Royal jelly-induced neurite outgrowth from rat pheochromocytoma PC12 cells requires integrin signal independent of activation of extracellular signal-regulated kinases

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ABSTRACT
We showed earlier that neurite outgrowth of rat pheochromocytoma PC12 cells was stimulated by royal jelly extract (PERJ) or its unique component, AMP N1-oxide, via adenosine A2a receptors. In this study, we found that stimulated neurite outgrowth occurred in medium supplemented with serum, but not in serum-free medium. The pentapeptide GRGDS, which includes the RGD sequence commonly shared by extracellular matrix (ECM) components, could attenuate the effect of serum, suggesting that integrin receptor signaling was essential for the neurite outgrowth induced by PERJ or AMP N1-oxide. PERJ or AMP N1-oxide also activated extracellular signal-regulated kinases 1 or 2 (ERK1/2); however, this activation was not associated with the neurite outgrowth. As it is known that Mn2+ induces neurite outgrowth from PC12 cells and activates ERK1/2 through integrin signals and that activation of ERK1/2 is essential for Mn2+-induced neurite outgrowth, a difference in the mechanism between Mn2+-induced and PERJ- or AMP N1-oxide-induced neurite outgrowth is suggested. Furthermore, we demonstrated that PERJ contained no ECM component-like substances. These results demonstrate that AMP N1-oxide and its analogues were the only entities in PERJ with neurite outgrowth-inducing activity and that they required integrin signaling in addition to activation of A2a receptors to induce neurite outgrowth.

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 (18, 20). We recently found that an extract of RJ induces outgrowth of neurites from cultured PC12 cells, a cell line obtained from rat pheochromocytoma cells (16), and identified AMP N1-oxide as one of the active components (17). AMP N1-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 specific protein of mature neurons, thus demonstrating that AMP N1-oxide can induce neuronal differentiation of PC12 cells (17). PC12 cells have become a principal model for the study about biological activities related to neuronal differentiation. A well-known molecular entity to affect PC12 cells is nerve growth factor (NGF). In response to the binding of NGF to receptors p75 (6) and TrkA (4, 19), PC12 cells stop dividing and extend neurites, differentiating into cholinergic cells similar to those found in the sympathetic neurons (10, 16). However, the neurite growth-promoting activity of AMP N1-oxide was found to be mediated by adenylate cyclase-coupled adenosine A2a receptors (17), the activation of which leads to an increase in the cAMP level to stimulate neurite outgrowth in cultured PC12 cells.

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(25). Furthermore, PC12 cells have been reported to respond to various molecules such as Mn$^{2+}$ (24, 34), cAMP analogs (28), and fibroblast growth factor-2 (9) by extending neurites. As the signal transduction pathways are mutually different among these molecules, the activity of AMP $N_1$-oxide may be influenced and/or modified by endogenous or exogenous active substances. In fact, previous report showed a synergistic effect between AMP $N_1$-oxide and NGF on the formation of neurites of longer length (17). The most important and plausible substances to interact with active components of RJ including AMP $N_1$-oxide are thought to be extracellular matrix (ECM) components such as fibronectin, laminin or collagen, because they bind to integrin receptors on neurons and transduce signals to elicit neurite outgrowth (3, 11).

Therefore, in this study, we examined the effects of ECM components contained in serum on RJ extract- or AMP $N_1$-oxide-induced neurite outgrowth and on phosphorylation of extracellular signal-regulated kinases (ERKs) of cultured PC12 cells. Our results demonstrated that integrin signaling was essential for the neurite outgrowth but independent of the activation of ERKs. Furthermore, the existence of ECM component-like substances in RJ and the interaction of the active component AMP $N_1$-oxide with other RJ components were investigated.

MATERIALS AND METHODS

Extract of RJ. RJ (originated from Apis mellifera, a generous gift from Api Co. Ltd., Gifu, Japan) was mixed with phosphate-buffered saline (PBS, pH 7.4) (25% w/v) and shaken slowly overnight at 4°C. The mixture was centrifuged at 12,000 $\times$ g for 10 min at 4°C, and the supernatant (PBS-extract of RJ: PERJ) was further diluted with the culture medium and used for experiments. The dilution of RJ is expressed as that from the original product. The 125-fold-diluted PERJ was previously determined to be optimal for neurite outgrowth of PC12 cells (17), and was thus used for the experiments.

