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ADP-RIBOSYLATION OF THE rho/rac PROTEINS INDUCES GROWTH INHIBITION, NEURITE OUTGROWTH AND ACETYLCHOLINE ESTERASE IN CULTURED PC-12 CELLS

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SUMMARY Botulinum ADP-ribosyltransferase C3 (C3 exoenzyme) was purified to homogeneity and added to cultured rat pheochromocytoma PC-12 cells. Incubation with this exoenzyme caused inhibition of cell growth and induced neurites as well as acetylcholine esterase in these cells. These changes were dependent on the amount of the enzyme added to the culture, which correlated with the in situ ADP-ribosylation of the rho/rac proteins in the cells. Preincubation with a specific anti-C3 exoenzyme monoclonal antibody inhibited both the ADP-ribosyltransferase activity and the neurite-inducing activity of the enzyme preparation. These results suggest that C3 exoenzyme affected the cellular function of the rho/rac proteins by ADP-ribosylation to induce these changes in the cells.

Two classes of guanine nucleotide binding proteins (G-proteins) are present in mammalian tissues. One is the G-proteins of heterotrimer complex containing an Mr 39,000-52,000 GTP-binding α subunit. The other is the low molecular weight G-proteins with Mr of 20,000-30,000 of which ras p21 is the archetype. Whilst the role of the former class of proteins as intermediates in transmembrane signalling has been well documented, little is known about the transduction pathway of the latter class of proteins (1,2). This is partly due to lack of adequate biochemical tools for analysis of these proteins such as cholera and pertussis toxins for Gs and Gi/Go, respectively. Type C and D Clostridium botulinum produces a novel type of ADP-ribosyltransferase, botulinum ADP-ribosyltransferase C3 (C3 exoenzyme), which modifies products of the rho and rac genes (3-9). Since the rho and rac genes are homologues of the ras oncogene(8,10) and this ADP-ribosylation occurs at an asparagine residue in the putative effector domain of the molecules (11), we predicted that this modi-

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fication affects signal transduction via these proteins and thereby causes alterations in some cell functions. Here, we report that incubation with botulinum C3 exoenzyme induces neurite outgrowth and acetylcholine esterase in cultured rat pheochromocytoma cell line, PC-12 cells. Several lines of evidence indicates that the ADP-ribosylation of the rho/rac proteins in situ is responsible for these changes in the cells.

MATERIALS AND METHODS

Materials. [α-^{32}P]NAD (36 Ci/mmol) was purchased from New England Nuclear. Acetylthiocholine iodide, N^6-2'-O-dibutyryl adenosine-3',5'-cyclic monophosphate (dibutyryl cAMP) and 5,5'-dithiobis-2-nitrobenzoic acid were obtained from Sigma. Butyrylthiocholine iodide was from Nakarai Tesque, Kyoto, Japan. Actinomycin D was from P.L Biochemicals. 2.5 S Nerve growth factor (NGF) was obtained from Wako Pure Chemical, Osaka, Japan. C3 exoenzyme was purified to homogeneity from the culture filtrate of Clostridium botulinum type C strain 003-9 and an anti-C3 exoenzyme monoclonal antibody (C302-1) was prepared as described previously (9). All other chemicals used were of reagent grade.

Cell Culture. PC-12 cells were plated at a density of 10^5 cells on a poly-L-lysine-coated 35 mm plastic dish, and grown at 36°C in DMEM containing 10% heat-inactivated horse serum, 5% heat-inactivated fetal calf serum and antibiotics as described (12). After 2 days, cells were washed and cultured in a fresh medium containing either C3 exoenzyme, NGF, or dibutyryl cAMP. At indicated times, number of cells possesing neurites was scored. A neurite was defined as a process extending from the cell over one cell body diameter in length and displaying a growth cone at its tip as described (13).

For measurement of cell proliferation, cells were plated at a density of 10^5 in a well of a 24-well plate without poly-L-lysine coating. After 2 days, cells were washed and cultured in a fresh medium containing either 100 μg/ml of C3 exoenzyme or a vehicle. At indicated times, cells were detached from the plate by repeated pipetting, and the number of viable cells was measured by trypan blue dye exclusion test.

ADP-ribosylation of rho/rac proteins and acetylcholine esterase assay. PC-12 cells were cultured in poly-L-lysine coated 35 mm plastic dishes with indicated concentrations of C3 exoenzyme for 3 days. After neurite formation was determined, cell monolayers were then washed three times with phosphate buffered saline, and cells were scraped by a rubber policeman in 1 ml of 10 mM sodium phosphate, pH 7.0. Cells were homogenized by sonication, and total cell homogenates were used for ADP-ribosylation reaction (9) and for measurement of acetylcholine esterase activity. For ADP-ribosylation, homogenates (22 μg protein) were incubated with 50 ng of C3 exoenzyme and [^{32}P]NAD as described previously (9). Acetylcholine esterase activity was measured colorimetrically in duplicates by the method of Ellman et al. (14).

