Enhancement of Neurite Outgrowth in PC12 Cells Stimulated with Cyclic AMP and NGF by 6-Acylated Ascorbic Acid 2-*O*-α-Glucosides (6-Acyl-AA-2G), Novel Lipophilic Ascorbate Derivatives

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It has been shown that ascorbate (AsA) and its stable derivative, ascorbic acid 2-*O*-α-glucoside (AA-2G), do not elicit neurite outgrowth in PC12 cells. However, these ascorbates are synergistically enhanced by both dibutyryl cyclic AMP (Bt_2cAMP)- and nerve growth factor (NGF)-induced neurite outgrowth in this model. In the present study, the effects of a series of novel lipophilic ascorbate derivatives, 6-acylated ascorbic acid 2-*O*-α-glucosides (6-Acyl-AA-2G), on neurite outgrowth induced by Bt_2cAMP and NGF were examined in PC12 cells. We found that all the tested acylated ascorbate derivatives enhanced neurite formation induced by both agents in a dose-dependent manner. Of the 6-Acyl-AA-2G derivatives, 6-octanoyl ascorbic acid 2-*O*-α-glucoside (6-Octa-AA-2G) enhanced the Bt_2cAMP-induced phosphorylated MAPK p44 and p42 expression. A α-glucosidase inhibitor, castanospermine, completely abrogated the promotion of neurite outgrowth and MAPK expression by 6-Octa-AA-2G. Addition of 6-Octa-AA-2G (0.5 mM) to PC12 cells caused a rapid and significant increase in intracellular AsA content, which reached a maximum and was maintained from 12 to 24 h after the culture. These findings suggest that 6-Acyl-AA-2G is rapidly hydrolyzed to AsA within the cell and enhances neurite differentiation through the interaction with the inducer-activated MAPK pathway.

Key words ascorbate; lipophilic ascorbate derivative; neurite outgrowth; cAMP; MAP kinase

L-Ascorbic acid (AsA, vitamin C) has for many years been known to be a natural bioactive agent, and its multifarious activities have been reported in many biological systems. For example, AsA can increase enzyme activity[1—3]; act as an inducer of the collagen pathway[4]; and scavenge free radicals as an antioxidant during oxidant stress.[5,6] It has been shown that the most serious human diseases of the central nervous system (CNS) such as Parkinson's and Alzheimer's diseases are associated with oxidation processes mediated by free radicals.[7—9] AsA is the most abundant antioxidant of low molecular weight,[10] and normally accumulates to higher concentrations in the CNS than in most other tissues.[11] Some studies suggest that in response to various exogenous stimuli, AsA may act as a neuromodulator as well as an antioxidant.[12—14]

In most mammals, birds, and reptiles, AsA can be synthesized in the liver or kidney, but in primates such as humans as well as in guinea pigs, it must be acquired from the diet. A major problem of AsA is that it is very unstable in solution and is rapidly oxidized to dehydroascorbic acid and further oxidation products, even under normal culture conditions of neutral pH and 37 °C.[15] We have previously shown that a new derivative of AsA, ascorbic acid 2-*O*-α-glucoside (AA-2G), which is very stable under various oxidative conditions and which releases AsA adequately through enzymatic hydrolysis,[16] enhances cAMP-stimulated neurite outgrowth in PC12 cells (unpublished data).

We have recently succeeded in synthesizing a series of lipophilic monoacylated ascorbic acid 2-*O*-α-glucosides (6-Acyl-AA-2G) that exhibit radical scavenging activity.[17—19] We have also demonstrated that these compounds are superior to AA-2G or AsA with regard to skin permeability and are susceptible to enzymatic hydrolysis by tissue esterase and/or α-glucosidase to give AA-2G and AsA.[17]

The present work was undertaken to examine whether a series of 6-Acyl-AA-2G compounds could modulate neurite formation in the presence or absence of dibutyryl cyclic AMP (Bt_2cAMP) and nerve growth factor (NGF) in PC12 cells. We report herein that 6-Acyl-AA-2G, which is incorporated into the cells and enzymatically releases AsA, synergistically enhances both Bt_2cAMP- and NGF-elicited neurite outgrowth. In addition, we found that although this action occurs in the same manner as with AsA and AA-2G, these compounds alone do not result in this enhancement of neurite outgrowth. We also show evidence for the involvement of activation of the MAPK cascade in this effect of ascorbate.

