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Induction of neurite outgrowth of PC12 cells by an inhibitor of vacuolar H^+-ATPase, bafilomycin A_1

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Bafilomycin A_1, a selective inhibitor of vacuolar H^+-ATPase, induced neurite outgrowth of PC12 cells dose- and time-dependently: more than 50% of the cells extended neurite-like spikes after 24 h treatment with 100 nM bafilomycin A_1. Its dose-response ran roughly parallel to that of a bafilomycin A_1-induced lysosomal pH increase. It was inhibited by LiCl, an inhibitor of the phosphorylation of microtubule-associated proteins and, like nerve growth factor (NGF)-induced neurite outgrowth, it was also inhibited by cycloheximide and actinomycin D. But, unlike the NGF-effect, it was not associated with rapid induction of *c-fos*.

Bafilomycin A_1; Neurite outgrowth; Nerve growth factor; Vacuolar H^+-ATPase; *c-fos*; PC12 cell

1. INTRODUCTION

Vacuolar H^+-ATPases are multi-subunit enzymes which hydrolyze ATP, generating a proton gradient that is used for acidification of vacuolar compartments within cells [1] as lysosomes, endosomes, the Golgi complex and secretory granules including synaptic vesicles. The acidification of such organelles has been considered to play numerous and critical roles in maintaining the normal function of the endocytic and exocytic pathways [2]. However, it remains unclear whether the acidification of these organelles is responsible for other cellular processes such as cell proliferation and differentiation.

Recently, Bowman et al. reported that bafilomycins, which are macrolide antibiotics with a 16-membered lactone ring isolated from *Streptomyces* spp. [3], selectively inhibited the vacuolar H^+-ATPases of *Neurospora crassa* vacuoles, bovine chromaffin granules and *Zea mays* vacuoles [4]. Bafilomycin A_1 is a powerful reagent for exploring the possible functions of acidic organelles in the regulation of cellular processes. Using bafilomycin A_1 we investigated the possible roles of acidic organelles in the PC12 cell differentiation. PC12 is a cloned pheochromocytoma cell line isolated from the rat adrenal medulla, which is induced to differentiate into sympathetic neuron-like cells by NGF and other inducers [5,6]. During the course of the study we found that bafilomycin A_1 unexpectedly induced neurite outgrowth of PC12 cells.

Here we report the characterization of the bafilomycin A_1-induced neurite outgrowth of PC12 cells and discuss a possible mechanism of action.

Abbreviations: NGF, nerve growth factor; Bt_2cAMP, dibutyryl adenosine 3′:5′-cyclic monophosphate; MAP, microtubule-associated protein.

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2. MATERIALS AND METHODS

2.1. *Materials*

Bafilomycin A_1 and mouse 7S nerve growth factor (NGF) were kindly provided by Professors K. Altendorf (University of Osnabrück, Germany) and K. Hayashi (Gifu College of Pharmacy, Japan), respectively. Plasmid pc-fos1 was supplied by the JCRB gene bank and dibutyryl adenosine 3′:5′-cyclic monophosphate (Bt_2cAMP) was obtained from Sigma. Fluorescein dextran (Mr 70,000, anionic) was obtained from Molecular Probes.

2.2 *Cell culture*

Rat pheochromocytoma PC12 cells were cultured in Dulbecco's modified Eagle's medium (Flow Laboratories) supplemented with 10% fetal calf serum (M. A. Laboratory) and 5% horse serum (Cosmo Bio, Japan) at 37°C in 5% CO_2.

2.3. *Neurite outgrowth assay*

Cells were plated in 24-well culture dishes at a density of 5×10^4 cells/ml and assayed on the following day. After incubation with or without additives for the period indicated in the figure legends, the extent of neurite outgrowth was estimated as follows: a neurite was defined as a process whose length was more than 1.5-times of that of the cell body judged from the photographs taken under phase contrast microscopy (Olympus, Tokyo). About 150 cells were counted per well, and the percentage of cells with neurites was calculated.