RESULTS

Fig. 1 shows morphology of PC-12 cells treated for 4 days with C3 exoenzyme, NGF or dibutyryl cAMP. As compared to control cells (panel a), the cells treated with C3 exoenzyme were polygonal in shape and extended several neurites (panel b). As reported previously (15,16), both NGF and dibutyryl cAMP induced similar changes in these cells (panel c and d). However, the cells treated with C3 exoenzyme had smaller cell bodies and exhibited neurites with more bendings and ragged surface than those treated with the two agents. The neurite forma-

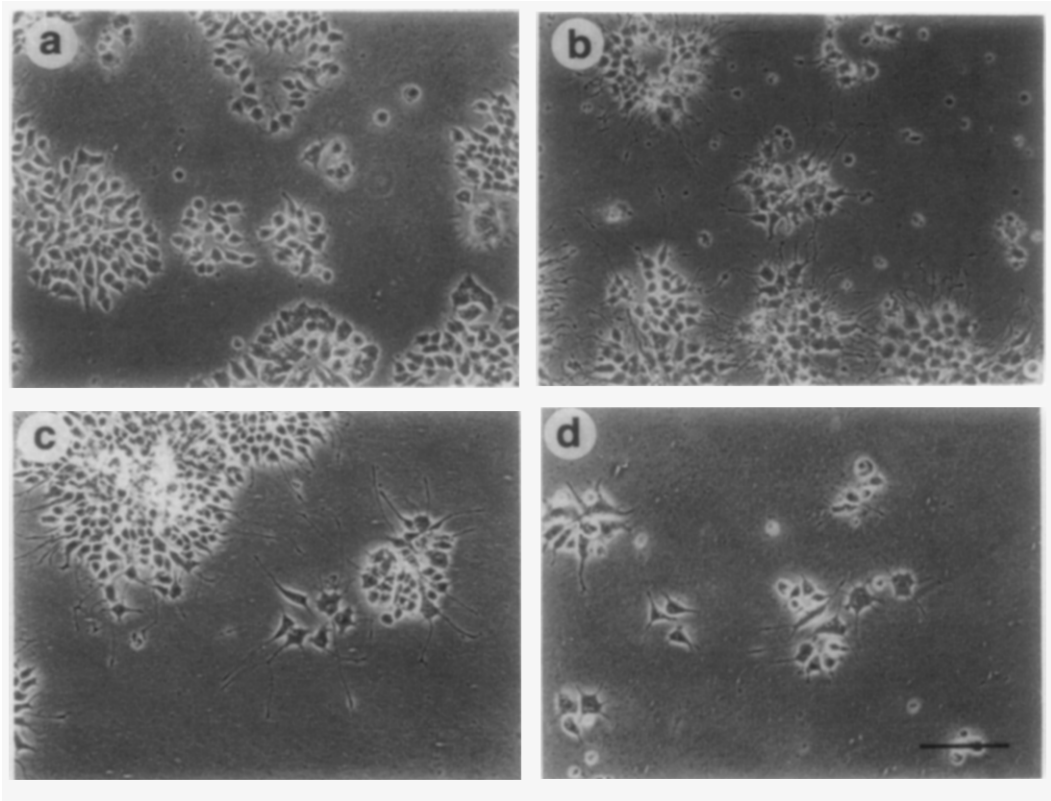

Fig. 1. Morphology of PC-12 cells incubated with botulinum C3 exoenzyme, NGF and dibutyryl cAMP. Cells were cultured in a medium without any additions (a) or with 100 μg/ml of C3 exoenzyme (b), 50 ng/ml of NGF (c), or 1 mM dibutyryl cAMP (d). After 4 days of culture, photographs of each culture were taken. Scale bar, 100 μm.