MATERIALS AND METHODS

Materials Collagen I was purchased from Nitta Gelatin Inc. (Osaka, Japan), and NGF from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Horse serum (HS) was obtained from Life Technologies, Inc. (Rockville, MD, U.S.A.). Fetal calf serum (FCS) and RPMI-1640 were obtained from ICN Biomedicals Inc. (Aurora, OH, U.S.A.). Dibutyryladenosine 3′,5′-cyclic monophosphate (Bt_2cAMP) and castanospermine were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sodium L-ascorbate and trichloroacetic acid were from Ishizu Seiyaku, Ltd. (Osaka, Japan). Antibody against MAPK and secondary antibody for immunoblotting were obtained from New England Biolabs Inc. (Beverly, MA, U.S.A.). AA-2G was a gift from Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan). A series of novel stable lipophilic vitamin C derivatives, 6-*O*-acyl-2-*O*-α-D-glucophyranosyl-L-ascorbic acids (6-Acyl-AA-2G) were synthesized in our laboratory as described previously.[17] 6-*O*-Butyryl-2-*O*-α-D-glucopyranosyl-L-ascorbic acid, 2-*O*-α-D-glucopyranosyl-6-*O*-hexanoyl-L-ascorbic acid, 2-*O*-α-D-glucopyranosyl-6-*O*-octanoyl-L-ascorbic acid, 6-*O*-decanoyl-2-*O*-α-D-glucopyranosyl-L-ascorbic acid, and 6-*O*-dode-

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canoyl-2-*O*-α-D-glucopyranosyl-L-ascorbic acid are abbreviated herein as 6-Buty-AA-2G, 6-Hexa-AA-2G, 6-Octa-AA-2G, 6-Deca-AA-2G, and 6-Dode-AA-2G, respectively (Fig. 1).

Cell Culture and Treatments The culture conditions were described by Greene and Tischler.[20] In brief, PC12 cells (RIKEN Cell Bank, Tsukuba, Japan) were grown in RPMI 1640 medium supplemented with 5% FCS, 10% heat-inactivated HS, 100 units/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate, and incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

The cells were seeded in 6-well plates (Nunc, Roskilde, Denmark) coated with porcine tendon collagen, cultured for protein assay and intracellular AsA measurement, and seeded in 96-well plates (Nunc.) coated with the same collagen for neurite-outgrowth determination in defined medium, as described above. The effects of AsA or its derivatives on neuronal differentiation were determined in the presence or absence of Bt_2cAMP (0.5 mM) or NGF (10 ng/ml).

Determination of Neurite Outgrowth PC12 cells from stock cultures were suspended in phosphate buffered saline (PBS) and washed once with PBS. The cells were suspended in the RPMI 1640 medium described in the previous section and seeded in 96-well plates coated with porcine tendon collagen at a density of 0.4×10^4 cells/100 μl/well, and neurite formation was observed 24 h after treatments. The incubation was stopped by the addition of 1% glutaraldehyde, then stained by Giemsa's solution. The number of cells bearing neurites longer than one cell body diameter after treatments was divided by the total number of cells counted as 200—300 cells per well in triplicate cultures. The significance of the difference between means of the two groups was statistically analyzed by the Student's *t*-test.

MAPK Assay PC12 cells elicited with Bt_2cAMP were incubated in the presence or absence of AsA (0.01 mM) or 6-Octa-AA-2G (0.5 mM) for 24 h on 6-well plates at 10^6 cells/3.0 ml/well. The cells were harvested by scraping, washed twice with cold PBS, and then lysed with 100 μl sample buffer (62.5 mM Tris–HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 10% glycerol, 0.1% BPB, and 50 mM DTT) in 1.5-ml Eppendorf tubes. After boiling for 10 min and ice-cooling for 5 min, the solutions were sonicated and centrifuged for 74 min at $8500\times\boldsymbol{g}$. The protein assay of the supernatant was carried out by the method of Bradford with Coomassie brilliant blue R-250 (Bio-Rad Laboratories, Hercules, CA, U.S.A.). The protein extracts (25 μg) were determined by Western blot analysis. Each protein sample was separated on 10% SDS-polyacrylamide gel. The gel was blotted onto nitrocellulose transfer membranes (Millipore, Bedford, MA, U.S.A.) and blocked with bovine serum albumin in Tris-buffered saline (pH 7.4) with 0.1% Tween 20. Blots were incubated with primary antibody followed by secondary antibody conjugated with horseradish peroxidase. Localization of protein molecular size markers on the electrophoretic gel was detected by Coomassie brilliant blue G-250 staining. Immunodetection of p42/44 MAPK was performed by chemiluminescence using the ImmunoStar kit for Rabbit from Wako Pure Chemical Industries, Ltd.. Signal intensities of the immunoreactive bands were quantified by NIH Image version 1.55 software.