2.4. *Fluorescent measurement of intralysosomal pH*

The PC12 cell lysosomes were loaded with fluorescein dextran by incubating the cells for 48 h in medium containing fluorescein dextran

(5 mg/ml). The cells were then washed with fresh medium and incubated in media with and without bafilomycin A_1, respectively. The fluorescence from cells was viewed under an IMT-2-21-CA3 inverted fluorescence microscope (Olympus, Tokyo) through a SIT-camera after excitation at 450 and 490 nm and analyzed by an ARGUS-100/pH image processor (Hamamatsu Photonics, Hamamatsu). The fluorescence intensity ratios (490–450 nm) were converted to pH values using a calibration curve obtained in vitro as described previously [7,8].

2.5. *Detection of* c-fos *expression in PC12 cells*

Total RNA was prepared from 1×10^6 cells treated with additives by a previously described method [9], electrophoresed and analyzed by RNA blot hybridization using ^{32}p-labeled *c-fos* DNA as described [10].

3. RESULTS

3.1. *Bafilomycin A_1 induced neurite outgrowth of PC12 cells.*

When PC12 cells were cultured in the presence of bafilomycin A_1, neurite-like spikes extended as shown in Fig. 1B. The length of some neurites was more than 10-times the cell body after 24 h incubation with 100 nM bafilomycin A_1. The morphology of those induced by bafilomycin A_1 resembled that of NGF- or Bt_2cAMP-induced neurites as shown in Fig. 1 C and D.

The bafilomycin A_1-mediated neurite outgrowth occurred time- and dose-dependently as shown in Fig. 2. Fluorescein dextran fluorescence measurements indicated a dose–response quite similar to that of the bafilomycin A_1-induced lysosomal pH increase; the apparent lysosomal pH increased from a typical pH value of 5.0 ± 0.2 to 5.6, 5.9 and 6.6 after 1 h incubation with 20, 100 and 500 nM bafilomycin A_1, respectively (data not shown). The lag time (1–2 days) that is usually associated with NGF-mediated neurite outgrowth, was barely evident before the emergence of neurite-like spikes (Fig. 2) resembling Bt_2cAMP-mediated neurite outgrowth induction (data not shown).

These results were not due to a toxic effect, if any, of bafilomycin A_1 on PC12 cells, since none of the other metabolic inhibitors tested induced neurite outgrowth: they included actinomycin D, cycloheximide (inhibitors of macromolecule synthesis), azide and/or 2-deoxyglucose (inhibitors of ATP generation), cytochalasin B and colchicine (inhibitors of cytoskeletal protein polymerization) (data not shown). Furthermore, no induction was caused by any other H^+-ATPase inhibitors, including *N,N'*-dicyclohexylcarbodiimide (≤0.5 mM), *N*-ethylmaleimide (≤0.1 M) (for vacuolar-type ATPases), azide (≤10 mM), oligomycin (≤5 mM) (both

