific [125I]-PAMP binding was determined as a function of [125I]-PAMP concentration. The amount bound (B)/free (F) is plotted as a function of the amount bound. The best-fit line is drawn assuming a single class of sites. This experiment is representative of three others.
4. Discussion

Previously, we found that ADM bound with high affinity to human cancer cell lines and human skin fibroblasts with high affinity [17–19,21]. ADM increased cAMP, c-fos gene expression, and proliferation of rat glioma C6 cells [23]. Here, the effects of ADM and PAMP on teratocarcinoma cells were investigated.

Two types of ADM receptors have been characterized. One type of ADM receptor that binds ADM but not CGRP or CGRP [8–37] with high affinity is present in rat tissues endothelial cells and rat vascular smooth muscle cells [8,24–27]. A second type of ADM receptor was identified in bovine aortic endothelial cells, neuroblastoma cells, and L6 cells, which bind ADM, CGRP, and CGRP [8–37] with high affinity [34,43]. There may be additional ADM receptors such as the CGRP2 receptor, which binds Cys(ACM) [2,7] CGRP with high affinity but not CGRP [8–37,38].

Previously, CGRP1 receptors were identified on F9 teratocarcinoma cells [31]. CGRP caused chemotaxis and stimulated the growth of F9 cells. Here, with PA1 cells, CGRP and CGRP [8–37] inhibited binding of 125I-ADM with high affinity (IC50 values of 10 and 15 nM, respectively). In contrast, PAMP had little effect of 125I-ADM binding (IC50 > 1000 nM). With PA1 cells, the increase in cAMP caused by ADM was antagonized by CGRP [8–37]. These results suggest that ADM binds to CGRP1 receptors stimulating adenylyl cyclase in PA1 cells.

PAMP binds with moderate affinity to PA1 cells (Kd = 110 nM). Previously, in vascular smooth muscle cells, 125I-PAMP bound with moderate affinity (Kd = 35 nM) to a single class of sites (Bmax = 4.5 × 106/cell) [9]. Specific 125I-PAMP binding to vascular smooth muscle cell membranes was inhibited by GTPγS, suggesting that the PAMP receptor interacts with a G protein [9]. Here, PAMP inhibited the increase in cAMP caused by ADM. The PAMP binding site in PA1 cells may interact with a guanine nucleotide binding protein (G), inhibiting adenylyl cyclase. Also, PAMP induces hypotension via a pertussis toxin-sensitive mechanism, suggesting that G may be involved [37]. The binding receptors for ADM and PAMP may be distinct. ADM binds to a G protein-coupled receptor of approximately 70 000 Da; 125I-PAMP is cross-linked to a 90 000-Da protein with disuccinimidyl suberate [9]. Also, SST inhibited the increase in cAMP caused by ADM. Preliminary data (T. Moody, unpublished) indicate that PA1 cells have mRNA for SST2 receptors. SST2 receptors bind octreotide with high affinity and interact with G [3]. These results suggest that ADM, PAMP, and SST bind to distinct membrane proteins in PA1 cells.

ADM elevates cAMP and the increased cAMP may stimulate protein kinase (PK) A. Subsequently, PKA will phosphorylate protein substrates such as the cAMP response element binding protein, CREB, which can enter the nucleus and alter transcription of early oncogenes [40]. Here,

### Table 2
Specificity of binding

<table>
<thead>
<tr>
<th>Addition</th>
<th>125I-PAMP bound</th>
<th>125I-ADM bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2590 ± 236</td>
<td>728 ± 78</td>
</tr>
<tr>
<td>PAMP, 1 µM</td>
<td>1110 ± 109</td>
<td>735 ± 18</td>
</tr>
<tr>
<td>ADM, 1 µM</td>
<td>2785 ± 108</td>
<td>404 ± 100</td>
</tr>
<tr>
<td>CGRP, 1 µM</td>
<td>2756 ± 114</td>
<td>586 ± 74</td>
</tr>
<tr>
<td>Amylin, 1 µM</td>
<td>2527 ± 119</td>
<td>741 ± 18</td>
</tr>
<tr>
<td>Calcitonin, 1 µM</td>
<td>2297 ± 21</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

The total cpm bound ± SD of four determinations using PA1 cells (10⁶) is indicated; n.d., not determined. This experiment is representative of two others.