Measurement of Intracellular AsA Concentration

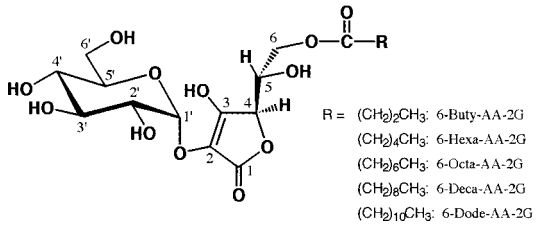

Fig. 1. Structure of 6-*O*-Acyl-2-*O*-α-D-glucopyranosyl-L-ascorbic Acid (6-Acyl-AA-2G)

PC12 cells were plated on 6-well plates at 10^6 cells/3.0 ml/well with AA-2G (0.5 mM) or 6-Octa-AA-2G (0.5 mM) in the presence or absence of Bt_2cAMP (0.5 mM), harvested by scraping, washed twice with PBS, and then extracted in ice-cold 5% trichloroacetic acid chronologically. The supernatant was subjected to HPLC analysis using an Inertsil ODS-3 column (ϕ4.6×250 mm, 5 μm, GL Sciences Inc., Tokyo), which was kept at 40 °C and eluted with 0.1 M H_3PO_4–KH_2PO_4 buffer (pH 2.0, containing 10 mg/l of EDTA) at a flow rate of 0.7 ml/min. The absorbance at 240 nm was monitored.

RESULTS

Effects of AsA, AA-2G, and 6-Acyl-AA-2G on Neurite Outgrowth in PC12 Cells NGF and cAMP are known to elicit neurite outgrowth in PC12 cells, one of the most widely used cell lines, as a model of neurite differentiation.[20—24] We confirmed that Bt_2cAMP increased neurite formation in PC12 cells in a dose-dependent manner after 24 h incubation, as originally described. The maximal increase was obtained by 1 mM Bt_2cAMP, and the percentage of cells with neurites was approximately 20%.[23] We have recently shown that a new derivative of AsA, ascorbic acid 2-*O*-α-glucoside (AA-2G), which is very stable under various oxidative conditions and releases AsA adequately through enzymatic hydrolysis,[16] enhances cAMP-stimulated neurite ougrowth in PC12 cells (unpublished data).

In the present study, we examined whether a series of novel lipophilic ascorbate derivatives, 6-acylated ascorbic acid 2-*O*-α-glucosides (6-Acyl-AA-2G), affects neurite outgrowth in PC12 cells, and compared these results with those of AsA and AA-2G. Of the 6-Acyl-AA-2G family having a straight-acyl chain of varying length from C_4 to C_{18}, 6-Buty-, 6-Hexa-, 6-Octa-, 6-Deca-, and 6-Dode-AA-2G were used in our experiments. The ascorbate derivatives were found to synergistically enhance the neurite formation induced by Bt_2cAMP (Fig. 2). However, none of the 6-Acyl-AA-2G compounds tested alone elicited neurite outgrowth. These characteristics are identical to those of AsA and AA-2G as shown in Figs. 2a and b. 6-Octa-AA-2G, for which the maximal increase was obtained at 0.1 mM, showed the strongest effects on neurite response, followed by 6-Buty-AA-2G and 6-Hexa-AA-2G. 6-Deca-AA-2G and 6-Dode-AA-2G were found to be less effective. In the presence of 6-Buty-, 6-Hexa-, or 6-Octa-AA-2G at a concentration rage of 0.01—0.1 mM, the percentage of cells with neurites (>20%) was higher than the maximum level of neurite outgrowth induced by Bt_2cAMP alone (Figs. 2c—e). In particular, in response to treatment with 0.1 mM 6-Octa-AA-2G, the frequency of neuritogenic cells increased to over 30%. In the presence of AA-2G at concen-