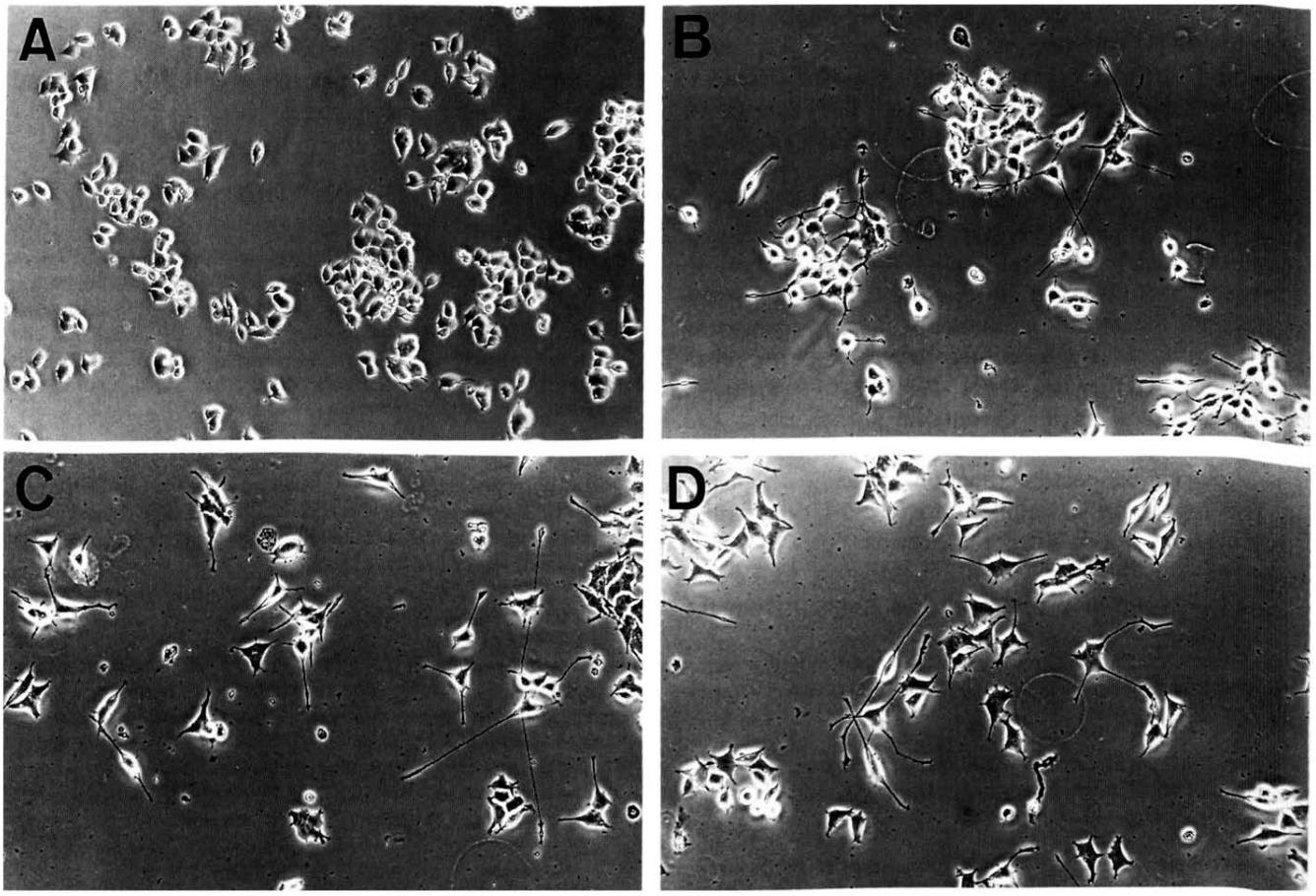

Fig. 1. Neurite outgrowth of PC12 cells induced by bafilomycin A_1, NGF and Bt_2cAMP. (A) control; (B) bafilomycin A_1 (100 nM); (C) NGF (500 ng/ml); (D) Bt_2cAMP (0.5 mM). Cells were treated for 16 h (A,B and D) or 2 days (C) ×200).

