AMP $N_1$-oxide, a unique compound of royal jelly, induces neurite outgrowth from PC12 cells via signaling by protein kinase A independent of that by mitogen-activated protein kinase

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Earlier we identified adenosine monophosphate (AMP) $N_1$-oxide as a unique compound of royal jelly (RJ) that induces neurite outgrowth (neuritogenesis) from cultured rat pheochromocytoma PC12 cells via the adenosine A$_{2A}$ receptor. Now, we found that AMP $N_1$-oxide stimulated the phosphorylation of not only mitogen-activated protein kinase (MAPK) but also that of cAMP/calcium-response element-binding protein (CREB) in a dose-dependent manner. Inhibition of MAPK activation by a MEK inhibitor, PD98059, did not influence the AMP $N_1$-oxide-induced neuritogenesis, whereas that of protein kinase A (PKA) by a selective inhibitor, KT5720, significantly reduced neurite outgrowth. AMP $N_1$-oxide also had the activity of suppressing the growth of PC12 cells, which correlated well with the neurite outgrowth-promoting activity. KT5720 restored the growth of AMP $N_1$-oxide-treated PC12 cells. It is well known that nerve growth factor suppresses proliferation of PC12 cells before causing stimulation of neuronal differentiation. Thus, AMP $N_1$-oxide elicited neuronal differentiation of PC12 cells, as evidenced by generation of neurites, and inhibited cell growth through adenosine A$_{2A}$ receptor-mediated PKA signaling, which may be responsible for characteristic actions of RJ.

Keywords: adenosine A$_{2A}$ receptor – 5-bromodeoxy uridine – neuronal differentiation – neurotrophic factor

Introduction

Royal jelly (RJ), which is fed to the queen honeybee, has been reported to have a variety of biological activities towards various types of cells (1–4). Although there are few reports so far showing the effects of RJ on the nervous system, we recently found that an extract of RJ induces neurites from cultured PC12 cells, a cell line of rat pheochromocytoma, and identified adenosine monophosphate (AMP) $N_1$-oxide as one of the active components (5). AMP $N_1$-oxide is a unique compound not found in natural products other than RJ; and it suppresses the proliferation of PC12 cells and stimulates the expression of neurofilament M, a protein of mature neurons, thus demonstrating that AMP $N_1$-oxide induces neuronal differentiation of PC12 cells (5), as does nerve growth factor (NGF), a well-known neurotrophic factor that affects PC12 cells. In response to the binding of NGF to receptors p75 and TrkA, PC12 cells stop dividing and extend neurites, differentiating into neurons similar to those found in sympathetic neurons (6). NGF triggers mainly two cascades of cellular signaling that mediate neurite outgrowth, neurofilament M expression and cell survival, i.e. mitogen-activated protein kinase...
(MAPK)/extracellular signal-regulated kinase 1 or 2 (ERK1/2) and phosphatidylinositol 3-kinase/Akt pathways (6,7).

The neurite outgrowth-promoting activity of AMP N1-oxide was found to be mediated by adenylate cyclase-coupled adenosine A2A receptors (5). Adenosine A2A receptor activation leads to an increase in the cAMP level followed by activation of protein kinase A (PKA) and extracellular signal-regulated kinase 1 or 2 (ERK1/2) in PC12 cells (8,9). Activation of ERK1/2 is a checkpoint to assess the activation of the Ras/MAPK cascade. Moreover cAMP elevation induces the development of neurites similarly as treatment with NGF (9). Forskolin, an activator of adenylate cyclase, increases the cAMP level, whose increase is in turn followed by neuronal differentiation (10). Also, the rescuing effect of A2A receptor-mediated cAMP/PKA signals was reported in the case of NGF-induced neurite outgrowth from PC12 cells impaired by suppression of the MAPK cascade (11).

Therefore, in this study, we focused on elucidating the intracellular signaling by AMP N1-oxide responsible for stimulation of neurite outgrowth and/or suppression of cell growth. Also, we speculated about the action of AMP N1-oxide in RJ toward the nervous system.

Materials and Methods

Materials
AMP N1-oxide was chemically synthesized (5). The adenosine A2A receptor antagonist ZM241385 and epidermal growth factor (EGF) were purchased from Sigma (St. Louis, MO). The PKA inhibitor KT5720 was purchased from Biomol GmbH (Hamburg, Germany). The MEK inhibitor PD98059 came from Wako, and the Trk inhibitor K252a, from Santa Cruz Biotechnology (Santa Cruz, CA). NGF was purified from mouse submaxillary glands as described previously (12).