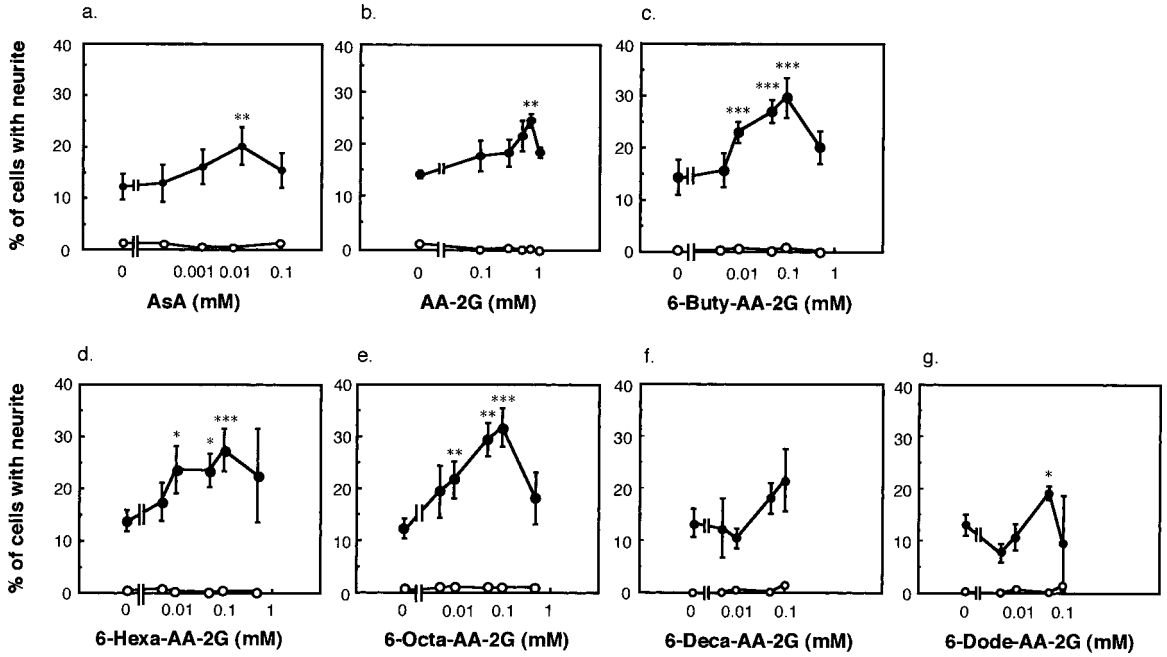

Fig. 2. Effects of AsA and Its Derivatives on Bt_2cAMP-Induced Neurite Outgrowth of PC12 Cells

PC12 cells were plated at 0.4×10^4 cells/well and cultured with AsA or its derivatives in the presence (●) or absence (○) of 0.5 mM Bt_2cAMP. The extent of neurite outgrowth was measured at 24 h and is expressed as the mean percentage of 200—300 cells from three separate experiments with triplicate cultures, with the bars showing the S.D. $*p<0.05$, $**p<0.01$, $***p<0.001$, compared with Bt_2cAMP 0.5 mM only.

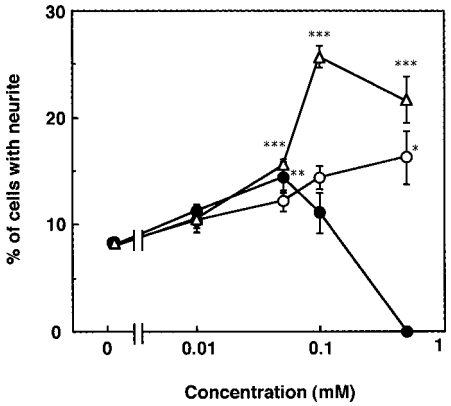

Fig. 3. Effects of AsA and Its Derivatives on NGF-Induced Neurite Outgrowth of PC12 Cells

PC12 cells were plated at 0.4×10^4 cells/well and cultured with AsA (●), AA-2G (○) and 6-Octa-AA-2G (△) in the presence of 10 ng/ml NGF. The extent of neurite outgrowth was measured at 24 h and is expressed as the mean percentage of 200—300 cells from three separate experiments with triplicate cultures, with the bars showing the S.D. $*p<0.05$, $**p<0.01$, $***p<0.001$, compared to NGF 10 ng/ml only.

trations of 0.3 and 0.5 mM, the percentage of cells with neurites was also higher than the maximum level of Bt_2cAMP-induced neurite outgrowth (Fig. 2b). In contrast, AsA (Fig. 2a) and 6-Deca- and 6-Dode-AA-2G (Figs. 2f, g) had an observed synergistic effect on Bt_2cAMP-induced neurite outgrowth, but the activities were weak and showed cytotoxicity at concentrations greater than 0.1 mM.