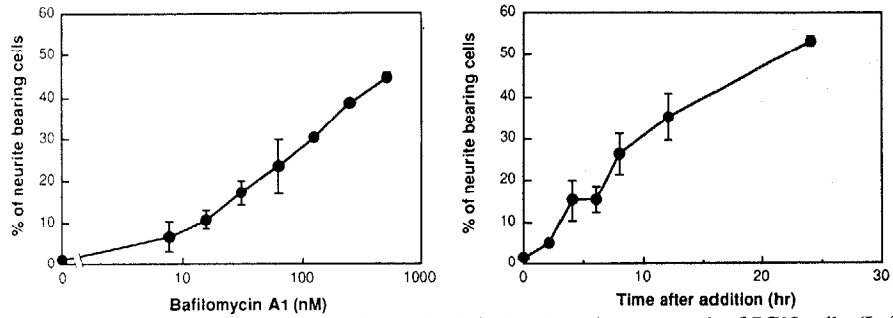

Fig. 2. Dose response (Left) and time-course (Right) of bafilomycin A_1-induced neurite outgrowth of PC12 cells. (Leftpanel) Cells were scored for neurite outgrowth as described in Materials and Methods after a 16 h incubation with bafilomycin A_1. Without the drug, the extent of neurite outgrowth was below 1%. The bafilomycin A_1 solvent, DMSO, did not induce any neurite outgrowth at 1% (v/v). Means ± SD of duplicates are shown. (Rightpanel) Cells were scored for neurite outgrowth at the indicated times after addition of bafilomycin A_1 (100 nM). Means ± SD of duplicates are plotted.

for mitochondrial F_oF_1-type ATPases) as well as vanadate (≤100 mM) (for E_1E_2-type ATPases) (data not shown).

Cells treated with 100 nM bafilomycin A_1 for more than 2 days became detached from the dish. However, the detached cells were still viable judging from the trypan blue dye exclusion test and could be re-differentiated with NGF (data not shown).

3.2. *LiCl inhibited the neurite outgrowth induced by bafilomycin A_1*

NGF-induced neurite outgrowth was inhibited by LiCl, an inhibitor of the phosphorylation of some microtubule-associated proteins (MAPs) [11]. As shown in Fig. 3, the bafilomycin A_1-induced neurite outgrowth was also markedly inhibited by LiCl in a dose-dependent manner. The inhibitory profile was quite similar to that of NGF- or Bt_2cAMP-mediated neurite induction. These results suggest that the bafilomycin effect was mediated by MAP phosphorylation and that the terminal responses of bafilomycin A_1-induced neurite outgrowth are probably the same as those of Bt_2cAMP or NGF. In support of this hypothesis, the bafilomycin effect was synergistically enhanced with Bt_2cAMP or NGF (data not shown), suggesting that bafilomycin A_1-induced neurite outgrowth is involved in the same cellular pathway as NGF- or Bt_2cAMP-mediated responses.

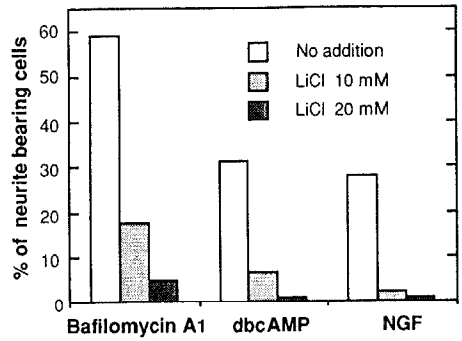

Fig. 3. The effect of LiCl on the neurite outgrowth induced by bafilomycin A_1, Bt_2cAMP or NGF. The neurite outgrowth was estimated after incubating PC12 cells with bafilomycin A_1 (100 nM, 20 h), Bt_2cAMP (0.5 mM 20 h) and NGF (500 ng/ml, 2 days) in the absence or presence of LiCl (10 mM or 20 mM). Bars represent the mean values of duplicates. Deviations were less than 5%

3.3. *Bafilomycin A_2-induced neurite outgrowth depended on the de novo syntheses of RNA and protein*

Since new RNA synthesis is required for NGF-mediated neurite generation (though not for regeneration) [12], the effects of inhibitors of RNA and protein synthesis on the bafilomycin A_1-induced neurite outgrowth were examined. As shown in Fig. 4, both actinomycin D and cycloheximide inhibited bafilomycin A_1- and NGF-induced neurite outgrowth, suggesting that bafilomycin A_1-induced effect is dependent on the de novo synthesis of RNA and protein.