Cell culture and assessment of neurite outgrowth. PC12 cells were maintained in Dulbecco’s Modified Eagle’s Medium supplemented with 15% (v/v) serum mixture (2 volumes of horse serum mixed with 1 volume of fetal bovine serum), as previously described (17). For assessment of the neurite outgrowth, PC12 cells (1–2 $\times$ 10$^5$ cells/cm$^2$) were plated on collagen-coated 6- or 24-well plates, which had been prepared by incubating the plates at room temperature for 2 h with hydrochloric solution (pH 3.0) containing 30 mg/mL collagen type IV (Nitta Gelatin, Osaka, Japan). Various reagents were added to the cells 1 day after plating, and morphological changes were then assessed under a phase-contrast microscope. GRGDS, a pentapeptide including the RGD sequence common to extracellular matrix (ECM) components (Peptide Institute, Osaka, Japan), was used to block RGD-mediated interactions between ECM components and integrin receptors (34). Neurite-bearing cells were defined as those having neurites 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 in them were counted. Involvement of the mitogen-activated protein kinase (MAPK) pathway was estimated by using a MAPK kinase (MEK) inhibitor, PD98059 (Wako Chemicals, Osaka, Japan).

Western blotting. PC12 cells were lysed with lysis buffer (20 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 2 mM EDTA, 1% NP-40, 10 $\mu$g/mL aprotinin, 10 $\mu$g/mL leupeptin, 50 mM NaF, 1 mM Na$_3$VO$_4$, 1 mM phenylmethyisulfonyl fluoride, 0.1% sodium dodecyl sulfate (SDS), and 1% Na deoxycholate). The lysates were centrifuged, and the protein concentration was determined with a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Each sample, containing 5 $\mu$g of protein, was electrophoresed by SDS-polyacrylamide gel electrophoresis (PAGE) on 7.5% gels. Proteins were transferred to a polyvinylidene fluoride membrane and blocked for 2 h at room temperature with 5% skim milk (Morinaga Milk Products, Tokyo, Japan) in 20 mM Tris-HCl, pH 7.4, containing 0.5 M NaCl and 0.05% Tween-20 (TTBS). Next the membranes were incubated with primary antibody (anti-MAP kinase antibody, anti-phospho MAPK antibody; New England Biolabs, Beverly, MA, USA), and then with alkaline phosphatase-conjugated secondary antibody (Promega, Madison, WI, USA) in TTBS containing 5% skim milk. Finally, the specific protein bands were developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indorylphosphate p-toluidine salt.

RESULTS

Effects of serum on PERJ-induced neurite outgrowth

PERJ (PBS-extract of RJ) diluted 125 times and AMP $N_1$-oxide (20 $\mu$M) purified from PERJ induced neurite outgrowth from PC12 cells cultured in the medium supplemented with the 15% serum mix-
ture. Both elicited process formation with similar morphological characteristics and caused a similar maximal % of neurite-bearing cells. The neurites induced by NGF, a well-known neurotrophic factor, became longer in a culture time-dependent manner; however, those induced by PERJ or AMP \( N_1 \)-oxide remained short even after a 3-day culture period (Fig. 1 IA, IIA). The difference in morphology between NGF- and PERJ/AMP \( N_1 \)-oxide-induced neurite genesis was presumed to be based on their different action mechanisms. Namely, NGF acts through TrkA and p75 receptor complexes, while PERJ and AMP \( N_1 \)-oxide, through adrenergic A2a receptors (17). A conspicuous change in morphology was found when the cells were cultured in serum-free medium. NGF increased the number of cells with long neurites, but PERJ or AMP \( N_1 \)-oxide did not induce any outgrowth of neurites at all (Fig. 1 IB, IIB). These results suggest that neurite outgrowth by NGF occurred irrespective of serum but that PERJ or AMP \( N_1 \)-oxide needed serum for the neurite outgrowth.

To confirm this serum effect, we further examined the effect of changing the serum concentration. A significant increase in the % of neurite-bearing cells occurred in a serum concentration-dependent manner over 6% (Fig. 2A), suggesting the requirement of some components of serum in mechanism of PERJ-induced neurite genesis. Similar serum-dependency is observed in Mn\(^{2+}\)-induced neuritegenesis, which is based on integrin signaling (21, 24). Therefore, we tested the involvement of ECM components in serum as ligand for integrin receptors by using GRGDS, a pentapeptide including the RGD sequence common to ECM components. PERJ-induced neuritegenesis was significantly attenuated