Similar results were observed with NGF-induced neurite extension in PC12 cells (Fig. 3). 6-Octa-AA-2G markedly enhanced NGF-induced neurite outgrowth, while AsA and AA-2G did only a little. AsA showed cytotoxicity at concentrations higher than 0.1 mM. Additional experiments showed the synergistic effects of neurite extension in response to AsA or its derivatives on Neuro 2a cells induced by Bt_2cAMP (data not shown).

Activation of MAPK by 6-Octa-AA-2G cAMP has been reported to stimulate the phosphorylated MAP kinases, p44 and p42, and induces neuronal differentiation in PC12.[20—23] We examined in the present study whether neurite outgrowth enhanced by AsA or 6-Octa-AA-2G is associated with MAPK activation in Bt_2cAMP-induced PC12 cells.

In PC12 cells, expression of phosphorylated p44/42 by Bt_2cAMP or 6-Octa-AA-2G alone was observed, and a synergistic effect of Bt_2cAMP and 6-Octa-AA-2G on this expression was clearly observed (Fig. 4). Although AsA synergized with Bt_2cAMP, the MAPK phosphorylation was lower than that of 6-Octa-AA-2G. A similar result was also observed in NGF-elicited cells (data not shown).

A α-glucosidase inhibitor, castanospermine, was found to completely abrogate the synergistic action of 6-Octa-AA-2G on MAPK expression (Fig. 5a) and neurite outgrowth (Fig. 5b) in Bt_2cAMP-treated PC12 cells at concentrations of 0.5 and 1 μM. The MAPK phosphorylation agreed well with the frequency of neuritogenic cells. These results suggest that formation of the active substance from 6-Octa-AA-2G requires hydrolysis by α-glucosidase.

Uptake and Metabolism of AsA Derivatives, AA-2G and 6-Octa-AA-2G, in PC12 Cells As intact 6-Octa-AA-2G cannot induce the extension of neurite outgrowth as described above, we investigated the uptake and metabolism of AsA derivatives, AA-2G and 6-Octa-AA-2G, in PC12 cells. The intracellular metabolites from AA-2G or 6-Octa-AA-2G were analyzed by HPLC.

We found that treatment with 0.5 mM 6-Octa-AA-2G results in a rapid accumulation of intracellular AsA, reaching a stable level (17.8 nmol/10^6 cells) after 12 h incubation which was maintained even after 24 h (Fig. 6). This level was nearly a twofold increase over that of AA-2G (9.5 nmol/10^6 cells) at

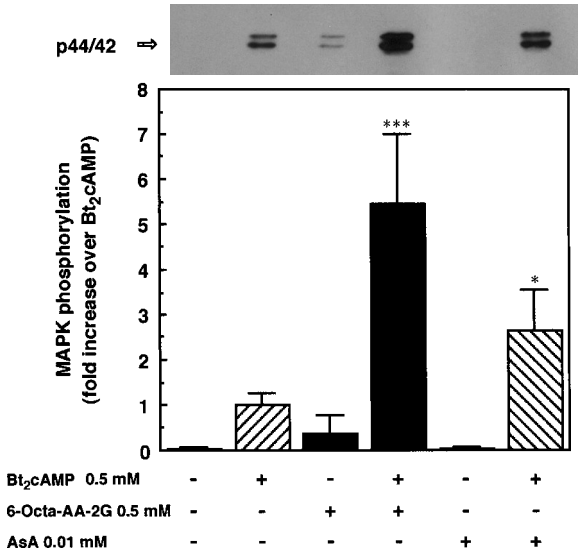

Fig. 4. Enhanced Expression of p44/42 Phosphorylation with AsA or 6-Octa-AA-2G in Bt_2cAMP-Induced PC12 Cells

PC12 cells were plated at 1×10^6 cells/well and incubated with the indicated reagents for 24 h. The cells were harvested, and the protein extracts prepared from these cells were subjected to Western blot analysis as described under Materials and Methods. Immunoreactive bands were visualized by the NIH Image version 1.55 software, and all data are normalized to the -fold increase over basal level as represented by cells induced with Bt_2cAMP alone. Similar experiments were performed three times. $*p<0.05$, $***p<0.001$, compared with the presence of Bt_2cAMP only.