tion by C3 exoenzyme occurred in a time-dependent manner (Fig. 2a). It became evident 12 to 24 h after the addition of the enzyme and increased time-dependently thereafter. Cells possessing neurites increased in number and formed neurites extended in length. After 4 days of culture with 100 μg/ml of C3 exoenzyme, more than 80% of cells showed neurite outgrowth, and most neurites were more than 50 μm in length as shown in Fig. 1b. Neurite induction by NGF showed similar time course with a lag time (data not shown). On the contrary, neurite formation by dibutyryl cAMP occurred without a lag time and reached plateau within 24 h of incubation. Thus, neurite formations by C3 exoenzyme and dibutyryl cAMP were different in their time courses. They were also different in their sensitivities to actinomycin D. Addition of 100 nM actinomycin D to culture medium almost completely inhibited neurite formation by C3 exoenzyme but hardly affected that by cAMP. When cells were treated with various concentrations of actinomycin D, it inhibited neurite outgrowth by C3 exoenxyme in a concentration dependent manner with the IC_{50} value of about 30 nM (data not

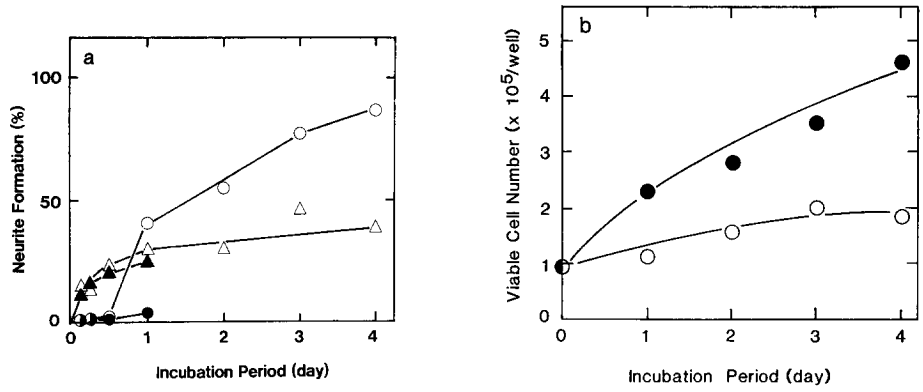

Fig. 2. Time courses of neurite outgrowth (a) and growth inhibition (b) by C3 exoenzyme in PC-12 cells. a, PC-12 cells were cultured with 100 μg/ml of C3 exoenzyme or 1 mM dibutyryl cAMP in the presence or absence of 100 nM actinomycin D. At indicated times, neurite formation was measured. o, cells treated with C3 exoenzyme only. •, cells treated with C3 exoenzyme and actinomycin D. Δ, cells treated with dibutyryl cAMP only. ▲, cells treated with dibutyryl cAMP and actinomycin D. Neurite formation in cells treated with actinomycin D was not examined after 2 days due to the toxicity of the compound. A typical result of three experiments is shown. b, PC-12 cells were cultured in the presence (o) or absence (•) of 100 μg/ml of C3 exoenzyme and cell growth was determined as described under "Materials and Methods". Each point represents the mean of duplicate experiments.

shown). These results suggest that neurite formation by C3 exoenzyme requires an RNA synthesis-dependent process similar to that observed for NGF (15), whereas that by cAMP occurrs without such process as reported previously (16). During this neurite induction by C3 exoenzyme, the growth of the treated cells was suppressed as shown in Fig. 2b. Under the present culture conditions, control PC-12 cells proliferated with a doubling time of 24 to 36 h. When C3 exoenzyme was added to the culture at 100 μg/ml, it inhibited the growth of the cells to less than 30% of the control. These growth inhibition and neurite induction by C3 exoenzyme appeared reversible. When cells treated with the enzyme were washed and cultured in a fresh medium without the enzyme, they lost neurites with a half life of about 2 days, became round in shape and resumed growth.

Fig. 3 shows the dose dependent effects of C3 exoenzyme on PC-12 cells. The neurite induction by C3 exoenzyme occurred dependent on the amount of the enzyme added to the culture as shown in Fig. 3a. The calculated EC_{50} was about 20 μg/ml. At the lower concentrations, the lag period became longer and no induction was observed below 1 μg/ml concentration. C3 exoenzyme also induced acetylcholine esterase in PC-12 cells in a dose-dependent manner (Fig. 3b). This occurred in a manner similar to that observed for neurite induction, and the specific activity of the enzyme increased two-fold after treatment with 100 μg/ml of C3 exoenzyme for 3 days. This enzyme induction was, however, much smaller than that by NGF. In the paralell experiment using the same batches of