Assessments of Neurite Outgrowth and Cell Growth
PC12 cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% horse serum and 5% fetal bovine serum (6). For assessment of neurite outgrowth, cells were plated at 10^4–10^5 cells/well in six well plates coated with collagen. The cells were exposed to test agents 1 day after plating, and morphological changes were observed under a phase-contrast microscope. Neurite-bearing cells were defined as those with processes longer than the length of the cell body. Four areas, each containing 100–200 cells, were randomly selected in each well, and the neurite-bearing cells were counted. Cell proliferation was evaluated by counting the total cell number, or by incorporation of 5-bromodeoxyuridine (BrdU) (Sigma) as described (13). Cells were cultured on cover glasses coated with poly-L-lysine and collagen in medium containing test samples. The cultures were incubated for another 4 h after the addition of BrdU (10 μM), fixed with 4% paraformaldehyde, and reacted with Alexa Fluor™-conjugated anti-BrdU antibody (Sigma). For estimating total cell number, cell nuclei were stained with propidium iodide (Invitrogen, Carlsbad, CA).

Western Blotting
PC12 cells were lysed with lysis buffer [20 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl, 2 mM EDTA, 1% NP-40, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 50 mM NaF, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 0.1% sodium dodecyl sulfate (SDS) and 1% Na deoxycholate]. The lysates were centrifuged, and the protein concentration of each supernatant was determined with a BCA Protein Assay Kit (Pierce, Rockford, WI). Each sample (5 μg of protein) was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on a 10% gel. Proteins were transferred to a polyvinylidene fluoride membrane and blocked with 5% skim milk (Morinaga Milk Products, Tokyo, Japan). Next the membranes were incubated with primary antibody at 4°C overnight, washed and then reacted with alkaline phosphatase-conjugated secondary antibody at room temperature for 1 h (Promega, Madison, WI). Finally, the protein bands were developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indoryl-phosphate p-toluidine salt. The primary antibodies used were those against p44/42 MAPK, phospho-p44/42 MAPK, cAMP-response element-binding protein (CREB) and phospho-CREB (Cell Signaling Technology, Danvers, MA).

Immunoprecipitation
AMP N1-oxide or NGF was added to PC12 cell cultures, which were then incubated for 10 min, 1 h or 2 h. The cells were washed with ice-cold PBS, and lysed with the lysis buffer. The cell lysates were incubated overnight at 4°C with anti-Trk antibody (C-14; Santa Cruz Biotechnology), followed by incubation with protein A-Sepharose beads (Amersham Pharmacia Biotech, IL) for 1 h at 4°C. The pellet was then collected, washed with the lysis buffer, and subjected to SDS-PAGE. Phosphorylated TrkA was detected by Western blotting with anti-phosphotyrosine antibody (Santa Cruz Biotechnology).
Correlation Between Antimitotic Activity and Neurite Outgrowth-promoting Activity

AMP N1-oxide at concentrations between 1 and 33 µM induced neurite outgrowth from PC12 cells in a dose-dependent manner (Fig. 1A) similarly as shown earlier (5). On the other hand, when the antimitotic effect of AMP N1-oxide on PC12 cells was examined by using the BrdU-labeling method, the ratio of BrdU-positive cells to total cells decreased with an increase in the concentration of AMP N1-oxide (Fig. 1B). At the highest concentration tested (33 µM), AMP N1-oxide reduced the ratio of BrdU-positive cells to nearly 2%, but induced the maximal neurite outgrowth. Namely, the rate of cell proliferation was inversely related to the degree of neurite outgrowth. In other words, the antimitotic activity of AMP N1-oxide may be critical for the neurite outgrowth-promoting activity by this molecule.

Phosphorylation of CREB and MAPK by AMP N1-oxide

We first examined the phosphorylation of CREB in response to AMP N1-oxide, since we had earlier shown that the actions of AMP N1-oxide were mediated by the adenylcyclase-coupled adenosine receptor A2A (5). As Fig. 2A shows, phosphorylation of CREB was increased dose dependently by AMP N1-oxide up to 500 µM, and diminished by the selective A2A receptor antagonist ZM241385 (Fig. 2B), suggesting that the phosphorylation of CREB was evoked by AMP N1-oxide through A2A receptor-mediated PKA activation.