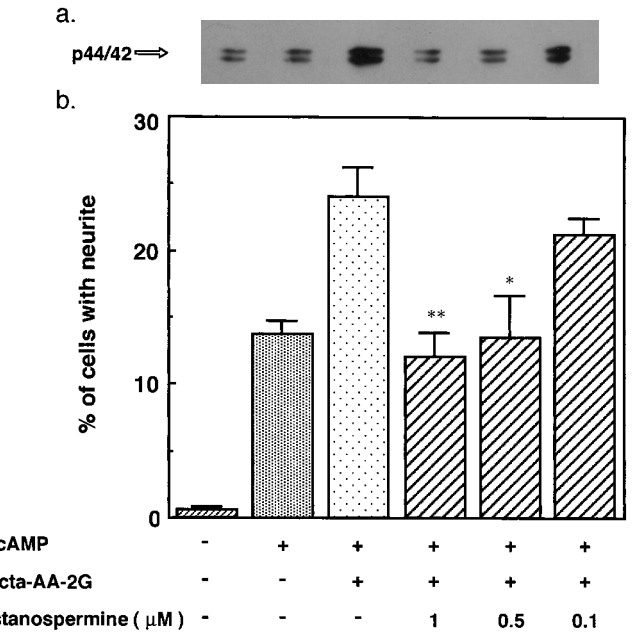

Fig. 5. Abrogation by Castanospermine of the Enhancement by 6-Octa-AA-2G of Neurite Outgrowth and Phosphorylation of p44/42 in Bt_2cAMP-Induced PC12 Cells

PC12 cells were plated at 1×10^6 cells/well (a, for expression of phosphorylation of p44/42) or 0.4×10^4 cells/well (b, for neurite outgrowth) and incubated with Bt_2cAMP (0.5 mM) and 6-Octa-AA-2G (0.5 mM) in the presence or absence of castanospermine. a; The cells were harvested after 24 h of incubation, and the protein extracts prepared from these cells were subjected to Western blot analysis. Similar experiments were performed three times. b; The extent of neurite outgrowth was measured at 24 h and is expressed as the mean percentage of 200—300 cells from three separate experiments with triplicate cultures, with the bars showing S.D. $*p<0.01$, $**p<0.001$, compared with the presence of Bt_2cAMP and 6-Octa-AA-2G.

the same treated concentration and time. When the cells were incubated with AA-2G, the AsA content gradually increased with the passage of incubation time, reaching a maximum

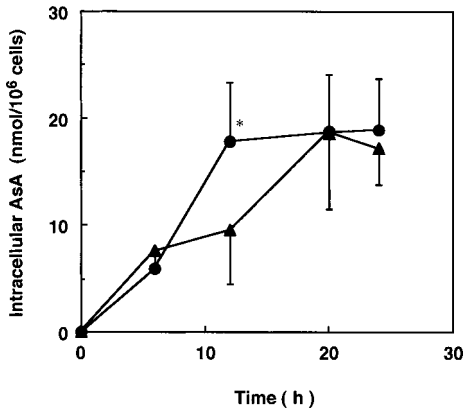

Fig. 6. Time Course of Intracellular AsA Content in Bt_2cAMP-Induced PC12 Cells Cultured with AA-2G or 6-Octa-AA-2G

PC12 cells were plated at 1×10^6 cells/well and incubated with AA-2G (0.5 mM, ▲) or 6-Octa-AA-2G (0.5 mM, ●) in the presence of Bt_2cAMP (0.5 mM). Each point is the mean of four or five determinations, with the bars showing S.D. $*p<0.01$, compared with presence of Bt_2cAMP and AA-2G.