The removal of cycloheximide after 8 h restored bafilomycin A_1-induced neurite outgrowth (data not shown).

3.4. *Bafilomycin A_1-induced neurite outgrowth did not depend on* c-fos *superinductiion*

The rapid superinduction of *c-fos* proto-oncogene expression is one of the major early events associated with NGF-induced neuronal differentiation in PC12 cells [13] and is considered to be responsible for long-term responses to NGF including neurite outgrowth [14]. However, *c-fos* induction was not detected 2 h after the addition of bafilomycin A_1 (Fig. 5, lanes 2–4), contrary to NGF where *c-fos* superinduction was detected by northern blot hybridization within 30 min (Fig. 5, lane 5). This indicates that the bafilomycin A_1-effect is not mediated by *c-fos* superinduction.

4. DISCUSSION

The mechanism of neurite extension of neuronal cells has been studied in model systems using neuroblastoma (NG108-15) or pheochromocytoma (PC12) cells and various inducers, including NGF and Bt_2cAMP. Accumulating evidence suggests that protein kinase C and adenylate cyclase systems are activated in response to NGF and other inducers [15–17]. However no causal

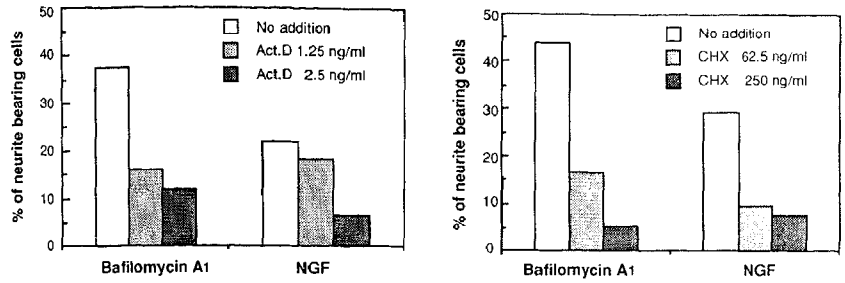

Fig. 4. Effects of actinomycin D (left) and cycloheximide (right) on the induction of neurite outgrowth by bafilomycin A_1 and NGF. Cells were scored for neurite outgrowth after incubation for 20 h (bafilomycin A_1, 100 nM) or 2 days (NGF, 500 ng/ml). Actinomycin D (Act.D, 10 ng/ml) or cycloheximide (CHX, 250 ng/ml) added alone did not induce any neurite outgrowth. Values represent the mean values of duplicates. Deviation were less than 5% in every case.

relationships have been established yet between the activated second messenger systems and neurite outgrowth induction because of the pleiotropic effects of NGF and other factors.

The experimental system we described in this paper involving bafilomycin A_1 and PC12 cells may be useful to clarify the mechanism of neurite outgrowth induction during neuronal differentiation. The characteristics of the bafilomycin A_1-induced neurite outgrowth were as follows;(I) as with the Bt_2cAMP-effect, it was rapid, reversible (Fig. 2), and inhibited by LiCl an inhibitor of MAP-kinase (Fig. 3); (II) similar to the effect of NGF, it was depentent on the de novo synthesis of RNA and protein (Fig. 4). This is in contrast to the neurite outgrowth induced by protein kinase inhibitors such as staurosporine, which occurs independently of protein synthesis [18]; (III) unlike the NGF- and Bt_2cAMP-effect [12,13], outgrowth was not associated with *c-fos* superinduction (Fig. 5). In this respect, it is similar to *N-ras*-induced neurite outgrowth which is not associated with *c-fos* superinduction [18]. As it has been suggested that *c-fos* superinduction is mediated by protein kinase C activation [13], the last feature suggests little participation of protein kinase C in bafilomycin A_1-induced neurite outgrowth. In fact, K-252a, a protein kinase C inhibitor, did not affect bafilomycin A_1-induced neurite outgrowth although it inhibited the NGF-effect (manuscript in preparation). Taken together, the experimental system involving bafilomycin A_1 and PC12 cells possesses completely new features and constitutes a unique model system which should shed light on the mechanism of neurite outgrowth in neuronal cells.