![Image](image-url)
A2a receptor, a G protein-coupled receptor, in cultured PC12 cells (17). A2a receptor activation leads to increase of intracellular cAMP level followed by activation of MAPK/extracellular signal-regulated kinases 1 or 2 (ERK1/2) in PC12 cells (7, 28). Activation of ERK1/2 is one of the checkpoints to assess the activation of the classical Ras/MAPK cascade, which is usually triggered by an engaged tyrosine kinase receptor or G protein-coupled receptor and results in proliferation and/or differentiation. It is well known that the binding of NGF to TrkA receptors triggers cellular signaling responses including the activation of the Ras/MAPK pathway that mediates neuritogenesis (15). Therefore, phosphorylation of ERK1/2 was thought to be critical for neuritogenesis by PERJ and AMP N1-oxide. We found that PERJ, AMP N1-oxide or NGF induced phosphorylation of ERK1/2, which order resembled that of the neuritogenic activity. Therefore, we tested the phosphorylation of ERK1/2 in the cells cultured in serum-free medium. As expected, the degree of phosphorylation was weak or nonexistent (Fig. 3B), suggesting that the phosphorylation of ERK1/2 was also facilitated by integrin signaling due to some specific interactions.

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rum component(s). To examine the effect of MAPK activation on the neurite outgrowth, the influence of a MEK inhibitor (PD98059) was evaluated. The phosphorylation of ERK1/2 by PERJ was completely inhibited in the presence of PD98059, but the neurite growth was not affected, demonstrating that PERJ stimulated neuritogenesis without participation of the MAPK pathway (Fig. 4).

**Behavior of active molecules of PERJ on gel filtration**

Our previous report suggested that the neurite outgrowth-promoting activity of PERJ could be predominantly attributed to AMP N1-oxide and its analogues including adenosine N1-oxide (17). In this study, morphological changes such as spreading and neurite outgrowth induced by PERJ were similar to those by AMP N1-oxide when the cells were treated in the presence of serum (Fig. 1 IA). The maximal values (%) of neurite-bearing cells were also similar (Fig. 1 IIA). However, there remained the possibilities that 1) active molecules other than AMP N1-oxide and its analogues were present in PERJ and 2) AMP N1-oxide and its analogues bound to some components of RJ such as particular proteins and thus lost their activity and/or stability. Therefore, PERJ was gel-filtered on a column of Sephacryl S-200, and the eluates were assessed for their ability to induce neurite outgrowth and to activate ERK1/2. An aliquot of the eluates was subjected to SDS-PAGE for protein staining and Western blotting for ERK1/2 (Fig. 5). Activities for both neurite outgrowth and phosphorylation of ERK1/2 were similarly distributed between tube nos. 70 and 80 corresponding to a molecular weight below 10–20 kDa, in which little of proteins were eluted. A separate gel filtration analysis of AMP N1-oxide on the same column showed that the position of both activities was nearly identical to that of AMP N1-oxide itself. These results suggest that AMP N1-oxide or its analogues did not interact with any large PERJ molecules. However, the possibility that AMP N1-oxide or its analogues bound to some larger or smaller PERJ molecules to inactivate them was not excluded in this experiment.

**DISCUSSION**

Neuronal differentiation is a complex process in which many different signaling pathways may be involved (2, 13, 33). Integrin is a cell-surface receptor molecule composed of 2 different subunits, α and β. It binds the RGD sequence of ECM molecules such as fibronectin, laminin or collagen, and activates intracellular tyrosine kinases such as focal adhesion kinase (FAK), Src, and phosphatidylinositol 3-kinase (P13K), growth factor receptors, and/or ion channels to generate intracellular signals (12, 14, 30). FAK and Src are involved in activation of Ras/MAPK pathways (14). So far, 19 species of integrin α subunit, 8 species of β subunit, and 25 species of heterodimer complexes of α and β have been found.
Various integrin complexes have diversity in their binding to ECM components and transduce a variety of signals to regulate cellular events. Integrins α(III)β(I) and α(I)β(I) are usually expressed in PC12 cells (32).

In this study, culture dishes were coated with collagen type IV, which binds integrin α(I)β(I) complexes; but this integrin signal was not sufficient for neurite outgrowth transduced by PERJ, AMP N1-oxide or Mn2+ in the absence of serum (Fig. 1). Neurite outgrowth was induced by PERJ or Mn2+ only in the presence of serum, but attenuated by the coadministration of the peptide GRGDS, which inhibits interaction between ECM components and integrin receptors (Fig. 2B). As serum contains various extracellular components such as fibronectin, we suspect that PERJ required integrin signaling for neurite outgrowth, as in the case of Mn2+ (21). Mn2+ is reported to enhance the expression of α(V) subunit in PC12 cells, and generates integrin α(V)β(I) heterodimer that enhances signaling from ECM molecules (21, 24). NGF is known to facilitate expression of α(I) subunit and to generate integrin α(I)β(I) complex (36). We observed that NGF could induce neurite outgrowth even without serum (Fig. 1B), which finding may be explained as follows: 1) col-
ligen type IV coated on the culture dishes was enough to grow neurites, because collagen IV binds integrin α(1)β(1) complex inducible by NGF, and 2) TrkA signaling by NGF is much more potent than integrin signaling.