MAPK was also phosphorylated in a dose-dependent manner by AMP N1-oxide from 4 to 500 µM (Fig. 3A).

Figure 1. Reciprocal relationship between neurite outgrowth (A) and cell growth (B) of AMP N1-oxide-treated PC12 cells. PC12 cells were cultured for 1 day in medium containing AMP N1-oxide. A: the ratio of the number of cells with neurite to total cells was calculated. The values are expressed as the mean ± standard error (n = 3). Significant differences from non-treated cells were determined by one-way ANOVA with Tukey’s test, ***P < 0.001, **P < 0.01. B: PC12 cells were incubated for 4 h with BrdU (10 µM), fixed, and reacted with Alexa Fluor™-conjugated anti-BrdU antibody. The number of BrdU-positive cells was counted, and the ratio of positive cells to total cells was calculated. The values are expressed as the mean ± standard error (n = 4). Significant differences versus the non-treated control were determined by one-way ANOVA with Tukey’s test, ***P < 0.001, **P < 0.01.

Figure 2. Phosphorylation of CREB in PC12 cells after treatment with various agents. PC12 cells were cultured for 30 min in medium supplemented with various concentrations of AMP N1-oxide (A) or in medium supplemented or not with 20 µM AMP N1-oxide in the presence or absence of various concentrations of KT5720 or ZM 241385 for 30 min (B). The cell lysates were subjected to PAGE (5 µg protein/lane) and blotted onto membranes for Western blotting with anti-phosphorylated-CREB and anti-CREB antibodies.

The MEK inhibitor PD98059 or A2A receptor antagonist ZM241385 clearly inhibited the phosphorylation of MAPK, whereas the PKA inhibitor KT5720 did not affect it even when administered at concentrations higher than 2 µM (Fig. 3B), demonstrating that MAPK phosphorylation was evoked through A2A receptor-mediated MEK activation independent of PKA activity.

Involvement of MAPK or PKA Signaling in the Action Mechanism of AMP N1-oxide

Involvement of MAPK activation in neurite outgrowth was next examined. The MEK inhibitor PD98059 did not inhibit AMP N1-oxide-induced neurite outgrowth (Fig. 4A), suggesting the induced outgrowth to be independent of the MAPK signaling pathway. The suppression
of cell growth was not dependent on MAPK, either, since the growth of PC12 cells was also slow when cells were treated with AMP \(N_1\)-oxide and PD98059 at the same time. However, the PKA inhibitor KT5720 significantly reduced a percentage of the cells with the AMP \(N_1\)-oxide-induced neurites (Fig. 4A), and simultaneously attenuated the AMP \(N_1\)-oxide-induced suppression of cell proliferation (Fig. 4B). These observations suggest the participation of PKA signaling not only in the neuritogenic activity of AMP \(N_1\)-oxide but also in its cell growth-suppressing activity.

**Interaction of the Action Mechanism of AMP \(N_1\)-oxide with TrkA Signal Pathway**

Transactivation of mitogenic kinase receptors through G protein-coupled receptors was described earlier (14,15). Namely, adenosine and adenosine receptor agonists were reported to activate Trk receptors through a mechanism that requires the adenosine A2A receptor (16). Therefore, we examined whether TrkA was activated during neurite outgrowth by AMP \(N_1\)-oxide. However, we could not obtain any positive results indicating activation (data not shown). Furthermore, treatment of cells with a selective TrkA inhibitor, K252a, greatly reduced the phosphorylation level of MAPK of cells incubated with NGF, but the level obtained with AMP \(N_1\)-oxide was unaffected (data not shown). The neurite outgrowth by AMP \(N_1\)-oxide was also unaffected by K252a. Thus, TrkA activation seems not to be involved in the response of cells to AMP \(N_1\)-oxide.

![Figure 3. Phosphorylation of ERK1/2 in PC12 cells after treatment with various agents.](image)

![Figure 4. Effects of inhibitors of PKA and MAPK on the neurite outgrowth of AMP \(N_1\)-oxide-treated PC12 cells (A) and on suppression of growth of PC12 cells elicited by AMP \(N_1\)-oxide (B).](image)
Discussion

Adenosine plays an essential role in modulating neuronal function via adenosine receptors (17). In the central nervous system (CNS), the A2A receptor gene is strongly expressed and is suggested to be involved in the regulation of synaptic plasticity and to play a critical role in early neuronal development (18). In such a context, adenosine derivatives may have various effects on the CNS, and be expected to modulate brain functions.