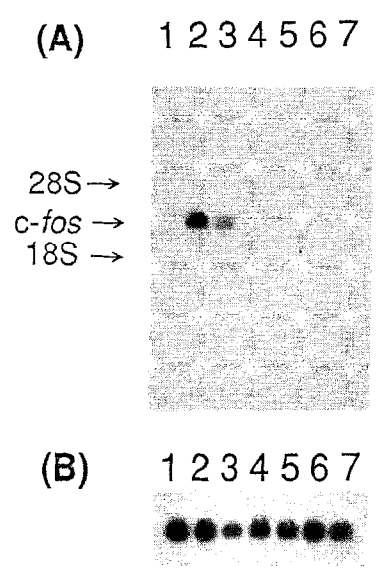

Fig. 5. Northern blot analysis of *c-fos* expression. (A) Total RNA (10 μg) isolated from PC12 cells treated with bafilomycin A_1 (200 nM) or NGF (500 ng/ml) was analyzed by northern blot for the expression of *c-fos* oncogene as described in Materials and Methods. (Lane 1) control; (lanes 2–4) bafilomycin A_1; (lanes 5–7) NGF. (Lanes 2 and 5) 30 min; (lanes 3 and 6) 60 min; (lanes 5 and 7) 120 min. The positions of 28 sRNA, 18 S RNA and *c-fos* are shown by arrows. (B) The same filter was probed with chicken ß-actin gene as an internal control.

It is not clear, however, how bafilomycin A_1 induced the neurite outgrowth. An increase in the internal pH of acidid organelles by bafilomycin A_1 was obvious from the decreased granular staining of PC12 cells with Acridine orange, and the dose–response of bafilomycin A_1-induced pH increases in lysosomes was quite parallel to that of bafilomycin A_1-induced neurite outgrowth (data not shown). These results are consistent with the explanation that the primary intracellular target of bafilomycin A_1 for neurite outgrowth is vacuolar H^+-ATPase. However, the increased pH of acidic organelles does not seem to be sufficient for neurite induction, since NH_4Cl and other basic substances that increase intra-vacuolar pH [7,20] did not induce neurite outgrowth (data not shown). In contrast to basic substances which affect only vacuolar pH, bafilomycin A_1, as an inhibitor of H^+-ATPase, should also decrease the membrane potential difference of acidic organelles generated by H^+-ATPase [21]. However, neither the combined addition of NH_4Cl and FCCP (to cancel both the pH- and membrane potential-differences) nor *N*-ethylmaleimide or *N,N'*-dicyclohexylcarbodiimide (both vacuolar H^+-ATPase inhibitors) induced neurite outgrowth (data not shown). These results are incompatible with the above explanation and suggest that the primary target of bafilomycin A_1 for neurite outgrowth is cellular components other than vacuolar H^+-ATPases. Another possibility is that vacuolar H^+-ATPases have additional functions other than H^+-

pumping, that are sensitive to bafilomycin A_1 and are closely associated with neurite outgrowth. Further investigations are required to elucidate the mechanism by which bafilomycin A_1 induces neurite outgrowth.

Although the precise mechanism of bafilomycin A_1 action on neurite outgrowth remains to be clarified, it is an intriguing hypotheses that a change in H^+-ATPase activity is involved in cell differentiation. The lysosomal pH of *ras*-transformed fibroblasts is reportedly higher than that of normal cells by 1 pH unit or more [22]. As *ras*-transformation of PC12 cells results in cell differentiation to neuronal cells [12, 23, 24], the increase of vacuolar pH may also be associated with *ras*-induced neurite outgrowth. The possible involvement of vacuolar H^+-ATPases is the regulation of cell differentiation is an issue that remains to be clarified.

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