Integrin signals activate the MAPK signaling pathway, which is required for Mn$^{2+}$-induced neurite outgrowth of PC12 cells (34). TrkA signals evoked by NGF also cause MEK activation followed by ERK1/2 phosphorylation necessary for neurite outgrowth (26). Thus, it is obvious that both Mn$^{2+}$- and NGF-induced neurite outgrowth require activation of ERKs. We already showed earlier that PERJ or AMP N$_1$-oxide elicits neurite outgrowth from PC12 cells via adenosine A2a receptors in the presence of serum (17). Activation of ERKs by PERJ and AMP N$_1$-oxide was achieved, as expected, from adenylylate cyclase-coupled A2a receptor signaling pathway (8, 26); and phosphorylation was more intense in the cells treated with serum than in those without serum, because activation of ERK1/2 through integrin signaling is additive (14). However, PERJ-induced neurite outgrowth was not inhibited by MEK inhibitor, suggesting that the neuritogenesis by PERJ progresses independently of the activation of MAPK signaling pathway (Figs. 3, 4). This mechanism may be supported by A2a signal-mediated activation of cAMP-response element-binding protein (CREB), which is known to induce neurite outgrowth without MAPK signaling (8). Integrins interact physically with ECM components and the cytoskeleton, thus providing a physical link between the extracellular environment and intracellular components that dictate its architecture (5). This feature may influence the neurite outgrowth without association with MAPK signaling. Growth cones of growing neurites require balance among cAMP levels, integrin function and activity of Rho GTPases to maintain motility and prevent collapse (22). Ultimately, present study demonstrates that interaction between adenosine A2a and integrin signals is essential for the PERJ-/AMP N$_1$-oxide-induced neurite outgrowth. This may be one of the mechanisms to establish functional neuronal circuit in vivo nervous systems, because regionally distributed ECM components critically guide neurite outgrowth after diffusible stimulation by AMP N$_1$-oxide in RJ taken as food. Both adenosine A2a and integrin signals are involved in the regulation of synaptic plasticity (11, 27) and thought to play a critical role in early neuronal development (11, 35). Therefore, in this point of view, taking RJ or AMP N$_1$-oxide may favor to maintain nervous systems healthy.

RJ is composed of 12–15% proteins, 10–12% carbohydrates, 3–7% lipids (including sterols and fatty acids), and traces of mineral salts and vitamins (29). Major proteins of RJ belong to one protein family designated major royal jelly proteins (MRJP), and the family consists of five main members (MRJP1, MRJP2, MRJP3, MRJP4, MRJP5) (1, 31). The MRJP gene family encodes a group of closely related proteins that share a common evolutionary origin with the yellow protein of Drosophila melanogaster (1). Most of other RJ proteins have not yet been clarified as to either chemical structure or physiological activity, suggesting the possible involvement of ECM component-like proteins in RJ that activate integrin receptors. Furthermore, 10-hydroxy-2-decenoic acid and 10-hydroxy-2-decanoic acid, an unsaturated and a saturated fatty acid, respectively, which characterize RJ (23) were shown to promote collagen production in skin fibroblasts (20). They may stimulate PC12 cells to synthesize collagen that binds and activates integrin receptors. However, PERJ did not show any superior effects on neurite outgrowth to AMP N$_1$-oxide (Fig. 1). Entities with activities of neurite outgrowth or ERK1/2 activation behaved chromatographically like a low-molecular-weight compound similar to AMP N$_1$-oxide (Fig. 5). These observations support our view that AMP N$_1$-oxide and its derivatives are the only entities of PERJ to express both activities for neurite outgrowth and activation of ERK1/2. PERJ did not have integrin ligand-like molecules.

In conclusion, we found that 1) integrin signaling was required for PERJ- or AMP N$_1$-oxide-induced neurite outgrowth, 2) phosphorylation of ERK1/2 was not always necessary for PERJ- or AMP N$_1$-oxide-induced neurite outgrowth, 3) PERJ contained neither integrin ligand-like substances nor large molecules binding to AMP N$_1$-oxide, and 4) the only active substances in RJ to induce neurite outgrowth and activate the MAPK signal pathway were AMP N$_1$-oxide and its analogues.

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REFERENCES