Here, we demonstrated that MAPK phosphorylation was evoked through A2A receptor-mediated MEK activation in a manner independent of PKA signaling (Fig. 3). Recently, Kim et al. (19) reported that secretin-induced neurite outgrowth of PC12 cells and that the activity was dependent on a cAMP-MAPK pathway. According to them, a PKA inhibitor suppressed the phosphorylation of MAPK, which is inconsistent with our results. MAPK can be phosphorylated in response to cAMP in a manner independent of PKA, whose phosphorylation is mediated by the Rap1-B-Raf pathway (20) or an Src family kinase (21). This pathway might be involved in the mechanism of AMP_N1-oxide-evoked MAPK activation.

Integrins activate the MAPK signaling pathway, which is required for Mn2+-induced neurite outgrowth of PC12 cells (22). TrkA signals evoked by NGF also cause MEK activation followed by MAPK phosphorylation necessary for neurite outgrowth (23). Thus, it is obvious that both Mn2+- and NGF-induced neurite outgrowth require activation of MAPK. However, AMP_N1-oxide-induced neurite outgrowth was independent of the activation of the MAPK signaling pathway (Fig. 4). This mechanism may be supported by A2A signal-mediated activation of CREB, which is known to induce neurite outgrowth without MAPK signaling (24). In this study, we found that the AMP_N1-oxide-induced CREB phosphorylation depended on A2A receptor-mediated PKA signaling (Fig. 2). However, we have not yet had direct evidence whether the CREB phosphorylation is correlated with neurite outgrowth or cell growth suppression elicited by AMP_N1-oxide.

Neuronal differentiation is accompanied by suppressed cell proliferation as reported earlier (25). Although the mechanism underlying cell growth suppression by AMP_N1-oxide is not known, we showed earlier that activities of both cell growth suppression and neurite outgrowth are mediated by the adenosine A2A receptor (5). Other investigators recently identified translin-associated protein X (TRAX) as a novel protein that interacts with the C-terminal part of the adenosine A2A receptor, and considered TRAX to be an essential molecule for mediating A2A receptor-induced inhibition of cell proliferation (26). It is likely that AMP_N1-oxide interacts with TRAX at higher affinity than does AMP. This hypothesis may explain the preferential neurite outgrowth by AMP_N1-oxide. Thus, AMP_N1-oxide may modulate the activities of various neurotrophic factors through TRAX and A2A receptors to a greater extent than AMP. Therefore, AMP_N1-oxide would be expected to have beneficial effects on the CNS when used in combination with other agents such as favorable neurotrophic factors.

AMP_N1-oxide is a unique compound not found in natural resources so far other than RJ. Therefore, it may be the molecule responsible for RJ-specific physiological actions on the CNS. Our study provides molecular-based evidence that RJ regulates neuronal functions through A2A receptor signaling enhanced by AMP_N1-oxide. We expect neuroprotective effects of AMP_N1-oxide on the brain as a potent agonist of A2A receptor, because particular ligands of A2A receptors including selective agonists are reported to protect neurons against kainate-induced excitotoxicity in vivo (27) or to attenuate the A1 receptor-mediated synaptic depression in the CA1 area of the hippocampus in vitro (28). In addition, A2A receptor antagonists can also reduce damage produced by combinations of sub-threshold doses of the endogenous excitotoxin quinolinic acid and free radicals (29). These observations suggest that up or down regulation of A2A receptor-linked signaling pathway appear to be promising for the prevention of neuronal damage (30). As A2A receptors are predominantly expressed in dendrites of medium spiny neurons of the striatum in rats, Cabeza et al. (31) demonstrated that A2A receptor agonist, CGS-21680, activated both ERK1/2 and CREB in the caudate-putamen, suggesting that AMP_N1-oxide modulates neuronal signaling in various brain regions including caudate-putamen.

Elucidation of the physiological roles of AMP_N1-oxide in brain function is important to develop RJ as an evidence-based complementary and alternative medicine. Taking RJ as a source of AMP_N1-oxide may be promising to maintain healthy brain functions.

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