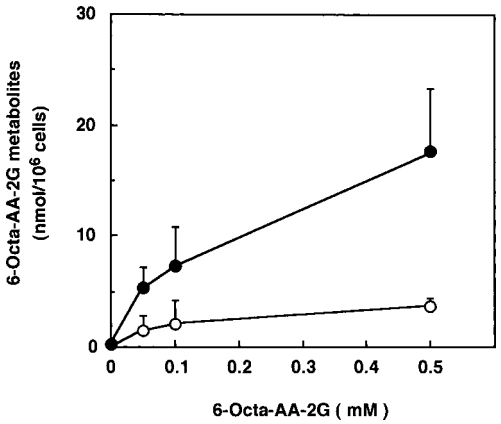

Fig. 7. Intracellular 6-Octa-AA-2G Metabolites (AsA and AA-2G) Detected in Bt_2cAMP-Stimulated PC12

PC12 cells were plated at 1×10^6 cells/well and cultured for 24 h in the presence of Bt_2cAMP (0.5 mM) and 6-Octa-AA-2G at the dose indicated in the figure. The metabolites AsA (●) and AA-2G (○) in PC12 cells were measured by HPLC. Each point is the mean of three determinations, with the bars showing S.D.

(18.7 nmol/10^6 cells) after 20 h. PC12 cells cultured in vitamin C-free medium did not synthesize AsA, as evaluated by HPLC analysis.

In Bt_2cAMP-treated PC12 cells incubated with 6-Octa-AA-2G at different concentrations, the intracellular AsA content increased in a concentration-dependent manner (Fig. 7). It is noteworthy that a small amount of AA-2G was detected in this experiment, but not in response to AA-2G treatment.

DISCUSSION

As the aged central nervous system is characterized by neuronal loss and cognitive impairments,[25] neurite outgrowth appears to be important to neuronal function. Our studies clearly show that ascorbic acid (AsA) synergistically acts on neurite outgrowth in Bt_2cAMP-induced PC12 cells (Fig. 2a), providing new insight into the process of recovery from neuronal damage. In addition, neuronal loss may be due to oxidative stress. AsA is known to be a reagent that can prevent oxidative injury, acting as an antioxidant. If an effective and

long-term supply of AsA is available to nerve cells, the cells show diminished oxidative stress. However, AsA is unstable under normal culture conditions, and more than 80% of the activity diminishes after 24 h of incubation at 37 °C.[15] A lot of stable AsA derivatives such as ascorbic acid 2-sulfate,[26] ascorbic acid 2-phosphate,[27] 2-*O*-octadecylascorbic acid (CV-3611),[28] and AA-2G[16] have been synthesized and used to study AsA activity. We recently synthesized a series of novel acylated ascorbic acid derivatives, 6-Acyl-AA-2G, to provide an effective and long-term supply of AsA,[17—19] and their effects on neurite outgrowth in Bt_2cAMP- or NGF-induced PC12 cells has been investigated.

6-Buty-, 6-Hexa-, and 6-Octa-AA-2G of the 6-Acyl-AA-2G family show prominent neurite extension on Bt_2cAMP-induced PC12 cells as compared with AsA (Fig. 2). The most remarkable effect was observed with 6-Octa-AA-2G. The proportion of cells with neurite outgrowth was much higher than the maximum level of Bt_2cAMP-induced neurite outgrowth. However, 6-Deca- and 6-Dode-AA-2G show weak synergistic effects on Bt_2cAMP-induced neurite outgrowth. At concentrations higher than 0.1 mM, these compounds exhibit cytotoxicity that may be caused by the detergent effect. As esterases are widespread in tissues *in vivo*, it seems that 6-Deca- and 6-Dode-AA-2G are rapidly hydrolyzed, thus not giving such cytotoxicity as that observed *in vitro*. In contrast, AA-2G shows maximal neurite extension at a high concentration (0.5 mM), although the maximal-effect concentration of 6-Octa-AA-2G was 0.1 mM. Similar results were observed on the neurite outgrowth induced by NGF (Fig. 3) as well as by IL-6 and bFGF (data not shown). The PC12 cells were also treated with AA-2G and a suitable fatty acid at a 1 : 1 molar ratio. The combinations gave no change in the response of neurite outgrowth compared with that of AA-2G alone. Furthermore, some low-molecular weight antioxidants such as 2-ME and GSH did not appear to play a role as an enhancer in PC12 cell neurite outgrowth. These results indicate that 6-Octa-AA-2G is a potential enhancer of neurite outgrowth, suggesting that the effects of AsA are not only due to its antioxidant status, but also to its specific structure.