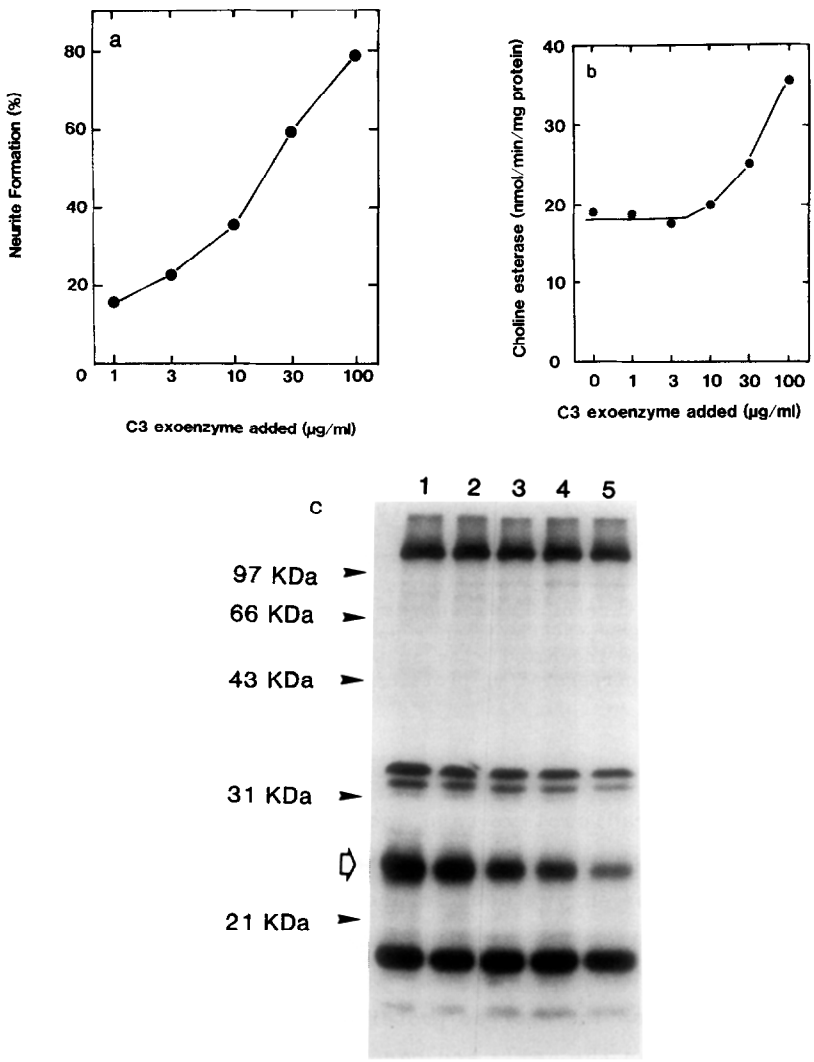

Fig. 3. Dose dependent effects of C3 exoenzyme on neurite formation (a), acetylcholine esterase activity (b) and in situ ADP-ribosylation (c) in PC-12 cells. In c, an autoradiogram of SDS-polyacrylamide gel electrophoresis of radiolabelled cell homogenates is shown. lane 1 to 5 represent the results with homogenates from cells pretreated with 0, 1, 10, 30 and 100 μg/ml C3 exoenzyme, respectively. The open arrow indicates the position of rho/rac proteins. Typical results of three experiments are shown.

cells, we found that about 3 to 4-fold induction over the basal enzyme activity was achieved by treatment with 50 ng/ml NGF for 3 days. In order to examine if ADP-ribosylation of the rho/rac proteins in the cells was responsible for these inductions, we made homogenates from cells pretreated with various amounts of C3 exoenzyme for 3 days, and incubated them with the enzyme and [^{32}P]NAD in vitro. As shown in Fig. 3c, an intense radioactive band was observed at Mr 22 K, the position corresponding to the ADP-ribosylated rho/rac proteins, on SDS-polyacrylamide gel electrophoresis of [^{32}P]-labelled homogenates from the control

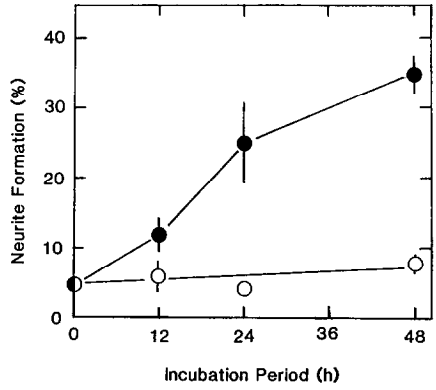

Fig. 4. Inhibition of C3 exoenzyme-induced neurite formation by an anti-C3 exoenzyme monoclonal antibody. C3 exoenzyme, 30 μg, was incubated with or without 300 μg of anti-C3 exoenzyme monoclonal antibody (C302-1) for 1 h at 37°C and overnight at 4°C. The mixtures were then filtered. Aliquots of the filtrates were taken and used for the in vitro ADP-ribosylation reaction using bovine adrenal cytosol as the substrate (5), and the rest was added to PC-12 cell culture at 30 μg of C3 exoenzyme/ml. Time course of neurite formation was followed for 2 days. Neurite formation in cells treated with C3 exoenzyme only (•) and with the mixture of C3 exoenzyme and the monoclonal antibody (o) is shown. Results are expressed as mean ± s.e.m. of three experiments. The activities of C3 exoenzyme incubated with or without the antibody were 32 and 360 nmol of ADP-ribose incorporated/mg protein/h, respectively.