Fig. 3. Specificity of binding. Specific 125I-ADM binding was determined as a function of ADM (○), CGRP (○), CGRP(8–37) (■) and PAMP (▲) concentration. The mean value ± SD of four determinations each repeated in quadruplicate is indicated.
ADM increased the c-fos mRNA 4-fold after 1 h and the increase in c-fos mRNA caused by ADM was reversed by PAMP. Similarly, ADM increased c-myc and c-jun mRNAs in PA1 cells (T. Moody, unpublished). The c-fos and c-jun may form a heterodimer and increase transcription of growth factor genes that have AP-1 sites [40]. PAMP inhibits the increase in cAMP caused by ADM. Previously, PAMP inhibited voltage-gated $\text{Ca}_{\text{2+}}$ channels through a pertussis toxin-sensitive G protein [37]. Also, PAMP-induced hypotension was inhibited by a pertussis toxin-sensitive drug [36].

ADM stimulated $[^{3}\text{H}]$thymidine uptake in PA1 cells and Swiss 3T3 cells [41]. Here, the increase in TCA-precipitable DNA caused by ADM was reversed by PAMP by using PA1 cells. These data suggest that PAMP may be a physiological antagonist of ADM. Previously, it was found that PAMP inhibited the proliferation of human neuroblastoma TGW cells [2]. It remains to be determined whether PAMP will function as an antagonist of ADM in vivo.

In addition to stimulating proliferation, ADM may have a role in development. In extraembryonic structures such as giant trophoblastic cells, high levels of ADM and PAMP are expressed [22]. High expression of ADM in trophoblastic cells at the implantation site has been reported [42]. Because ADM and PAMP are differentially expressed during development, they may regulate differentiation in a paracrine manner. Stem cells can differentiate into a wide variety of

Table 3

<table>
<thead>
<tr>
<th>Additions</th>
<th>Fmol/50 000 cells after 5 min</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>0.5 ± 0.2**</td>
</tr>
<tr>
<td>ADM, 10 nM</td>
<td>3.3 ± 0.9</td>
</tr>
<tr>
<td>ADM + 1000 nM PAMP</td>
<td>2.0 ± 0.2*</td>
</tr>
<tr>
<td>PAMP, 1000 nM</td>
<td>0.5 ± 0.1**</td>
</tr>
<tr>
<td>ADM + SST, 1000 nM</td>
<td>1.8 ± 0.5*</td>
</tr>
<tr>
<td>SST, 1000 nM</td>
<td>0.4 ± 0.2**</td>
</tr>
<tr>
<td>ADM + CGRP(8–37)</td>
<td>1.2 ± 0.4*</td>
</tr>
<tr>
<td>CGRP(8–37), 1000 nM</td>
<td>0.4 ± 0.1**</td>
</tr>
</tbody>
</table>

The mean value ± SD of four determinations is indicated $P < 0.01$; **$P < 0.05$; *from 10 nM ADM using Newman–Keul’s multiple comparisons test. This experiment is representative of three others.

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Fig. 4. ADM elevates cAMP. The ability of ADM (solid bar) and PAMP (shaded bar) to increase cAMP was determined as a function of concentration. The mean value ± SD of four determinations is indicated. This experiment is representative of three others.

Fig. 5. ADM and c-fos mRNA. C-fos mRNA was determined in PAI cells after (a, A) no additions, (b, B) 100 nM ADM, (c, C) 1000 nM PAMP, and (d, D) 100 nM ADM + 1000 nM PAMP. The c-fos mRNA (left) was determined by Northern blot (A–D), whereas the RNA loading (right) was determined by ethidium bromide staining (a–d). This experiment is representative of two others.
tissues, including teratocarcinoma [35]. The roles of ADM and PAMP in development need to be explored further.

In summary, teratocarcinoma cells bind ADM and PAMP specifically. ADM stimulates cAMP, c-fos mRNA, and the proliferation of PA1 cells, whereas PAMP inhibits the effects of ADM.

Acknowledgments

We thank Drs A. Martinez and S. Jakowlew for helpful discussions.

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