It is well known that the second-messenger cAMP mediates diverse cellular responses to external signals such as proliferation, regulation of metabolism, and gene transcription by activation of the cAMP-dependent protein kinase. Many studies have shown that cAMP mediates diverse cellular responses through stimulation of the MAP kinase cascade, including phosphorylation of some specific proteins mediated through two closely related MAP kinases, p44/42 in PC12 cells.[20—24] Activated MAP kinase is transferred to the nucleus, where it phosphorylates several transcription factors such as c-Myc, c-Jun, and $p62^{TCF}$.[29—31] MAP kinase also phosphorylates and activates the serine/threonine kinase, p90 ribosomal S6 kinase (p90RSK), which can amplify the effects of MAP kinase in the phosphorylation of transcription factors.[32] We found that ascorbate and AA-2G do not elicit neurite outgrowth in PC12 cells. However, these ascorbates synergistically enhance both Bt_2cAMP- and NGF-induced neurite outgrowth in a dose-dependent manner in PC12 cells. We expected that the synergistic effect of AsA would act as an amplification factor in the signal transduction system, as AsA alone does not induce neurite outgrowth in PC12 cells. We therefore investigated whether the lipophilic ascorbate derivatives activate the MAP kinase cascade through the expression of phosphorylated p44/42. Our results indicate that exposure of Bt_2cAMP-induced PC12 cells to 6-Octa-AA-2G or AsA increases the phosphorylation of p44/42 (Fig. 4). 6-Octa-AA-2G- and AsA-induced p44/42 phosphorylation reached maximal levels at 6 and 12 h after incubation, respectively (data not shown). Even after 24 h of treatment, the expression levels of phosphorylation p44/42 treated with 6-Octa-AA-2G were much greater than those of AsA (Fig. 4). However, the enhancement of neurite outgrowth and phosphorylation of p44/42 was completely blocked by castanospermine, an inhibitor of α-glucosidase, suggesting that formation of the active substance from 6-Octa-AA-2G requires hydrolysis by α-glucosidase (Fig. 5). These results indicate that 6-Octa-AA-2G is rapidly hydrolyzed to AsA and efficiently exerts the activity of AsA on PC12 cells, in contrast to native AsA, and acts as an enhancer of the MAPK signal cascade as mediated by cytokines.

The expression of phosphorylated p44/42 was slightly enhanced by 6-Octa-AA-2G alone, but the expression level was extremely lower than that of Bt_2cAMP (Fig. 4). On the other hand, AsA did not increase the phosphorylation of p44/42. The reason why the phosphorylation of MAPK was slightly enhanced by this ascorbate derivative as well as Bt_2cAMP, although 6-Octa-AA-2G alone did not elicit the neurite outgrowth, is obscure. However, speculation has focused on the matter that an appropriate amount of phosphorylation might be necessary to induce the gene expression involved in neurite outgrowth of PC12 cells or that beside phosphorylation, a second signal is required for activation of the gene expression implicated in the neurite outgrowth.

The effects of 6-Octa-AA-2G on neurite outgrowth were found to be greater than those of AA-2G at 24 h after treatment, although the intracellular AsA content was the same as that of AA-2G at that time (Fig. 6). However, the time-course experiments regarding intracellular AsA concentrations provided additional evidence that 6-Octa-AA-2G causes a rapid accumulation and continual supply of AsA in PC12 cells, thus constituting one possible mechanism by which 6-Acyl-AA-2G exerts a stronger effect on neurite outgrowth. In Bt_2cAMP-treated PC12 cells incubated with 6-Octa-AA-2G, the intracellular AsA content increased in a concentration-dependent manner (Fig. 7). In addition, it is noteworthy that AA-2G was detected in the cells, but not in response to AA-2G treatment. These results suggest that 6-Octa-AA-2G is incorporated in an intact form into PC12 cells and metabolized *via* AA-2G to AsA in these cells, while AA-2G is enzymatically hydrolyzed to AsA on the cell membrane or in the neighborhood and then incorporated in its metabolized form into the cells.

Our experiments indicate that 6-Acyl-AA-2G remarkably enhances Bt_2cAMP-induced neurite outgrowth in PC12 cells and that this enhancement is accompanied by MAPK activation. We also observed that 6-Octa-AA-2G is rapidly hydrolyzed to AsA within the cells and then efficiently exerts AsA activity, as an enhancer, on neurite extension. 6-Acyl-AA-2G, which is stable under nonenzymatic conditions and acts as an effective and long-term supply of AsA to cells, may be useful and superior to AsA in studies of neuron systems and in cerebral disease therapies in future.

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