cells (lane 1). This radioactive band decreased in intensity in homogenates from cells pretreated with C3 exoenzyme (lanes 2 to 5). This decrease occurred with the amounts of the enzyme used in pretreatment, and less than 30% of the radioactivity was found in the corresponding band in the homogenates from the cells pretreated with 100 μg/ml enzyme. These results suggest that, when cultured with C3 exoenzyme, the rho/rac proteins in PC-12 cells had undergone ADP-ribosylation in situ in the cells and become unable to be modified further in vitro. The above results further demonstrate that this in situ ADP-ribosylation in its dose dependency correlates well with inductions of neurite outgrowth and acetylcholine esterase, and suggest that they were the events caused by the same action of C3 exoenzyme. In order to confirm it further, we used C301-2 mouse monoclonal antibody against C3 exoenzyme. C3 exoenzyme was incubated with or without this anti-C3 exoenzyme antibody and then added to PC-12 cell culture. As shown in Fig. 4, the addition of the antibody not only inhibited the ADP-ribosyltransferase activity but also abolished neurite inducing activity of the enzyme preparation.

DISCUSSION

The present study has demonstrated that botulinum ADP-ribosyltransferase C3 added to PC-12 cell culture stopped the growth of the cells and induced neurite outgrowth as well as acetylcholine esterase in these cells. These inductions

occurred in correlation with in situ ADP-ribosylation of the rho/rac proteins in the cells and were abolished by suppression of the ADP-ribosyltransferase activity of the enzyme. These results indicate that the ADP-ribosylation affects cellular function of these G-proteins and caused induction of these properties. In addition to PC-12 cells, we observed that several other types of cultured cells undergo similar changes on incubation of C3 exoenzyme (Nishiki, T. (1989) the thesis to University of Osaka Prefecture). However, it may be argued that the amount used in our experiments is too large for such experiments. In the present experiment, we used 10 to 100 μg/ml of the enzyme to induce the changes in PC-12 cells. Although enzyme amount can be reduced in other cell types, it is still much greater than amounts of cholera and pertussis toxins used in similar experiments. This is probably due to the fact that the latter toxins have A-B toxin structure and are incorporated into cells via specific interaction of the molecules and cell surface receptors (17,18), while C3 exoenzyme lacks such a structure and may be incorporated into cells by nonspecific endocytotic process. Changes similar to those observed here was found when the minute amount of C3 exoenzyme was introduced into PC-12 cells by osmotic shock (19). However, the effects were transient and less significant than those in the present study.

Then, how does the ADP-ribosylation of the rho/rac proteins evoke signals to phenotypic expression? Our previous results demonstrated that the ADP-ribosylation occurrs in the putative effector domain of the rho protein but affects neither its GTPase nor its GTP-binding activity (11). Nonetheless, this ADP-ribosylation evoked several changes in PC-12 cells as shown in this study. These results suggest that the ADP-ribosylation makes the proteins active irrespective of whether it binds GTP or GDP, and triggers the transduction pathway mediated by them. Our study suggest that in PC-12 cells this transduction is linked to gene regulation in nuclei. However, it may exert its effects more directly in other cell systems. Chardin et al. (20) found that incubation of Vero cells with C3 exoenzyme caused the loss of actinomorphic structure and condensation of cell bodies within 45 min after the addition of the enzyme. These authors suggested that rho proteins may be directly involved in cytoskeletal assembly. The difference between the two findings may be due to the difference in the species of the rho/rac proteins present in these cells, because several rho and rac genes are present in mammals (8,21). Or the two phenomena were caused differently by the same signal(s) released by activation of the rho/rac proteins by ADP-ribosylation. The rho/rac genes are homologues of ras oncogenes and their products are G-proteins similar to ras p21. Induction of phenotypic changes similar to those reported here has been observed in PC-12 cells transfected with activated ras oncogenes (22). Whether the rho/rac proteins cause these changes by mechanism similar to ras p21 remains to be eluci-

dated. Although the rho/rac proteins are rich in nervous tissues (5), their role in nervous system remains unknown. Application of C3 exoenzyme in various systems may help to reveal it in future studies.

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